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Engineered Nanoparticles: Review of Health and Environmental Safety (ENRHES)



About the project *Engineered Nanoparticles: Review of Health and Environmental Safety*

This report is the result of an international collaborative review involving researchers from Edinburgh Napier University (ENU), the Institute of Occupational Medicine (IOM), the Technical University of Denmark (DTU), the Institute for Health and Consumer Protection of the European Commission's Joint Research Centre (JRC), and the Institute of Nanotechnology (IoN). The project was funded by a grant under the Seventh Framework Programme of the European Commission.

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CONTENTS

ACRONYMS AND SPECIES LIST	VII
ORGANISATIONS CONDUCTING THE REVIEW	XI
EXECUTIVE SUMMARY	XII
1 INTRODUCTION	1
1.1 Background	1
1.2 Aims and objectives	2
1.3 An overview of the four nanomaterial classes	2
1.4 Review structure	3
1.5 References	3
2 MATERIALS PRODUCTION AND USE	4
2.1 Introduction	4
2.2 Carbon fullerenes	4
2.3 Carbon nanotubes	5
2.4 Metals	7
2.5 Metal oxides	10
2.6 Industry survey	12
2.7 Summary	16
2.8 References	17
3 CHARACTERISATION OF NANOMATERIALS	19
3.1 Introduction	19
3.2 Challenges	19
3.3 Attribute-focused overview of characterisation techniques	22
3.4 Summary	30
3.5 References	31

4	EXPOSURE	34
4.1	Introduction	34
4.2	Carbon fullerenes	38
4.3	Carbon nanotubes	40
4.4	Metals	44
4.5	Metal oxides	45
4.6	Effectiveness of exposure control	47
4.7	Environmental exposure	48
4.8	Consumers' exposure to nanomaterials	51
4.9	Conclusions and recommendations	52
4.10	References	53
5	ENVIRONMENTAL FATE AND BEHAVIOUR	55
5.1	Introduction	55
5.2	Environmental fate and behaviour of nanomaterials in air	57
5.3	Environmental fate and behaviour of nanomaterials in water	59
5.4	Environmental fate and behaviour of nanomaterials in soil and sediment	64
5.5	Modelling approaches	67
5.6	Conclusions and recommendations	67
5.7	References	69
6	HUMAN TOXICITY	73
6.1	Introduction	73
6.2	Carbon fullerenes	74
6.3	Carbon nanotubes	90
6.4	Metals	121
6.5	Metal oxides	137
6.6	References	163
7	EPIDEMIOLOGY AND HUMAN EXPOSURE STUDIES	183

7.1	Epidemiology	183
7.2	Human exposure studies	190
7.3	Limitations and strengths of epidemiology	193
7.4	Recommendations for future epidemiological research in nanotechnology industries	195
7.5	References	197
8	ECOTOXICITY	201
8.1	Introduction	201
8.2	Carbon fullerenes	201
8.3	Carbon nanotubes	206
8.4	Metals	212
8.5	Metal oxides	217
8.6	Conclusions	227
8.7	References	232
9	RISK ASSESSMENT	238
9.1	Introduction	238
9.2	Applied risk assessment methodology	238
9.3	Carbon fullerenes	239
9.4	Carbon nanotubes	259
9.5	Metals nanoparticles	287
9.6	Metal oxide nanoparticles	307
9.7	Conclusions and recommendations	329
9.8	References	334
10	SUMMARY OF CONCLUSIONS AND RECOMMENDATIONS	350
10.1	Introduction	350
10.2	Materials production and use	350
10.3	Characterisation	351
10.4	Exposure	351

10.5	Environmental fate and behaviour	352
10.6	Toxicity	353
10.7	Epidemiology and human studies	355
10.8	Ecotoxicity	356
10.9	Risk assessment appraisal	361
10.10	References	366
APPENDICES		369
Appendix 1: Review methodology		369
A1.1	Co-ordinated information management	369
A1.2	Review activity	370
A1.3	Risk assessment appraisal	372
Appendix 2: Industry survey methodology and data		373
A2.1	Methodology	373
A2.2	Survey data	380

ACRONYMS AND SPECIES LIST

Acronym	Definition
ADME	Absorption, Distribution, Metabolism and Excretion
AES	Auger Electron Spectroscopy
AFM	Atomic Force Microscope
AgNP	Silver Nanoparticles
ATOF-MS	Aerosol Time-of-Flight Mass Spectrometry
AuNP	Gold Nanoparticles
hpf	Hours Post-fertilisation
BAF	Bioaccumulation Factor
BALF	Broncho Alveolar Lavage Fluid
BCF	Bioconcentration Factor
BET	Brunauer-Emmett-Teller
BSA	Bovine Serum Albumin
BSAF	Biota-sediment Accumulation Factors
C ₆₀	Fullerene
CAP	Concentrated Ambient Particles
CAT	Catalase (used as a marker for oxidative stress)
CFU	Colony Forming Units
CNF	Carbon Nanofibre
CNS	Central Nervous System
CNT	Carbon Nanotube
CPC	Condensation Particle Counter
CuNP	Copper Nanoparticles
CVD	Chemical Vapour Deposition
CYP2	Cytochrome P450 Family 2
DEP	Diesel Exhaust Particles
DLC	Diamond Like Carbon
DLS	Dynamic Light Scattering
DMA	Differential Mobility Analysis
DMSO	Dimethylsulfoxide (solvent)
DNA	Deoxyribonucleic acid
DNEL	Derived No Effect Level
DSC	Differential Scanning Calorimetry
DWCNT	Double-walled Carbon Nanotube
EC _x	Effective Concentration
ECR	Electron Cyclotron Resonance
ECWR	Electron Cyclotron Wave Resonance
EDS	Energy Dispersive Spectrometry
EDX	Energy Dispersive X-ray Spectroscopy
EELS	Electron Energy Loss Spectroscopy
EFM	Electrical Force Microscopy
ELPI	Electrical Low Pressure Impactor
EPR	Electron Paramagnetic Resonance
ESCA	Electron Spectroscopy for Chemical Analysis
ESR	Electron Spin Resonance
FCVA	Filtered Cathodic Vacuum Arc
FEV	Forced Expiratory Volume
FEF	Forced Expiratory Flow
FE-SEM	Field Emission Scanning Electron Microscopy
FFF	Field Flow Fractionation
FIB	Focused Ion Beam
FMPS	Fast Mobility Particle Sizer
Fpg	Formamidopyrimidine DNA glycosylase
FTIR	Fourier Transform Infrared Spectroscopy
FVC	Force Vital Capacity
g-C	Glassy Carbon

Acronym	Definition
GIT	Gastrointestinal Tract
GSH	Glutathione
HDMEC	Human Dermal Microvascular Endothelial Cells
HFCVD	Hot Filament Chemical Vapour Deposition
HiPCC	High Pressure Carbon Monoxide
Hpf	Hours post fertilisation
HPHT	High Pressure High Temperature
IARC	International Agency for Research on Cancer
IBA	Ion Beam Analysis
ICD	International Classification of Diseases
ICP	Inductively Coupled Plasma
IEP	Isoelectric Point
ISO	International Organization for Standardization
ITC	Information technology and communication
LC _x	Lethal Concentration
LD _x	Lethal Dose
LDH	Lactate Dehydrogenase
LFM	Lateral Force Microscopy
LOAEC	Lowest Observed Adverse Effect Concentration
LOAEL	Lowest Observed Adverse Effect Level
LOEL	Lowest Observed Effect Level
LOEC	Lowest Observed Effect Concentration
LPO	Lipid Peroxidation
MALS	Multi-Angle Light Scattering
MCDA	Multi-Criteria Decision Analysis
MEM	Minimal Essential Medium
MFM	Magnetic Force Microscopy
MRFM	Magnetic Resonance Force Microscopy
MS	Mass Spectrometry
MWCNT	Multi-walled Carbon Nanotube
MWCNT-COOH	Multi-walled Carbon Nanotube Carboxylated
MWCNT-OH	Multi-walled Carbon Nanotube Hydroxylated
nC ₆₀	Fullerene in aqueous suspension/solution
NEXAFS	Near Edge X-ray Absorption Fine Structure
NIOSH	National Institute of Occupational Safety and Health
NMR	Nuclear Magnetic Resonance
NOAEL	No Observed Adverse Effect Level
NOAEC	No Observed Adverse Effect Concentration
NOEC	No Observed Effect Concentration
NOM	Natural Organic Matter
NSOM	Near-Field Scanning Optical Microscopy
OECD	Organisation for Economic Cooperation and Development
OES	Optical Emission Spectrophotometry
PAA	Poly(acrylic acid)
PBS	Phosphate Buffered Saline
PBT	Persistent, Bioaccumulative and Toxic
PCS	Photon Correlation Spectroscopy
PEC	Predicted Environmental Concentration
PECVD	Plasma Enhanced Chemical Vapour Deposition
PEEM	Photoemission Electron Microscopy
PEN	Project on Emerging Nanotechnologies
PL	Photoluminescence
PLA	Pulsed Laser Ablation
PLV	Pulsed Laser Vapourisation
RNA	Ribonucleic Acid
PNEC	Predicted No Effect Concentration
RBM	Radial Breathing Mode
RBS	Rutherford Back Scattering

Acronym	Definition
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
REL	Recommended Exposure Level
ROS	Reactive Oxygen Species
RS	Raman Spectroscopy
SAED	Selective Area Electron Diffraction
SANS	Small Angle Neutron Scattering
SAXS	Small Angle X-ray Scattering
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SCM	Scanning Capacitance Microscopy
SDS	Sodium Dodecyl (Surfactant)
SECM	Scanning Electrochemical Microscopy
SEM	Scanning Electron Microscopy
SERRS	Surface Enhanced Resonant Raman Scattering
SIMS	Secondary-Ion Mass Spectrometry
SKPM	Scanning Kelvin Probe Microscopy
SMPS	Scanning Mobility Particle Sizing
SOD	Superoxide dismutase (used as a marker for oxidative stress)
SPM	Scanning Probe Microscopy
SQUID	Superconducting Quantum Interference Device
SRSAXS	Synchrotron Radiation Small Angle X-ray Scattering
STM	Scanning Tunnelling Microscope
SWCNT	Single-walled carbon nanotube
ta-C	Tetrahedral Amorphous Carbon
TBARS	Thiobarbituric acid (used as a marker for oxidative stress)
TEM	Transmission Electron Microscopy
TEOM	Tapered Element Oscillating Microbalance
TGA	Thermogravimetric Analysis
THF	Tetrahydrofuran (solvent)
TWCNT	Triple-walled carbon nanotubes
TXRF	Total Reflection X-ray Fluorescence Spectroscopy
US FDA	United States Food and Drug Administration
UV-Vis	Ultraviolet-Visible
XAES	X-ray Auger Electron Spectroscopy
XPS	X-ray Photoelectron Spectroscopy
XRD	X-ray Diffraction
XRDLB	X-ray Diffraction Line Broadening

Latin name	Common name/organism
<i>Ambystoma mexicanum</i>	Axolotl; Salamander
<i>Amphiascus tenuiremis</i>	Copepod
<i>Bacillus megaterium</i>	Bacteria
<i>Bacillus subtilis</i>	Gram Positive Bacteria
<i>Brachionus calyciflorus</i>	Rotifer
<i>Carassius auratus</i>	Carp
<i>Ceriodaphnia dubia</i>	Crustacean
<i>Chlamydomonas reinhardtii</i>	Algae
<i>Chydorus sphaericus</i>	Crustacean
<i>Cucurbita maxima</i>	Pumpkin
<i>Cyprinus carpio</i>	Carp
<i>Danio rerio</i>	Zebrafish
<i>Daphnia magna</i>	Crustacean
<i>Daphnia pulex</i>	Crustacean
<i>Desmodesmus subspicatus</i>	Algae
<i>Eisenia foetida</i>	Earthworm
<i>Eisenia veneta</i>	Earthworm
<i>Escherichia coli</i>	Gram Negative Bacteria
<i>Fucus serratus</i>	Algae

Latin name	Common name/organism
<i>Fundulus heteroclitus</i>	Killifish, Saltwater Minnow; Common Mummichog
<i>Hydra attenuate</i>	Cnidaria; Micro-Invertebrate
<i>Hyalella azteca</i>	Benthic crustacean
<i>Leptocheirus plumulosus</i>	Burrowing amphipod (common tube builder)
<i>Lumbriculus variegates</i>	Sediment Worm
<i>Micropterus salmoides</i>	Largemouth Bass
<i>Mytilus galloprovincialis</i>	Blue Mussel
<i>Oncorhynchus mykiss</i>	Rainbow Trout
<i>Phaseolus limensis</i>	Beans
<i>Phaseolus radiatus</i>	Mung Bean
<i>Porcellio scaber</i>	Woodlouse
<i>Pimephales promelas</i>	Fathead minnow
<i>Pseudokirchneriella. subcapitata</i>	Algae
<i>Pseudomonas putida</i>	Gram Negative Bacteria
<i>Spinacia oleracea</i>	Spinach
<i>Staphylococcus aureus</i>	Bacteria
<i>Streblospio benedicti</i>	Polychaete Worm
<i>Streptococcus agalactiae</i>	Bacteria
<i>Stylonychia mytilus</i>	Protozoan
<i>Tetrahymena pyriformis</i>	Ciliated Protozoan
<i>Tetrahymena thermophila</i>	Ciliated Protozoan
<i>Thamnocephalus platyurus</i>	Crustacean
<i>Triticum aestivum</i>	Wheat
<i>Vibrio fischeri</i>	Marine Luminescent Bacteria
<i>Xenopus laevis</i>	Frog

ORGANISATIONS CONDUCTING THE REVIEW

Edinburgh Napier University (ENU) is a modern University which includes three faculties, one of which is the Faculty of Health Life and Social Sciences. The Centre for Nano Safety at Edinburgh Napier University provides a multi-disciplinary environment with state of the art equipped laboratories to conduct cellular and molecular toxicity testing. This Centre is one of the members of the Safety of nanomaterials Interdisciplinary Research Centre (SnIRC) (<http://www.snirc.org>). Edinburgh Napier University nanotoxicology researchers have been involved in a number of FP6 projects, including the recently published 'PARTICLE_RISK', as well as FP7 projects such as NanoImpactNet, InLiveTox and ENPRA. Professor Vicki Stone, the Director of the Centre for Nano Safety, is Editor-in-Chief of the journal Nanotoxicology.

The Institute of Occupational Medicine (IOM) is a major independent centre of scientific excellence in the fields of occupational and environmental health, hygiene, and safety. IOM have established a leading presence on nanotechnology risk issues through a strong reputation in assessing the risks of respirable particles (e.g. coal dust, asbestos, metal oxides), by leading or participating in several of the key reviews commissioned by the UK Government in response to the Royal Society Report, through initiatives such as the Safety of Nanomaterials Interdisciplinary Research Centre (SnIRC) collaboration which was co-founded by IOM and Edinburgh Napier University. IOM also runs the SAFENANO initiative (<http://www.safenano.org>), the UK's only Micro and Nanotechnology (MNT) centre of excellence on nanoparticle risk, facilitating the responsible development of safe nanomaterials by integrating focussed research, review activities, expert opinion and best practice training. IOM has been involved in a number of FP6 projects, including PARTICLE_RISK, and is playing a major role in FP7 projects including NANOMMUNE, NanoImpactNet, NanCore, ObservatoryNANO and ENPRA.

The Technical University of Denmark (DTU) is one of the largest university groups in Europe specialising in environment and resources through its Department of Environmental Engineering (DTU Environment). DTU Environment aims to develop technical and sustainable solutions to minimise the impact of society on the environment and humans through research and teaching at an international level. Research conducted at DTU Environment includes, among other topics, environmental chemistry and ecotoxicology. DTU Environment was awarded the Stockholm Water Prize in 1992 and is a member of NanoDTU, an interdisciplinary network for about 180 nanoscientists at DTU. DTU Environment have been involved in a number of European FP6 and FP7 projects, including COMPRENDO, RiskBridge and NanoImpactNet, as well as the development of an inventory of products based on nanotechnology commissioned by the Danish EPA.

The European Commission Joint Research Centre's Institute for Health and Consumer Protection (<http://ihcp.jrc.ec.europa.eu/>) provides independent scientific and technical support to the development and implementation of EU policies on chemicals, food and consumer products. This includes work on risk assessment methodologies, testing and non-testing methods (e.g. QSARs) and testing strategies. Furthermore, the IHCP focuses on nanobiosciences (<http://nmi.jrc.ec.europa.eu/>) including nanoparticle synthesis and activation, advanced surface characterisation, and health aspects related to nanoparticles. It also provides advice on how to address nanomaterials under the chemicals policy (REACH). Staff members are contributing to the OECD Working Party on Manufactured Nanomaterials (WPMN) and the ISO/CEN standardisation work. Moreover, the Nanobiosciences unit is involved in a number of European FP6 and FP7 projects including ENPRA, NanoImpactNet, NanoReTox, NanoTher, NHECD and NanEx.

The Institute of Nanotechnology (ION), founded in 1997, is an independent not-for-profit organisation whose core activities are focused on education and training in nanotechnology. IoN was one of the world's first nanotechnology information providers and is now a global leader, working closely with governments, universities, researchers, companies and the general public to educate and inform on all aspects of nanotechnology. IoN staff members sit on a number of influential eu and international nanotechnologies panels, and have produced several important milestone publications. IoN works closely with industry including a very proactive linkage with smes. It has a database of over 71000 people actively involved in nanotechnology research and has a membership base of over 12000.

EXECUTIVE SUMMARY

Engineered Nanoparticles: Review of Health and Environmental Safety presents a comprehensive and critical scientific review of the health and environmental safety of four classes of nanomaterials: fullerenes, carbon nanotubes (CNT), metals and metal oxides. The review considers sources, pathways of exposure the health and environmental outcomes of concern, followed by a risk assessment based upon this information. The report includes an illustration of the state-of-the-art as well as on-going work, while identifying knowledge gaps in the field. Prioritised recommendations have been developed and set in the context of informing policy makers in the development of methods to address exposure as it relates to the potential hazards posed by engineered nanoparticles, and in the development of appropriate regulation.

The review first provides context for the materials chosen, in term of the **production techniques, applications and market value**. This is supplemented with the findings of an industry survey carried out in an attempt to gather up to date information on the quantities of various types of nanomaterials produced and used, the type of products in which they are used, any exposure data gathered and risk assessment practices employed. Whilst useful to an extent, the survey received a limited response and does not provide a complete overview of nanomaterial production and use worldwide.

The review subsequently highlights the essential role which **nanoparticle characterisation** plays in a variety of overlapping contexts ranging from fundamental and applied research, through process and product quality control and commercialisation, to health and environmental protection. The review highlights the effort being made towards improving the characterisation basis for toxicological studies, such as identifying the key physico-chemical characteristics of nanoparticles and how they can be measured. The body of literature published confirms that there is now a consensus that thorough and accurate particle characterisation is an essential part of assessing the potential toxicity of nanoparticles in biological systems. Appropriate and common characterisation of test materials is important to ensure that results are reproducible, and also to provide the basis for understanding the properties of nanoparticles that determine their biological effects. Some of the key physico-chemical parameters influencing the biological activity of nanoparticles remain unknown or to be fully understood at this point. Hence, the characterisation of test materials should be as thorough as possible and broad in scope. The review identifies the basis of a minimum set of characteristics that should be measured for test materials used in toxicity studies. In addition to composition, these include size and shape, state of dispersion, surface area, and surface chemistry.

In the context of **exposure assessment**, the review shows that there is, in general, a paucity of published data. For the materials of interest, eleven studies were identified which have reported measured exposure data. All of these are in the occupational setting, while no studies have reported consumer exposures or exposures in the environment. All but one of the studies have reported inhalation exposure only; one study reported dermal exposure and no studies reported ingestion exposure. Most of the studies were carried out in university settings, however, some industrial settings were also found. A wide range of instruments and approaches were used and exposures were reported in terms of number, mass and surface area concentrations, as totals and differentiated as a function of size. Most studies showed some evidence of elevated exposures although these were often associated with ineffective or deliberately disabled control systems. Studies which have assessed the effectiveness of respiratory filters have shown that, as theory predicts, they are efficient collectors of nanoparticles. Exposures are clearly plausible in occupational, consumer and environmental settings throughout the lifecycle of materials and products. The review also considers modelling studies and identified two which provide useful information relating to environmental and consumer exposure. Recommendations are made for further occupational, consumer and environmental exposure assessment to support effective risk assessment and characterisation.

Similarly, the review highlights the general paucity of data in the area of **environmental fate and behaviour**, which represents a major obstacle in developing a holistic view of the fate

and transport of nanomaterials within the environment and therefore environmental exposure. Current knowledge of transport of nanomaterials within air, soil and water compartments is rooted in aerosol and colloid science. This background is used to provide preliminary information from which further understanding of nanoparticles' fate and transport can be developed. With respect to the aquatic environment, one consistent finding is that most nanomaterials interact with natural organic matter and other materials found in the environment, and that this influences the fate and transport of nanomaterials in water and may also be of significance for their biological effects. The review identifies the need for systematic studies to be undertaken on different types of nanomaterials using a range of physico-chemical parameters (e.g. size, shape, form, surface area), in order to generate data which will support development of reliable and truly relevant models. Predictive modelling of emission scenarios and subsequent transport pathways will play an important role in furthering understanding of this area.

A substantial appraisal of the **toxicity of nanoparticles** is presented for each nanomaterial class. The review evaluates the toxic potential of the four classes of nanomaterials and identifies the underlying mechanisms driving each of their toxicities, and determines whether any generalisations can be made regarding nanomaterials as a whole. In addition, the review reveals material or particle specific attributes that are particularly relevant in driving nanomaterial toxicity, therefore allowing identification of key characteristics that can influence safety. In an attempt to achieve this, available information regarding the exposure conditions and characteristics of nanomaterials used within the described studies has been outlined. The review highlights discrepancies regarding the dose metrics used when expressing the concentration of particles exposed to cells or animals, specifically whether dose is based upon the mass, surface area, or particle number administered. This is of relevance as it has been repeatedly demonstrated that the toxicity of particles is related to their size, so that as particle size decreases, toxicity generally increases, which is thought to be driven by their surface area. However, nanomaterials are a diverse group of materials, and it has become evident that other particle dimensions are also important in driving their toxicity, such as length. This has been clearly demonstrated in using both *in vitro* and *in vivo* studies of CNT. Surface functionalisation of nanomaterials also alters their potential toxicity (e.g. fullerenes), although at this time the mechanisms of such changes are not understood. Furthermore, the tendency of nanomaterials to agglomerate or aggregate is of concern, and has encouraged investigation into improving nanomaterial suspensions, including the use of dispersants, solvents, surface modification or mechanical processes.

As exposure to nanomaterials is expected to primarily occur through dermal, inhalation, ingestion or injection routes, the focus of the toxicological review employs studies using the lungs, skin, gastrointestinal tract (GIT), or blood as routes of entry, with the inclusion of both *in vitro* and *in vivo* models for each route. However, the realisation that nanomaterials can distribute from their exposure site, within the blood or even nerves, means that nanomaterial toxicity may be exerted at a number of targets including, for example, the liver, brain, spleen, and kidneys.

The review has considered the latest studies which have sought to assess the toxicity of nanomaterials including the utilisation of both *in vivo* (within mice and rats) and *in vitro* models (using cell lines and primary cells). The evidence-base from particle toxicology combined with the use of models provides a useful series of protocols to allow benchmarking of new nanomaterials to the relative potential toxicity of other substances of known hazard. Cell lines are frequently used to investigate the effects of potentially toxic substances. The types of cells considered in the review are diverse and represents a wide range of organ and cell types, including tumour derived and transformed cells. Their response is often representative of the *in vivo* response, but careful comparisons and controls are required to ensure relevance. The review highlights the limitations in using *in vitro* cytotoxicity (cell death) for risk assessment purposes, even if benchmarked against a material of 'known' risk, is questionable, as very few particle-induced diseases are associated with acute immediate cell death. Instead, sub-lethal effects measured *in vitro* are shown to have useful potential for risk assessment purposes. Measures of cytotoxicity are, more useful to ascertain sub-lethal concentrations for further investigation rather than explicitly for risk assessment purposes. Most pathological particles act via the induction of cellular and molecular changes such as

oxidative stress and/or the induction of inflammation, both of which can lead to disease. These endpoints have therefore been assessed with greatest interest and highest priority, when assessing the toxicity of nanomaterials.

When appraising the available **epidemiology and human studies**, the paucity of data relating to CNT, fullerene, metal and metal oxide nanoparticles, has required a broader approach. This draws upon the depth and breadth of knowledge available for a small number of nanoparticles which have been manufactured at the industrial scale for decades. The results from the discussed epidemiology studies of the carbon black industry indicate some adverse effects of exposure to carbon black dust on respiratory health. However, the main findings are reassuring in that respiratory symptoms and lung function appear to be primarily associated with current exposure rather than being caused by cumulative exposures. A mortality study by Sorahan *et al.* 2001 clearly indicates no strong and little suggestive evidence of excess non-malignant respiratory disease associated with working in the carbon black industry. Despite the fact that two of the five factories investigated generated evidence that there was excess mortality from lung cancer, the study has failed to link this disease to carbon black exposure. Although the available TiO₂ industry epidemiology studies provide little information to evaluate the health risks associated with ultrafine particle manufacture, as most work has focused on larger particles, it is unlikely that exposure to a true ultrafine or nanoparticle dust explains the variations in lung cancer mortality between studies and factories. The review considered the limited number of relevant epidemiological studies that assess particle number in ambient air which conclude that; (i) there are adverse health effects associated with the ultrafine fraction of respirable particles, with effects indicated on mortality in the general population and panels of susceptible individuals and (ii) death was related to particle numbers in the nano-size range. Overall, the findings of the review of human studies suggest that nanoparticles are capable of inducing physiological and inflammatory responses in humans.

The review of the literature on the **ecotoxicity of nanoparticles** for each of the four groups of nanomaterials has addressed aquatic toxicity, terrestrial toxicity, bioaccumulation, and degradability. Aquatic ecotoxicity is further sub-divided into studies dealing with fish, crustacean, algae and other taxa (covering studies on bacteria, non-crustacean invertebrates, and amphibians) with the view to providing data for risk assessment purposes. Due to the strong focus on regulatory use of the ecotoxicity data for the risk assessment aspects of the review, a special effort has been put into translating the effects, found in the reviewed papers, into the terminology traditionally used in risk assessment, e.g. EC_x- and LC_x-values and NOEC/LOEC-values. Large differences in behaviour, fate and effects, even in standardised test systems, were encountered for different metals and metal oxides within each class. Hence, as for the toxicology review, the ecotoxicity review of metals and metal oxide nanoparticles have considered studies pertaining to specific substances rather than considering them as one group of substances. The review identifies the range of studies that have been carried out with aquatic and terrestrial species and those carried out towards the base-set organisms used in the REACH risk assessment procedures for chemicals (fish, crustacean and algae). More studies are available using bacterial groups and, though they do not report the findings in traditional ecotoxicological endpoints, these studies may be of value for mechanistic interpretations of ecotoxicity in both the aquatic and the terrestrial environment. The review highlights how initial studies used different solvents to suspend C₆₀, but how more recent studies avoided the use of any solvents since, as for the mammalian toxicology studies, it has been demonstrated that not only C₆₀/solvent interactions may affect toxicity, but also solvent degradation products may be responsible for some of the observed effects. Major knowledge gaps are identified within persistence and bioaccumulation of fullerenes since no structured studies, aimed to investigate this, have been reported in the reviewed literature. Only a few ecotoxicological studies of the effects of CNT on aquatic and terrestrial species have been carried out. The review identified studies on other taxa (ranging from bacteria and protozoans to amphibians), but the high variability in these studies means that it is not possible to draw any common conclusion on the effects of CNT on this basis. It was identified, however, that a number of studies do not find adverse effects after exposure to CNT at often very high concentrations. The review highlights that degradability of CNT still remains to be studied and testing difficulties in relation to obtaining, handling, purification and solubilisation are likely to have an influence in the very limited number of studies available for

environmental risk assessment (i.e. ecotoxicity, persistency, and bioaccumulation). The review identifies that the major part of the published ecotoxicology literature deals with toxic effects of silver and copper nanoparticles and general conclusions on the toxicity of these are reported. Only a very few studies have dealt with bioaccumulation of metal nanoparticles even though this is a topic of high concern when considering past experiences with metals which, by definition, are not degradable. However, the review highlights that changes in the metal speciation can occur depending on redox conditions, salt content etc. The ecotoxicity studies of aluminium, gold, cobalt, and nickel nanoparticles have also been reviewed. However, the literature on these metals can best be described as extremely limited. Although general conclusions on metal oxide ecotoxicity are hampered by the large diversity of materials, the review presents summaries for three individual metal oxides types (TiO₂, ZnO and SiO₂) and identifies a number of trends. The review highlights the importance of considering the effect of functionalisation on bioavailability and hence toxicity and bioaccumulation of nanoparticles, which remains to be fully studied.

The penultimate chapter presents a basic **risk assessment**, inspired by the REACH Guidance, for the four types of nanomaterials under review based on the information provided by preceding chapters of the review. It includes an assessment for both the human health and the environment, limited to the extent that the available data allows. For each nanomaterial uncertainties and additional work needed to complete the assessment are also described.

Each of the four groups of nanomaterials under review - fullerenes, carbon nanotubes, metals and metal oxides –different forms of the substances are included, e.g. fullerenes with different functionalisation, or single and multi walled carbon nanotubes. In particular, the metals and the metal oxide nanoparticles - like those in the conventional/'bulk' form - cannot be considered as one group in terms of risk assessment due to the chemical, toxicological and ecotoxicological diversity between substances within one group. Therefore for metals and metal oxides the most data rich substance(s) were chosen as case studies in the development of a simple risk assessment approach. The case studies are presented as a purely scientific exercise which allows the exploration of key questions associated with the risk assessment of nanomaterials, and should not be used in any other way. These case studies do not reflect any opinion of the European Commission.

The limited availability of information (which does not comply comprehensively with the REACH requirements in terms of detailed information on the use, exposure, and data on inherent properties), means that the risk assessments are commensurate with an assessment as carried out under the old chemicals legislation (for 'existing substances') where the regulatory authorities conducted an assessment based on the available information. In order to follow this format, information has been extracted from previous chapters of the review and assimilated into a risk assessment. On the basis of the identified information, the risk assessments are carried out following both a quantitative and a qualitative approach. For human health, the quantitative approach requires establishing exposure values for the various routes of exposure (inhalation, dermal and oral) for consumers and workers and the establishment of a Derived-No-Effect Level (DNEL), typically based on extrapolation of animal data to the human situation by using appropriate assessment factors. For the environmental assessment, the quantitative approach requires the determination of the Predicted Exposure Concentration (PEC) and the Predicted No Effect Concentration (PNEC) for each environmental compartment. PEC and PNEC are then compared to identify any risk for environmental compartments. For both human health and the environment, the application of assessment factors is based on the REACH guidance. Qualitative risk characterisation was carried out in the event where no exposure value and/or no dose descriptors were available or estimated.

The risk assessments show a significant lack of measured and modelled exposure data of nanoparticles, for humans (occupational and consumer exposure) and for the environment. The limited amount of published measured data for human exposure may be due to the difficulties associated with the measurement of ultrafine or nanoparticles, the decision regarding which metric(s) to use, and their distinction from background particles. For the environment, this is further complicated by the challenges of "identifying" nanoparticles in

environmental matrices. A few relatively simple exposure models have been used, however more sophisticated reliable models for predicting exposure to nanoparticles have not been identified. The risk assessment highlights that it is highly recommended to further establish good exposure data for all relevant exposure routes and targets, via measurements as well as to develop validated exposure models. Establishment of exposure data should address the issues related to a proper characterisation. It is also important to further study the interaction of nanoparticles with environmental matrices (e.g. natural organic matter, sediments, etc.), affecting the environmental fate and transport, and thus the exposure for aquatic and soil organisms.

For human health, it seems that the risk of metal and metal oxides is largely driven by the size and therefore surface area of the nanoparticles, and it seems that chemistry may (e.g. silver) or may not (e.g. TiO_2) influence the toxicity, possibly depending (at least partly) on the formation and toxicity of free metal ions. For the carbon-based nanoparticles it seems very relevant in addition to consider the three dimensional structure of the nanoparticle (e.g. fibre-like characteristics), the chemical composition (e.g. impurities from their production) and not least the various surface modifications, which are often added deliberately to promote a certain effect (e.g. increase water solubility). A particular challenge (both in terms of measuring exposure and assessing risks) is introduced by the fact that exposure data often refer to a distribution of particles of different characteristics and different sizes, whereas toxicity tests are often performed for nanoparticles of limited size ranges and of one type. In addition, nanoparticles will often aggregate to agglomerates (both relevant for exposure assessment and toxicity testing) and as evidenced in the risk assessments, it is not always clear what the agglomeration state was in the relevant studies. Even if known it is difficult to make general conclusions on how this will influence the toxicity and therefore the risk.

For the environment, it was not possible to determine an influence of the size or the shape on the ecotoxicity for any of the groups of investigated nanoparticles, although many studies lack particles of different characteristics allowing such comparisons to be made. The effects may however be affected by agglomeration and aggregation, especially at the very high concentrations used in the tests. Toxicity of metals and metal oxides seems to be driven by chemical composition, but the effect of coatings (e.g. in consumer products mostly coated nanomaterials are used) on their toxicity was not sufficiently studied. For example, coating can reduce or block the release of toxic ions from silver nanoparticles thus reducing their toxicity. Moreover, coating and surface functionalisation may improve the metal and metal oxide nanoparticle dispersion stability and hydrophilicity and consequently may increase the possibility of transport over long distances in the environment. The effects of carbon-based nanomaterials on organisms are influenced by functionalisation and the level of impurities (especially in CNTs).

The review considers one of the key issues in nanoparticle safety assessment, namely the prospect and validity of scaling risk information from bulk substances. It is often discussed to which degree the risks of nanoparticles can be assessed based on the toxicity of the bulk/normal substances; i.e. whether the risk of the bulk/normal substances can simply "be scaled" to the nanoform taking into account the smaller size of the particle or whether the small sizes triggers "nano-specific" behaviour/effects. To date no firm conclusion can be drawn which would be applicable to all nanoparticles. However, when considering whether scaling is possible, it seems to be a prerequisite that if the 'chemistry' (at least partly) drives the toxicity, it needs to be the same chemistry in the bulk/normal form as well as in the nanoform before such scaling can be considered. This already introduces some reservations for carbon-based nano-materials, which have surface modifications deliberately added to give them in order to generate specific properties. For more chemically 'inert' particles, it may be possible to draw conclusions on their behaviour and scaled from larger inert particles simply based on the shape. However, this needs further investigation beyond the possibilities in this review.

For human health, there are indications that (some of) the toxic effects of nanosized TiO_2 can simply be scaled based on surface area considerations from the toxicity of the micro-sized TiO_2 . For silver, there is still too little known about the toxico-kinetics to give a fair judgement of this question. For the carbon-based nanomaterials, it does not seem obvious that the

toxicity observed for the nanoforms could be found based on scaling from any normal/bulk state of carbon-materials. These observations should rather be seen as reflections than firm conclusions. For the environment, it is interesting to note that the toxicity seen for nanosized ZnO is indicated by some authors to be related to the release of zinc ions (Zn^{2+}) just as the toxicity of the bulk form of ZnO. Scaling may therefore be possible for this substance. The influence of increased surface area of nano-forms with respect to the bulk form is to be verified on the amount and efficiency of ion leaching. However this does not include coated nanomaterials, which have peculiar properties. Concerning silver nanoparticles, no conclusion can be drawn yet, even if toxicity of silver seems to be related to Ag^+ ions. In conclusion, it seems possible to predict (part of) the toxicity of some nanomaterials based on the toxicity of the bulk/normal form, but this is not possible for all types of nanomaterials.

In **conclusion**, the review's findings strongly supports the further development of thorough characterisation (including proper considerations of agglomeration/aggregation) of the nanoparticles in exposure media when conducting exposure assessment, as well as in the generation of data for determining exposure to both humans and the environment as well as assessing hazardous properties. This is a crucial prerequisite for carrying out a meaningful assessment of the risks. Further testing strategies are required to be established to cover all relevant endpoints needed for a risk assessment. At present, carrying out risk assessment of nanoparticles can only sensibly be done on a case-by-case basis. Only when more data becomes available may it be possible to group nanomaterials according to their physical, chemical and/or biological properties or mode of action, so that testing could be done for representatives of each group.

1 INTRODUCTION

1.1 BACKGROUND

Nanotechnology is a new and fast emerging field that involves the design, production and use of structures at the nano-scale i.e. 1 to 100 nanometres (nm) (BSI 2007). Nanotechnology is a sector of the material manufacturing industry that has already created a multibillion \$US market, and is widely expected to grow to 1 trillion \$US by 2015. Nanoparticles are defined as particles with all three external dimensions in the nanoscale, while nano-objects are discrete pieces of material with one or more external dimensions in the nanoscale, such as nanotubes. These definitions are provided by the British Standards Institute (BSI) (PAS 136, BSI 2007). To put the size of nanoparticles into perspective, a human hair is typically 80000 nm wide, a red blood cell has a diameter of 7-8000 nm, while virus particles are similar in size to many nanoparticles, with maximum dimensions of 10 to 100 nm.

Due to their small size, nanoparticles exhibit novel properties that are often vastly different from their bulk counterparts (larger sized particles with the same chemical composition), such as high tensile strength, low weight, high electrical and thermal conductivity, and unique electronic properties, the discovery of which has led to widespread interest in their potential commercial and industrial applications. Nanoparticles tend to be more reactive than the corresponding conventional forms due to two main properties: i) per unit mass, nanoparticles have a much higher surface area and thus a greater proportion of constituent atoms exposed to the environment on the surface; and ii) quantum effects appear to become more important at the nano-scale, particularly for nanoparticles at sizes of less than 10 nm, resulting in constrained bonds which are more likely to be disrupted.

Many applications of nanotechnology involve the use of both nanoparticles and nano-objects. In fact, numerous nanoparticles are already on the market, in products such as paints, sunscreens, cosmetics, nanomedicines, self-cleaning glass, industrial lubricants, advanced tyres, semiconductors and food. This proliferation of nanotechnology has prompted concerns over the safety of engineered nanoparticles where exposure to humans and/or the environment occurs intentionally or accidentally.

In 2004, the Royal Society and the Royal Academy of Engineering published, at the request of the UK government, a major review of the opportunities and uncertainties of nanotechnologies (RS/RAE 2004). This was one of the first reports to highlight the potential risks to health and the environment that may arise from exposure to nanomaterials, especially nanoparticles (which included nano-objects such as nanotubes). Since then, more than 50 national and international reviews carried out by government departments, industry associations, insurance organisations and researchers have considered nanoparticle risk issues. These reviews have provided a remarkably consistent view about the nature and the potential risks of nanoparticles, which may be summarised as follows:

- There are potential risks to human health and the environment from the manufacture and use of nanoparticles;
- There is a lack of knowledge about what these potential risks might be and how to deal with them;
- The lack of data makes it difficult for manufacturers, suppliers and users to have effective risk management processes and to comply with their regulatory duties;
- All of the stakeholders (regulators, companies) need to start to address these potential risks now.

Since publication of the joint Royal Society and Royal Academy of Engineering report (RS/RAE 2004), there has been a significant increase in research activity in the UK and internationally, intended to fill these gaps. A great deal of emphasis has recently been placed on the need for research in the field of nanoparticle risk assessment, particularly evident in the European Union through the Sixth and Seventh Framework Programme calls, and initiatives elsewhere around the world. Outputs from this research can further contribute to the field's evidence base through the conduct of timely and comprehensive reviews that

assimilate the wealth of scientific data on health and environmental implications of manufactured nanomaterials alongside knowledge of materials' production, application and resulting potential new exposure pathways.

1.2 AIMS AND OBJECTIVES

The overall aim of the ENRHES project was to perform a comprehensive and critical scientific review of the health and environmental safety of four classes of nanomaterials: fullerenes, carbon nanotubes (CNT), metals and metal oxides. The review considers sources, pathways of exposure, the health and environmental outcomes of concern, illustrating the state-of-the-art and identifying knowledge gaps in the field, in order to coalesce the evidence which has emerged to date and inform regulators of the potential risks of engineered nanoparticles in these specific classes.

The specific objectives of the ENRHES project were to review information on:

- production, use and exposure to the target engineered nanomaterials;
- persistence, bioaccumulation, toxicity (i.e. PBT) and interactions of the engineered nanoparticles in living and environmental systems;
- differences in toxicity posed by variations in physico-chemical characteristics.

The final objective of the project was to perform a coherent evaluation of the feasibility of conducting a regulatory risk assessment for each material type and perform basic risk assessments to the extent possible based on the information presented within the review.

The final report provides an overview of the current state of knowledge concerning exposure to nanoparticles and ongoing work in the area. Prioritised recommendations have been developed and set in the context of informing policy makers in the development of methods to address exposure as it relates to the potential hazards posed by engineered nanoparticles, and in the development of appropriate regulation.

1.3 AN OVERVIEW OF THE FOUR NANOMATERIAL CLASSES

The review focusses on four classes of nanomaterials: fullerenes, carbon nanotubes, metal and metal oxides and often uses selected specific nanomaterials within each class as an illustration. In cases where published data from a specific nanomaterial substance is used, the review authors have endeavoured to include available information describing the nature of the substance (surface functionalisation, presence of contaminants, etc.) which may influence the effects being investigated.

Fullerenes are closed cage structures consisting of 60 or more carbon atoms, with each carbon atom bonded to three others. Fullerenes are similar in structure to graphite but contain pentagonal rings in addition to hexagonal rings, which allow the cage to close. Fullerenes are often depicted as discrete particles, but in reality they often crystallise into larger particles. As such, exposure is often to clusters of crystals, termed nano or colloidal fullerenes. Buckminsterfullerene (C_{60}), which consists of 60 carbon atoms arranged in a sphere with a van der Waals diameter of approximately 1 nm, is the most common type of fullerene whose properties and behaviour feature frequently in our review of the fullerenes class of nanomaterials. Surface functionalisation can lead to a wide variety of fullerenes that vary in their physico-chemical properties as well as biological activity. In addition, contamination with solvents, amorphous carbon or other materials may also influence their behaviour.

Carbon nanotubes (CNT) are a form of carbon, first discovered by Iijima (1991), similar in structure to C_{60} but elongated to form tubular structures 1-2 nm in diameter. CNT can be produced with very high aspect ratios (ratio of length and width) and range in length from a few micrometres up to millimetres. There are principally three types of CNT: i) single-walled carbon nanotubes (SWCNT) which consist of a single layer of carbon atoms (single molecule) arranged in a cylinder; ii) double-walled carbon nanotubes (DWCNT) which consist of a two concentric single-walled tubes; and iii) multi-walled carbon nanotubes (MWCNT) which

comprise of multiple stacked single-walled tubes, with diameters up to 20 nm and length greater than 1 mm. As with the fullerenes, surface functionalisation can lead to a wide variety of CNT that vary in their physico-chemical properties as well as biological activity. In addition, contamination with metal catalysts, fullerenes, amorphous carbon or other materials may also influence their behaviour.

Metal and metal oxide nanoparticles are based on a number of diverse elements, including aluminium, chromium, cobalt, copper, iron, magnesium, nickel, palladium, tin, titanium and zinc. However, with regards to research into the environmental, health and safety of these materials, studies to date have largely focused on a number of key substances in relation to their potential applications. For metals, the greatest number of studies have concentrated on silver nanoparticles, largely due to their application in products such as clothing and wound dressings as a result of their unique microbial properties. Studies relating to gold and copper nanoparticles have also shown high prevalence. In terms of metal oxides, titanium dioxide (TiO₂) and zinc oxide (ZnO) nanoparticles have been widely studied due to their high utilisation in products such as sunscreens. Cerium dioxide (CeO₂) and silicon dioxide (SiO₂) nanoparticles have also attracted research attention due to their widespread application. As a result, the properties and behaviour of these metals and metal oxides feature frequently in our review of these classes of nanomaterials. Surface modification/coatings can lead to a wide variety of metal and metal oxide particles that vary in their physico-chemical properties as well as biological activity. Studies on quantum dots and multi-element nanoparticles were outwith the scope of our review and consequently have not been included.

Exposure to nanoparticles depends upon the formulation of nanoparticles during production, their use in products, and their potential release at the end disposal. Nanoparticles may be attached to surfaces (e.g. surface coatings), dispersed in solids (e.g. CNT in plastics), suspended in liquids (e.g. TiO₂ in sunscreen lotion), exist as a powder, or be airborne (e.g. during production of nanoparticles). In this review, we have focused mainly on airborne nanoparticles, powders and nanoparticles suspended in liquids. Furthermore, being a review study, the project only focuses on first generation, passive nanomaterials (materials designed to perform one task), as information on future generation nanomaterials (e.g. active nanostructures for multi-tasking, such as drug delivery devices) is not yet available.

1.4 REVIEW STRUCTURE

Chapter 1 sets out the background, principal objectives and material classes considered in the review. Chapter 2 provides a general background to nanomaterials production and use, and is supplemented by the results obtained from an industry survey. The state of the art in physico-chemical characterisation techniques, exposure assessment, and environmental fate and behaviour are detailed in chapters 3, 4 and 5, respectively. Chapter 6 describes the state-of-the-art of the human toxicity of the four nanoparticle classes, with the epidemiology and data from human studies of carbon black, titanium dioxide and ultrafine particles discussed in chapter 7. Chapter 8 reviews the ecotoxicological literature. Chapter 9 builds on the preceding chapters and presents basic risk assessments, for selected nanomaterials using the available data. Finally, a collation of each chapter's conclusions and recommendations is presented in chapter 10. Appendices describe the methodological approaches employed for the review and industry survey.

The chapters adopt a consistent and systematic approach, structured according to the four material types. Italicised text has been used within sub-sections to summarise findings prior to the chapters' overall conclusions and recommendations.

1.5 REFERENCES

BSI. 2007, *Terminology for nanomaterials*, PAS 136:2007, British Standards Institute, UK.

Iijima, S. 1991, "Helical microtubules of graphitic carbon", *Nature*, vol. 354, pp. 56-58.

RS/RAE. 2004, *Nanoscience and nanotechnologies: Opportunities and uncertainties*, The Royal Society and The Royal Academy of Engineering, London, UK.

2 MATERIALS PRODUCTION AND USE

2.1 INTRODUCTION

The production of nanoscale materials is usually very different to the production of bulk chemicals and materials. Many nanoscale materials are currently being produced solely at the laboratory scale and thus there is a need for scale-up before many applications can be commercialised. Those that are being produced on a commercial scale and which use a manufacturing process that claims to be more “environmentally beneficial” require rigorous life cycle analysis to show that the process gives rise to net benefits over the life of the material or product.

There are a number of techniques for producing the different types of material, all with various degrees of quality, speed and cost. Differing techniques are required for the production of thin films (e.g. chemical vapour deposition), to nanoparticles (e.g. milling, flame pyrolysis, laser ablation), to nanograined materials (e.g. mechanical processing), and carbon nanotubes and fibres (e.g. electro-explosion, electrospinning). All of these techniques are concerned with producing new or enhanced materials either by a “top down” approach, for example by etching to create circuits on the surface of a silicon micro chip, or a “bottom up” approach, such as self-assembly.

One of the significant difficulties in the production of free-standing nanoscale materials is their tendency to agglomerate (a natural process in which particles simply stick loosely together), aggregate (particles/crystals stick irreversibly together) or grow into larger crystal structures. Novel properties exhibited at the nano-scale can be lost due to these processes; however in some cases agglomeration may be desirable for particular applications. New production and metrology methods are required to understand such process and their impact on manufacturing.

The production of carbon nanotubes and graphene is of significant current interest and has a wide range of potential applications. However, due to a lack of understanding of growth mechanisms, and potential environmental susceptibility, selective and uniform production with specific dimensions and uniform properties has yet to be achieved.

Production is one of the biggest challenges that nanotechnology companies currently face. Producing in small quantities is proving to be expensive, and hence, to achieve better margins and obtain economies of scale, the production has to be scaled up. Quality issues and industrial utility are important factors, however with the increase in the scale of production, the manufacturing technology itself matures and the production cycle improves. Mass manufacture is still to be realised by many companies, but there are examples of large scale manufacturing processes in place for materials such as fumed silica and carbon black, both of which are produced in many thousands of tonnes per year by, for example, Evonik (formerly Degussa) and Cabot.

2.2 CARBON FULLERENES

2.2.1 Production methods

Fullerenes spontaneously occur in nature from origins as simple as the flames produced by burning methane, ethylene, and benzene. In the laboratory, they can be produced using two carbon electrodes in a helium or neon atmosphere. An arc is generated between the electrodes, and fullerenes are generated. Carbon soot is also produced in the reaction and can be removed by a solvent. There are issues in the consistency of manufacture of fullerenes, but one bulk production method - combustion synthesis - can produce 95% pure fullerenes (NPL and IoN 2008).

2.2.2 Applications

Chemically-modified carbon fullerenes are being developed for targeted drug delivery. The fullerene encases a tiny dose of a particular drug. By controlling the functionalisation of the fullerene, the drug can remain encased until it reaches the site where the drug is required. Using a chemical “trigger”, the fullerene can then release the drug in location (Vogelson 2001). Another suggested application of fullerenes is to use them as 'molecular ball bearings', allowing surfaces to glide over one another, acting as a lubricant. However, nano-onions or bucky-onions (fullerenes with shells around them) have shown even better performance (Holister *et al.* 2003). Fullerenes with metal atoms attached to them might function as catalysts, increasing the rate of important chemical reactions. It is also known that fullerene compounds with added potassium act as superconductors at very low temperatures (Nakamura and Sawamura 2001). Reinforcing a polymer matrix with fullerenes strengthens and lowers the density of the resulting material. Dupont concentrated on stretchability and tensile strength of modified polymers and Exxon holds several patents on fullerenes applied into polymers.

In medical applications, it is believed that fullerenes could be put to work as nanoscale chemical sponges. For example, following a head injury, free radicals produced in the brain can kill off nerve cells and fullerenes appear able to encase free radicals. This in turn could reduce the damage to the delicate brain tissue (Tagmatarchis and Shinohara 2001).

2.2.3 Market Data

The fullerenes market is worth around \$58.5 million in 2007. The energy sector is the most prominent at present for fullerenes finding their way into fuel cells, solar cells and batteries. This will continue to grow, with significant growth also expected in the information technology and communications (ITC) and automotive sectors (TTC 2008; BCC Research 2007).

2.3 CARBON NANOTUBES

2.3.1 Production Methods

Carbon nanotubes are usually made by carbon-arc discharge, laser ablation of carbon, or chemical vapour deposition. The three main techniques are described below:

- Chemical vapour deposition (CVD) – involves heating up a precursor carbon gas (e.g., methane, carbon monoxide, or acetylene) with a plasma or a heated coil and reacting it with a metallic oxide surface catalyst, like nickel or iron; can be used to make both single- and multi-walled nanotubes, however the multi-walled tubes are of higher quality; can be scaled up for commercial production;
- Arc discharge – involves a “plasma-based process” using a high temperature vapour discharge from one solid carbon electrode to make multi-walled nanotubes on another carbon rod; a metal catalyst is added to create single-walled nanotubes;
- Laser ablation or pulsed-laser vapourisation uses a high-powered laser beam (continuously applied or pulsed) to vapourise powdered graphite with a metal catalyst; only creates single-walled nanotubes; produces smaller quantities than the other two methods, but at a higher purity.

Table 2.1 provides a comparison of nanotube production technologies. The CVD technique is the most commonly used for making nanotubes. Companies such as CNRI, Nanocyl, NanoLab, Nanoamor, and Shenzhen Nanotech use CVD; MER, Nanocarblab, NanoLedge use arc discharge; ILJIN uses both CVD and arc discharge. Large quantities of nanotubes are produced by Mitsui in Japan and in France by Arkema. The production methods have not yet been mastered and thus nanotubes have yet to be produced in mass quantities. Some single-walled carbon nanotube (SWCNT) producers may be moving away from the older methods and using fluidised beds and other high throughput methods, in order to scale production with relatively low costs.

Table 2.1: Comparison of nanotube production technologies (extracted from Garland, 2009).

Properties	Arc Discharge	Laser Ablation	Gas Phase Process	Vapour Deposition
Nanotube generator	Requires high voltage arc discharge	Requires expensive high energy lasers	Catalytic particles	Catalytic particles
Process	Batch	Batch	Continuous	Continuous or Semi Batch
Diameter of SWCNT	1.2-1.4 nm	1.2-1.4 nm	0.8-1.4 nm	0.8-1.4 nm
Length of Nanotubes	1-10 μm	1-10 μm	μm or longer	Up to 20 cm
Yield	~ 50%	~ 70%	50%	~ 97-99%
Quality of Nanotubes	Produces largely defect free nanotubes	Produces largely defect free nanotubes. Considered highest quality nanotubes	Produces SWCNT and MWCNT; hard to separate	Produces tubes with some defects. Number of defects declining
Production Quantities	Could exceed 10 g per day	Less than 1 g per day	In full operation 500-2000 kg per day	Theoretically can produce kg or more per day

Depending on the method of synthesis, impurities in the form of catalyst particles, amorphous carbon, and non-tubular fullerenes are also produced. Thus, subsequent purification steps are required to separate the tubes from other forms of non-tubular carbon. Purification involves chemical processes like acid reflux, filtration, centrifugation, and repeated washes with solvents and water. Typical nanotube diameters range from 0.4 to 3 nm for SWCNT, and from 1.4 to more than 100 nm for MWCNT. It has been established that a nanotubes properties can be tuned by changing its diameter.

SWCNT are presently produced only on a small scale and are extremely expensive. High-purity samples cost about \$750 per gram, and samples containing substantial amounts of impurities cost about \$60 per gram. The past few years have also seen a substantial increase in the number of other companies producing commercial quantities of nanotubes, and the speculative forecasting regarding their possible uses. For example, Nanocyl in Belgium is capable of producing 10 kilograms per day, NanoLedge in France is capable of producing 120 grams per day, Nanothinx in Greece produces 100 grams per day, and Nanocarblab in Russia is capable of producing 3 grams per day (Garland 2009).

2.3.2 Applications

The potential applications and uses of nanotubes are wide and varied, including:

- Carbon nanotube-enhanced plastics (Garland 2009);
- Electromagnetic interference/radio frequency interference (EMI/RFI) shielding (Yang, 2007);
- Antistatic materials (Garland 2009);
- Flexible fibres and advanced polymers, owing to their mechanical properties (Avallone *et al.* 2006);
- Medical and health applications to treat diseases at a cellular level (Wong and Kam, 2005);
- Electrical energy storage and hydrogen storage (An *et al.* 2001);
- Scanning probe microscopy (Rothschild *et al.* 1999).

2.3.3 Market Data

There is a great demand in the market for carbon nanotubes, especially in the electronics and polymers sectors; production and price are restraints at present but this is changing. A kilogram of carbon nanotubes used to cost up to €1000, but now, as a result of targeted research and development activities, companies have managed to significantly lower the price-per-kilogram of nanotube product, thereby enabling the development of new, industrial applications.

In most cases, CNT are used as an additive to add value to existing products or to develop new products such as field emission displays. The advantage as an additive is usually an enhancement of the properties at a low loading of nanotubes. This low loading also offers new possibilities like transparency in coatings.

One of the biggest challenges facing the carbon nanotube producers is the ability to obtain significant quantities of the desired type of carbon nanotube. Expensive, small scale production of nanotubes as well as clumping, lack of binding to the bulk material, and temperature effects are therefore key barriers to their application in the industry (Garland, 2009).

The market for carbon nanotubes was approximately \$168.5 in 2008. The ITC market is likely to see the biggest penetration to 2015, with the performance enhancing properties allowing electronics manufacturers to meet demanding market needs. Their incorporation into the displays market will increase demand by 2010, with a revenue forecast in the ITC market of \$1.096 billion by 2015. While in the longer run, electronics will continue to dominate nanotube applications as broader use in semiconductors occurs, strong opportunities are also expected in CNT-based products using chemical vapour deposition technology (Garland 2009).

In 2006, the WTEC - Carbon Nano Tubes Study Panel concluded that the global production capacity was 69.1 kg hr⁻¹ and 271 tonnes yr⁻¹ (WTEC 2006).

2.4 METALS

Metal (and metal oxide) nanoparticles are of great scientific interest as they often exhibit different properties to those of the bulk material. The properties of many materials change as their size approaches the nanoscale. For example, the higher surface area versus volume ratio with decreasing size of nanoparticles results in increased chemical reactivity compared with the bulk material. A good example of this is gold – it is a non-reactive element at the micro and macroscale, but nanoparticles of gold are used as a catalyst.

2.4.1 Production Techniques

There are several methods to fabricate metal nanoparticles such as combustion synthesis, mechanochemical processing, chemical precipitation, sol-gel processing, chemical vapour deposition, laser ablation; however attrition and pyrolysis are the most commonly used techniques.

The attrition method involves macro- or microscale particles being ground in a ball mill at room temperature for 100 hours, and annealed at 1400°C. Some of the produced particles are in the nanoscale range, and these are separated from the larger particles. This is a “top-down” approach.

In pyrolysis, a vapourous precursor (liquid or gas) is forced through a small opening at high pressure and burned. The resulting solid can be filtered to recover nanoscale oxide particles from by-product gases. This method produces aggregates, rather than individual particles.

However, neither method is particularly efficient. Thermal plasmas are often used to produce metal nanoparticles – powder of micrometer-sized particles can be easily evaporated in the high temperatures of the plasma (up to 1000 K). The nanoparticles are formed upon cooling

while exiting the plasma region. There are several types of thermal plasmas used to produce metal and metal oxide nanoparticles, for example direct current plasma jet, direct current arc plasma and radio frequency (RF) induction plasmas.

In the arc plasma reactors, the energy necessary for evaporation and reaction is provided by an electric arc, which forms between the anode and the cathode. Oxygen quenching is used to rapidly cool the vapours produced by the arc plasma process. This ensures the quality of the nanoparticles produced.

In the RF induction plasma technique, an electromagnetic field is generated by an induction coil and couples to the plasma. The plasma gas does not come in contact with electrodes, thus eliminating possible sources of contamination and allowing the operation of such plasma torches with a wide range of gases. The injected feed droplets remain in the plasma for a very short time; the droplets must be small enough to ensure complete evaporation. The RF plasma method has been used to synthesise different metal nanoparticle materials, carbides and nitrides of titanium and silicon (NPL and IoN 2008).

During sol-gel precipitation-based synthesis particles or gels are formed by hydrolysis-condensation reactions, which involve first hydrolysis of a precursor, followed by polymerisation of these hydrolysed precursors into particles.

Microemulsion methods create nanometer-sized particles by confining inorganic reactions to nanometer-sized aqueous domains that exist within an oil. The drawback of this method is that it produces small reaction volumes, thereby resulting in low production volumes, low yields, and an expensive process.

A number of methods exist for the synthesis of nanoparticles in the gas phase. These include gas chemical vapour condensation, condensation processing, microwave plasma processing and combustion flame synthesis. In these methods the starting materials are vapourised.

Precipitation or co-precipitation can be used if reaction conditions and post-treatment conditions are carefully controlled. Precipitation reactions are among the most common and efficient types of chemical reactions used to produce inorganic materials at industrial scales.

Also it is important to note that carbon blacks are formed by incomplete combustion of a hydrocarbon feedstock such as oil or gas.

2.4.2 Applications

Metal nanoparticles are currently being employed in a wide-range of applications, including:

- Catalysts, particularly in the automotive industry (e.g. ceria in diesel) (Companies include Rhodia Electronics and Catalysis Inc. and INERGY Automotive Systems);
- Wound dressings containing nanoparticles of silver are already on the market and can be used in a number of other anti bacterial products (Panacek *et al.* 2006);
- Metal nanoparticle-enhanced plastics are used in electromagnetic interference/radio frequency interference (EMI/RFI) shielding (Yang *et al.* 2007; Patent IPC8 Class: AB22F500FI - USPC Class: 428546);
- Remediation of polluted land (zero valent iron).

2.4.3 Market Data

In 2007 the total nanoparticle market in general was worth around \$1.6 billion. Nanoposts predicts this market will be worth \$20.5 billion in 2015 (TTC 2008; BCC Research 2007). According to European Commission figures, industrial investment in nanotechnology in Europe lags behind that of the US and Japan. The economic and social benefits of nanotechnology will only be realised when it is incorporated in volume products. This requires its widespread utilisation in industry. There are many companies in Europe (over a third of which are based in Germany) exploring nanotechnology opportunities (Garland 2009) and (NPL and IoN 2008).

Many of these materials are made by sole producers however some nanomaterials are made by 5 or more companies in Europe including cobalt, copper, gold and silver nanoparticles (NPL and IoN 2008).

Silver nanoparticles, one of the most applied types, are discussed in more detail below.

2.4.3.1 Silver nanoparticles

The Project on Emerging Nanotechnologies (PEN) estimates that there at least 235 products available to the public containing nano silver including toothpaste, wound dressings and hair removal products (Garland 2009). Mueller and Nowack (2008) indicated that the primary applications of nanosilver would be textiles, cosmetics, sprays, metal products plastics and paints. Use as food supplement has also been reported commercially (PEN 2009). Examples of nano silver based products on the market are outlined in Table 2.2.

Table 2.2: Applications of silver nanotechnology on the market (Garland, 2009)

Company	Product	Web*
Smith and Nephew	Nanocrystalline silver wound dressing	http://wound.smith-nephew.com/uk/node.asp?NodeId=3774
Dermion Corp	Toothpaste incorporating nanocrystalline silver	-
GECKOLINE Sportswear GmbH	UV cotton textiles with nano silver coating	http://www.geckoline.com/dummy-4.0/textiles-nano-silver-coating.html
JR Nanotech plc	Nanoscale silver coated fabrics for preventing foot odour	http://www.jrnanotech.com/acatalog/More_Info.html
Nanohorizons Inc	Anti-microbial nanosilver for leisure equipment.	http://www.nanohorizons.com/SmartSilver™.html
Axomed S.r.l.	Non-invasive hair removal products	http://www.axomed.com/

* Date of access: 15th October 2009.

Silver production (largely non-nano) in 1999 was estimated at 15.5 million kilograms worldwide, with Mexico and the US leading the list of producers. It is estimated that approximately 2.5 million kilograms of silver in various forms is lost to the environment in the US every year, and that 29% of that amount is released to water and 68% to land. The most prevalent release routes are purportedly from smelting operations, photographic processing supplies, manufacturing of electrical components and wires, coal combustion, electroplating operations, and cloud seeding. NIOSH estimates that 70000 people are exposed to silver in the workplace each year and inhalation is the most important route of exposure (Monica 2008).

Maynard and Michelson (2006) reported that 20% of the nanotechnology enhanced products on the market contained antimicrobial properties (many of which will contain silver nanoparticles) and more recently, it has been estimated that silver nanoparticle production is at the level of 500 tonnes per annum production present reported by Mueller and Nowack (2008).

Studies have investigated the environmental impacts of nanosilver, however predicting the impacts to the environment is complicated (Kulinowski 2008).

2.5 METAL OXIDES

2.5.1 Production techniques

The production techniques for metal oxide nanoparticles are largely the same as those outlined for metal nanoparticles. Companies worldwide are now actively manufacturing metal oxide nanoparticles, which have applications in areas as diverse as cosmetics, coatings, solar cells, and plastics (Lux Research 2008). The key manufacturing companies are outlined in Table 2.3.

Table 2.3: Key companies manufacturing metal oxide nanoparticles (extracted from Lux Research, 2008)

Company	Details
Oxonica	Manufactures Envirox cerium oxide catalyst, which aims to reduce fuel emissions; also developing doped titanium dioxide for plastic stabilisers and sunscreen
Cerion Technology	Formed in 2006 by former members of Kodak's nanomaterials group; producing cerium dioxide nanoparticles for diesel fuel additives
Nanophase Technologies	Manufacturing nanopowder metal oxides, homogeneous mixed metal oxide nanopowders; partnerships with BASF, BYK, Rohm and Haas, Behr, and Cognis
Altair Nanotechnologies	Uses nanoparticles to develop lithium-ion battery electrodes; produces titanium dioxide nanoparticles through a joint venture, AlSher Titania, with Sherwin-Williams
Sokang Nano	Producing titanium dioxide, zinc oxide, and silicon dioxide nanoparticles; seeking foreign investment of \$3 million to \$7 million
Nanogate	Develops tribological (low-friction) and easy-to-clean coatings based on ceramic nanoparticles; offers materials, toll coatings, and licenses to its patents
Evonik	Makes ceramic nanoparticles for paints, coatings, and personal care applications, as well as for materials for advanced lithium-ion batteries
PPG	Has used ceramic nanoparticles to create scratch-resistant automobile coatings and self-cleaning windows; recently acquired NanoProducts

2.5.2 Applications

Metal oxide nanoparticles are used in a wide range of applications, including:

- Nanoparticles attached to textile fibres in order to create smart and functional clothing. Examples of functional materials are water- and stain-repellent textiles (Vigneshwaran *et al.* 2006);
- Microscale titanium dioxide (TiO₂) is used to make white pigments for paints, paper, plastics, and printing inks. However, when the average particle size of TiO₂ is reduced to <100 nm, TiO₂ becomes transparent to visible light and is a strong absorber of UV light;
- The newer transparent sunscreens are a direct result of this size effect (Jaroenworarluck 2006);
- Depending on the angle of incidence of light, nanoparticles of TiO₂ also cause some components of visible light to be reflected and refracted differentially. Several market applications take advantage of these properties, including "metallic" paint formulations, in cosmetics, plastics, and as the active ingredient in self-cleaning coatings (companies include Pilkington Ltd.);
- Metal oxide nanoparticles are also used as pigments in the inkjet and photography industries (companies include Nanosolar Inc. in Germany and the USA);
- Metal oxide nanoparticles are being used in the healthcare industry, with many more potential uses under development (NPL and IoN 2008);

- Nanoparticles of superparamagnetic iron oxide have been used as MRI contrast agents for a number of years (Atanasijevic *et al.* 2006).

2.5.3 Market Data

Many metal oxide nanomaterials are made by sole producers. However, some materials are made by five or more companies in Europe, including cerium oxide, indium tin oxide, tin oxide, titanium dioxide, zinc oxide, zirconium oxide (NPL and IoN 2008).

Titanium dioxide nanoparticles are discussed in more detail below.

2.5.3.1 Titanium dioxide nanoparticles

Titanium dioxide nanoparticles are transparent, able to absorb and reflect UV light, and have found applications in sunscreens. There are a variety of other applications, including:

- self-cleaning exterior surfaces;
- coat glazing, since it has sterilising and anti-fouling properties;
- cementitious products;
- protective coatings possessing anti-bacterial and anti-fungal properties;
- indoor air treatment by visible light catalysis;
- degrading many different pollutants such as nitrous oxides and volatile organic compounds.

Examples of nano titanium dioxide products on the market are outlined in Table 2.4.

A recent study examined trends in nano-TiO₂ production and suggested that global TiO₂ nanoparticle production numbers (5000 MT yr⁻¹ on the low end and 64000 MT yr⁻¹ on the high end (Mueller and Nowack 2008) might be low, as the current U.S. production alone could be as high as 40000 MT yr⁻¹ (Robichaud *et al.* 2009).

Table 2.4: Applications of titanium dioxide nanotechnology on the market

Company	Product	Web*
GfE Medizintechnik GmbH	Nanoscale titanium-coated synthetic implants.	http://www.gfe.com/opencms2/opencms/en_gfe-medical.de/Technology.html
Altair Nanotechnologies	TiNano Spherehollow-microporous titanium dioxide structures	http://www.altairnano.com/
Solaronix	Nanocrystalline titanium oxide for solar cells	http://www.solaronix.ch/products/titania/
Sachtleben Chemie GmbH	Nano titanium dioxides	http://www.sachtleben.de/include/2_12_0_EN.html
Orionsolar Photovoltaics Ltd.	Nanocrystalline titanium oxide for solar cells	http://www.3gsolar.com/what-is-dsc.html
Tayca Corp	Titanium dioxide nanopowder	http://www.urban.ne.jp/home/tokosaku/intoro.html
Chengyin Technology Co., Ltd	Nanostructured titanium dioxide in sunscreen, antimicrobial, antistatic and photocatalysis	http://www.shanghuinano.com/Enx/changin3-11.asp?id=8
Micronisers Pty, Ltd.	Nanoscale titanium dioxide	http://www.micronisers.com/overview.html
Evonik	UV filter for cosmetics	http://www.evonik.com
Kemira	White pigment	http://www.kemira.com/en/solutionsproducts/Pages/SelectSeparationMining.aspx

* Date of access: 16th October 2009

2.6 INDUSTRY SURVEY

As part of the ENRHES project, a survey was carried out to determine the quantities of various types of nanomaterials currently produced and used, as well as the type of products in which they are used. The survey was open from 04/02/2009 to 06/03/2009 and announcements and invitations to participate were sent to industry associations including CEFIC, the CIA, the NIA, ENTA and the Institute of Nanotechnology's database and its media contacts.

Submitted surveys totalled 44, however many of these were incomplete and missing vital data for use. A total of 13 survey responses were deemed to have relevant information. The industry associations had suggested that the survey was very similar to the UK's Voluntary reporting scheme and that many companies were hesitant to provide information. The data gathered from the survey is available to view in full in Appendix 2.

The survey highlighted that many companies do not describe their products generally as nano, preferring often to describe them as ultrafine. The survey data is incomplete with CNT seeming to dominate, which does not relate to volume of product in the market at present or for the foreseeable future i.e silica and carbon black products.

There is therefore concern that at least some of the information requested was company confidential and so what was received was limited and based on product of limited commercial quantity and value. It can be concluded therefore that the survey is not representative of manufacture and use of nanomaterials in the UK, EU or the US.

A distillation of the data is provided below.

The data was provided primarily from commercial organisation < 250 employees, however the response breakdown was as follows:

- 7 respondents from Commercial organisations < 250 employees;
- 3 respondents from Commercial organisations > 250 employees;
- 1 respondent from an Association;
- 2 respondents from Universities.

The respondents are involved in a cross section of business areas including: micro fabrication, standardisation, research and development (R&D) regarding fluid dynamics, research and education, thin film coatings, power cables and flexible pipes, nano medicine, additives of lubricants and fuels, R&D and manufacture of powders for forensic applications, chemistry, specialty chemicals, nanotube and applications and chemicals for the pulp and paper industry.

The organisations are manufacturing or using the following materials:

- Nickel
- Titanium dioxide
- Iron oxide
- Double walled carbon nanotubes
- Silicon Oxide
- Silicon dioxide
- Multi-walled Carbon Nanotubes
- Single-walled Carbon Nanotubes
- Yttrium oxide
- Zirconium oxide
- Nanoclay (aluminium silicon oxide)
- Tungsten disulphide
- Magnetic materials

Engineered Nanoparticles: Review of Health and Environmental Safety

These materials were manufactured in different forms in countries such as Germany, UK, France, USA, Greece and Sweden; imported from UK, Denmark, Italy, USA, France and Japan; and used in Belgium, UK, Romania, Denmark, India, France, USA and Singapore.

The quantities of materials indicated by respondents in the survey were:

- Materials Manufactured or Imported 1-10 kg (Multi-walled Carbon Nanotubes and Single-walled Carbon Nanotubes);
- Materials Manufactured or Imported 10-100 kg (Single-walled Carbon Nanotubes);
- Materials Manufactured or Imported 100-1000 kg (Multi-walled Carbon Nanotubes);
- Materials Manufactured or Imported 1-10 T (Multi-walled Carbon Nanotubes);
- Materials Manufactured or Imported > 1000 T (Silicon dioxide);
- Materials with unidentified usage level (Titanium dioxide, Single-walled Carbon Nanotubes, Yttrium oxide, Zirconium oxide, Multi-walled Carbon Nanotubes);
- Materials used < 1 kg (Double walled carbon nanotubes and Tungsten disulphide)
- Materials used 1-10 kg (Iron oxide and Nano Clay);
- Materials used 10-100 kg (Nickel, Silicon oxide, Silicon dioxide and Magnetic materials);
- Materials used > 1000 T (Iron oxide).

Survey respondents indicated the following Sector of Use categories:

- Other activity related to manufacturing of chemical products (Titanium dioxide, Iron oxide, Zirconium oxide and Yttrium oxide);
- Other activity related to manufacturing of chemical products, Research and Development (Multi-walled Carbon Nanotubes, Single-walled Carbon Nanotubes and Nano Clay);
- Industrial manufacturing (Silicon oxide, Multi-walled Carbon Nanotubes, Tungsten disulphide, and Magnetic materials);
- Manufacture of pulp, paper and paper products (Silicon dioxide);
- Manufacture of textiles, leather, fur (Multi-walled Carbon Nanotubes);
- Printing and reproduction of recorded material (Magnetic materials);
- Manufacture of bulk, large scale chemicals (Iron oxide);
- Manufacture of fine chemicals (Nano Clay and Magnetic materials);
- Chemical formulation and/or re-packaging (Silicon dioxide);
- Manufacture of rubber products (Multi-walled Carbon Nanotubes);
- Manufacture of plastic products, including compounding and conversion (Nickel, Double walled carbon nanotubes and Multi-walled Carbon Nanotubes);
- Manufacture of computer, electronic and optical products, electrical equipment (Silicon dioxide, Multi-walled Carbon Nanotubes and Single-walled Carbon Nanotubes);
- Building and construction work. (Silicon dioxide);
- General Manufacturing (Magnetic Materials);
- Health services (Titanium dioxide and Multi-walled Carbon Nanotubes);
- Private households (general public, consumers) (Titanium dioxide and Silicon dioxide);
- Public domain (administration, education, entertainment, services, craftsmen) (Titanium dioxide);
- Recycling (Titanium dioxide).

Survey respondents indicated the following Process categories:

- Other (Titanium dioxide, Iron oxide, Multi-walled Carbon Nanotubes, Single-walled Carbon Nanotubes, Zirconium oxide and Yttrium oxide);
- Use in closed process, no likelihood of exposure (Multi-walled Carbon Nanotubes);
- Use in closed systems, continuous process with occasional controlled exposure (Multi-walled Carbon Nanotubes);

Engineered Nanoparticles: Review of Health and Environmental Safety

- Used in closed batch process (synthesis or formulation) (Nickel, Silicon dioxide, Multi-walled Carbon Nanotubes, Single-walled Carbon Nanotubes and NanoClay);
- Use in batch and other processes where opportunity for exposure arises (Silicon dioxide, Multi-walled Carbon Nanotubes, Tungsten disulphide and Magnetic materials);
- Mixing or blending in batch processes for formulation of preparation and articles (Silicon dioxide, Multi-walled Carbon Nanotubes, Single-walled Carbon Nanotubes and Magnetic materials);
- Transfer of substance or preparation into small containers (Silicon dioxide and Multi-walled Carbon Nanotubes);
- Roller application or bursting of adhesive and other coating (Silicon dioxide);
- Spraying outside industrial settings and/or applications (Silicon dioxide);
- Use as laboratory reagent (Double walled carbon nanotubes);
- Using materials as fuel sources, limited exposure to unburned product to be expected (Iron oxide);
- Lubrication at high energy conditions in partly open processes (Iron oxide);
- Heat and pressure transfer fluids in dispersive use but closed systems (Magnetic materials);
- Potentially closed processing operations at elevated temperature (Multi-walled Carbon Nanotubes and Single-walled Carbon Nanotubes);
- Open processing and transfer operations at elevated temperatures (Silicon oxide);
- High (mechanical) energy work-up of substances bound in materials and/or articles (Magnetic materials).

Survey respondents indicated the following Product categories:

- Other (Titanium dioxide, Multi-walled Carbon Nanotubes, Single-walled Carbon Nanotubes, Nano Clay, Zirconium oxide and Yttrium oxide);
- Other - Optical Fibers (Silicon oxide);
- Other - Forensics (Silicon dioxide);
- Adhesives, Sealants (Magnetic materials);
- Base metals and alloys (Magnetic materials);
- Coatings, paints, fillers, putties, thinners (Silicon dioxide);
- Building and construction preparations not covered elsewhere (Silicon dioxide);
- Fuels (Iron oxide);
- Metal surface treatment products (Nickel and Tungsten disulphide);
- Non-metal-surface treatment products (Silicon dioxide);
- Heat Transfer Fluids (Magnetic materials);
- Products such ph-regulators, flocculants, precipitants, neutralisation agents, other unspecified (Silicon dioxide);
- Laboratory Chemicals (Multi-walled Carbon Nanotubes and Single-walled Carbon Nanotubes);
- Lubricants, greases, and release products (Iron oxide);
- Metal working fluids (Magnetic materials);
- Pharmaceuticals (Multi Wall Carbon Nanotubes);
- Polymer preparations and compounds (Double wall carbon nanotubes, Multi Wall Carbon Nanotubes and Single Wall Carbon Nanotubes);
- Semiconductor (Multi Wall Carbon Nanotubes, Single Wall Carbon Nanotubes and Magnetic materials).

Survey respondents indicated the following Article categories:

- Other - Polymer chips (Nickel and Iron Oxide);
- Other articles - Fingerprint development powders (Silicon dioxide);
- Other articles (Titanium dioxide, Single Wall Carbon Nanotubes, Nano Clay, Zirconium oxide and Yttrium oxide);
- Passenger cars and motor cycles (Iron oxide, Multi Wall Carbon Nanotubes and Tungsten disulphide);
- Other vehicles (Iron oxide, Multi Wall Carbon Nanotubes and Tungsten disulphide);
- Machinery and mechanical applications thereof (Tungsten disulphide);
- Electrical and electronic products (Silicon dioxide, Multi Wall Carbon Nanotubes and Magnetic materials) ;
- Electrical batteries and accumulators (Double wall carbon nanotubes, Multi Wall Carbon Nanotubes and Single Wall Carbon Nanotubes);
- Electrical and electronic products : household appliances (Multi Wall Carbon Nanotubes);
- Metal Products (Magnetic materials);
- Paper Products (Multi Wall Carbon Nanotubes and Single Wall Carbon Nanotubes);
- Paper products: newspaper and packaging (silicon dioxide);
- Constructional articles and building materials for outdoor use (Titanium dioxide).

Responses to the survey's questions concerning risk assessment and usage of good practice guidelines were:

- EC guideline: 2 organisations using for Titanium dioxide, 2 for Multi Wall Carbon Nanotubes, 2 for Single Wall Carbon Nanotubes, 1 for Zirconium oxide and 1 for Yttrium oxide;
- ISO guidelines: 1 organisation using for Titanium dioxide, 1 for Iron Oxide;
- BSI guidelines: 1 organisation using for Titanium dioxide, 1 organisation using Iron Oxide; 1 organisation using silicon dioxide;
- Good practice guidelines currently under development with 2 organisations for Titanium dioxide, 2 for Silicon dioxide, 2 for Multi Wall Carbon Nanotubes, 2 for Single Wall Carbon Nanotubes, 1 for Zirconium oxide and 1 for Yttrium oxide.

2.7 SUMMARY

Current data suggests that in 2007 the fullerenes market was worth \$58.5 million and the market for carbon nanotubes was approximately \$168.5 in 2008. The market for nanoparticles as a whole was worth around \$1.6 billion in 2007. This market is anticipated to expand further since many nanoscale materials are currently being produced solely at the laboratory scale with further work required for scale-up before many nanomaterial manufacturing techniques and applications can be fully commercialised. For example, carbon nanotubes have a wide range of potential applications, however high volume manufacture for this nanomaterial is still to be realised by many companies.

Once such nanomaterials can be generated in sufficient volumes, there is also a need to integrate the nanomaterial into products or applications, and at present there is a lack of fundamental knowledge regarding the ability to process such nanomaterials. There are also technological challenges in the areas of molecular manufacturing, quality assurance and the eventual programmability of nanodevices.

In terms of developing from raw nanomaterials to usable systems/devices, issues include the stability and durability of materials and reproducibility of the system for mass production. It is necessary to improve both the synthetic control in producing nanomaterials for applications and to improve selectivity in their mode of action. In terms of up scaling to industrial economic processing, the costs of production, when developed, also need to be kept low. Price and cost of the end unit is driven by efficient and effective cheap synthetic chemistry that can be scaled to industrial production. Validation of reliability is also important as this gives a "usable" end product. However there is a problem in correlating limited testing with assurance of total quality. Breakthroughs are expected in uniformity of composition and self assembled systems compositional purity, this relates to better and more consistent end-product performance.

As part of the ENRHES review, a survey was carried out to determine the quantities of various types of nanomaterials produced and used, the type of products in which they are used, any exposure data gathered and risk assessment practices employed. The survey was open from 04/02/2009 to 06/03/2009 and announcements and invitations to participate were sent to industry associations including CEFIC, the CIA, the NIA, ENTA, the Institute of Nanotechnology's database and its media contacts. However many of the responses were incomplete and missing vital data for use. Only 13 survey responses were deemed to have relevant information. The survey data was provided primarily from commercial organisations with less than 250 employees, from a cross section of business areas. The industry associations had suggested that the survey was very similar to the UK's voluntary reporting scheme and that many companies were hesitant to provide information. There was concern that at least some of the information requested was company confidential and so the information received was limited, and was based on products of limited commercial quantity and value. The survey also highlighted that many companies do not describe their products generally as nano, preferring often to describe them as ultrafine.

The survey data does not provide a complete overview of nanomaterial production and use worldwide. Although the survey suggests that CNT dominate the market, this does not reflect the actual predominance of silica and carbon black products currently in the market place.

2.8 REFERENCES

An, K.H., Jeon, K.K., Kim, W.S., Park, Y.S., Lim, S.C., Bae, D.J. and Lee, Y.H. 2001, "Characterization of supercapacitors using singlewalled carbon nanotube electrodes", *Journal of the Korean Physical Society*, vol. 39, pp. S511-S517.

Atanasijevic, T., Shusteff, M., Fam, P. and Jasanoff, A. 2006, "Calcium-sensitive MRI contrast agents based on superparamagnetic iron oxide nanoparticles and calmodulin", *Proceedings of the National Academy of Sciences*, vol. 103, no. 40, pp. 14707–14712.

Avallone, E., Baumeister III, T. and Sadegh, A.M. 2006, *Marks' Standard Handbook for Mechanical Engineers*, 11th edn, McGraw-Hill, New York, US.

BCC Research. 2007, *Nanofibres: Technologies and Developing Market*, Report NAN043A.

Choi, O. and Hu, 7. 2008, "Impact of Silver Nano-particles on Wastewater Treatment", *ACWA Annual Conference Presentation*, Department of Civil and Environmental Engineering, University of Missouri, Columbia, USA.

Garland, A. 2009, "The Global Market for Carbon Nanotubes to 2015: A realistic market assessment", *Nanoposts*. Accessed at: <http://www.nanoposts.com/index.php?mod=nanotubes> (16th October 2009).

Holister, P., Román Vas, C. and Harper, T. 2003, *Fullerenes Technology White Paper nr. 7*, Cientifica Ltd. Accessed at: <http://www.clubofamsterdam.com/contentarticles/01%20Nanotechnology/Fullerenes.pdf> (16th October 2009)

Jaroenworoluck, A., Sunsaneeyametha, W., Kosachan, N., Stevens, R., 2006. Characteristics of silica-coated TiO₂ and its UV absorption for sunscreen cosmetic applications. *Surface and Interface Analysis*, 38 (4), pp. 473-477

Kulinowski, K. 2008, *Environmental Impacts of Nanosilver - An ICON Backgrounder*, ICON. Accessed at: http://icon.rice.edu/resources.cfm?doc_id=12722 (16th October 2009)

Lux Research. 2008, *Nanomaterials State of the Market Q3 2008: Stealth Success, Broad Impact*, LRNI-SMR-08-01, Lux Research, New York, US.

Maynard, A. and Michelson, E. 2006, *Projects on Emerging Technologies*, Woodrow Wilson International Center for Scholars.

Monica, J. 2008, "Nano-Silver EHS Backgrounder", Porter Wright Morris and Arthur LLP. Accessed at: <http://www.nanolawreport.com/2008/07/articles/nanosilver-ehs-backgrounder/> (16th October 2009)

Mueller, N.C. and Nowack, B. 2008, "Exposure Modeling of Engineered Nanoparticles in the Environment", *Environmental Science and Technology*, vol. 42, pp. 4447–4453.

Nakamura, E. and Sawamura, M. 2001, "Chemistry of η⁵-fullerene metal complexes", *Pure and Applied Chemistry*, vol. 73, no. 2, pp 355–359.

National Physical Laboratory and Institute of Nanotechnology (NPL and IoN). 2008, *European Nanotechnology Capabilities Paper*, 21669/08/NL/EM.

Panacek, A., Kvítek, L., Pucek, R., Kolar, M., Vecerova, R., Pizúrova, N., Sharma, V.K., Nevecna, T. and Zboril, R. 2006, "Silver colloid nanoparticles: synthesis, characterization, and their antibacterial activity", *Journal of Physical Chemistry Part B*, 24, vol. 110, no. 33, pp. 16248-53

Patent: SHIELDING BASED ON METALLIC NANOPARTICLE COMPOSITIONS AND DEVICES AND METHODS THEREOF - IPC8 Class: AB22F500FI - USPC Class: 428546
Inventors: Gregory A. Jablonski Michael A. Mastropietro Christopher J. Wargo.

Project on Emerging Nanotechnologies (PEN), 2009. Woodrow Wilson International Center for Scholars. Accessed at: <http://www.nanotechproject.org/> (16th October 2009).

Robichaud, C.O., Uyar, A.E., Darby, M.R., Zucker, L.G. and Wiesner, M.R. 2009, "Estimates of Upper Bounds and Trends in Nano-TiO₂ Production As a Basis for Exposure Assessment", *Environmental Science and Technology*, vol. 43, no. 12, pp. 4227–4233.

Rothschild, A., Cohen, S.R., Tenne, R. 1999, "WS₂ nanotubes as tips in scanning probe microscopy", *Applied Physics Letters*, vol. 75, no. 25, pp. 4025-4027.

Tagmatarchis, N. and Shinohara, H. 2001, "Fullerenes in medicinal chemistry and their biological applications", *Mini Reviews in Medicinal Chemistry*, vol. 1, no. 4, pp. 339-348.

Technology Transfer Centre Ltd. (TTC). 2008, *Nanomaterials and Markets 2008-2015*.

Vigneshwaran, N., Kumar, S., Kathe A., Varadarajan, P., Prasad, V., 2006, "Functional finishing of cotton fabrics using zinc oxide–soluble starch nanocomposites", *Nanotechnology*, vol. 17, pp. 5087-5095

Vogelson, C. 2001, "Advances in drug delivery systems", *Modern Drug Discovery*, vol. 4, no. 4, pp 49–52.

Wong, N, and Kam, S. 2005, "Carbon nanotubes as multifunctional biological transporters and near-infrared agents for selective cancer cell destruction" *Proceedings of the National Academy of Sciences*, vol. 102, no. 33, pp 11600–11605.

Yang, Y., Gupta, M.C., Dudley, K.L., 2007, "Studies on electromagnetic interference shielding characteristics of metal nanoparticle- and carbon nanostructure-filled polymer composites in the Ku-band frequency", *Micro and Nano Letters*, vol. 2, no. 4, pp. 85–89.

3 CHARACTERISATION OF NANOMATERIALS

3.1 INTRODUCTION

Nanoparticle characterisation plays an essential role in a variety of overlapping contexts ranging from fundamental and applied research, through process and product quality control and commercialisation, to health and environmental protection. Fibre-like aerosols present distinct challenges when characterised using many of today's routine measurement techniques that focus on spherical particles. Moreover, not all particles with the same 'apparent' composition have the same potential to cause harm. As with other chemical substances, the importance of, and relationship between, the wide range of physico-chemical characteristics for nanoparticles is an important aspect in understanding their toxicology and remains to be fully elucidated. The implementation of reliable findings from experimental studies into regulatory frameworks with the desired objective of protecting human and environmental health is also subject to the limitations of inadequately characterised materials and the complexity of mixtures of particles in 'real world' exposures.

It is established that nanomaterials exhibit properties and behaviour that can be very different compared to the bulk-scale materials of the same chemical identity. Knowledge of size, shape, and surface-related properties has been used to account for many of the observed differences. It is widely acknowledged that adequate characterisation of a nanomaterial is necessary to accompany any toxicity study. Particularly in cases where nanomaterials (e.g. carbon nanotubes) can be produced by different processes yielding notionally the same material, but which exhibit quite different properties.

Zuin *et al.* (2007), amongst others, highlight that toxicity studies on carbon nanotubes (CNT), fullerenes, metal oxides (e.g. titanium dioxide, iron oxide), silica and quantum dots (QD), require adequately characterised nanomaterials to interpret potential causes of biological effects. They suggest that, ideally, each toxicological assay should be accompanied by a detailed characterisation of all the physico-chemical properties of the investigated material that could have biological relevance.

3.2 CHALLENGES

A number of challenges are associated with realising the needs for nanoparticle characterisation mentioned above. These challenges are now highlighted along with an overview of the state-of-the-art techniques which may be employed for nanomaterial characterisation.

3.2.1 Representativeness of the analyte

Aliquots of nanoparticles used in toxicity studies are generally of a small quantity, but nevertheless should be representative of the sample material. Powers *et al.* (2007) have discussed in detail powder sampling along with some of the common errors associated with sample preparation and how they can be overcome.

Nanoparticle properties in liquid suspensions tend to change with time and surrounding environment. The physico-chemical properties of nanoparticles, as characterised prior to exposure, may change once the particles are added to biological media for toxicity studies. Hence, the need of characterisation at different experimental stages is important to understand the characteristics of nanoparticles at the time of exposure.

The tendency of nanoparticles to agglomerate both in dry and solution media is another significant technical challenge encountered while dealing with nanomaterials. Size, specific surface area, number concentration and size distribution, which at this time are still some of the key parameters that are important in nanotoxicity assessment, are modified as the particles agglomerate (see for example Borm *et al.* 2006 and Teeguarden *et al.* 2007). This may call into question the inference of potential distinct hazards posed by nanoparticles based on knowledge from non-nano forms of the materials.

3.2.2 When and where to measure?

Whilst characterisation of nanomaterials as-produced or as-supplied is the most direct and currently realistic approach to obtaining physico-chemical information about the material being studied, this data may not appropriately represent the properties of the material when in contact with the environment in which it is being observed, for example in air or physiological environments of *in vivo* or *in vitro* assays. Exclusive reliance on pre-determined (often estimated) characterisation parameters will limit the comparability of studies and confidence in the interpretation of results.

Characterising nanomaterials before and after administration in an experimental system provides the highest quality of data on dose and material properties that are related to observed responses. Characterisation after administration is particularly advantageous where the possibility of physico-chemical changes in the material before and after administration exists. Examples of potential changes include aggregation, physisorption or chemisorption of biomolecules and changes in surface chemistry. While characterisation after administration is considered a goal to work towards, it is recognised that in many cases, improved characterisation at the point of administration will be the more realistic and feasible option in the shorter term. It is recognised that in many cases characterisation at the point of administration will remain to be essential for the comparison of studies.

As highlighted by Powers *et al.* (2006), the most challenging time at which to characterise a nanomaterial is at the point(s) of interaction with the organism. At present, this requires invasive techniques which usually cannot be used without compromising the integrity of the organism and possibly invalidating the test.

Whilst many of the techniques described in more detail below can be applied to the characterisation of nanomaterials after administration, a significant challenge here is that concentration levels are usually very low and may be below the technique's limit of detection. Spatially-resolved analysis is common in materials science, but the presence of complex biological matrices renders this task more demanding for both detecting and quantifying the actual concentration in specific tissues or cell components.

Prior to the realisation of non-invasive techniques with high sensitivity and high specificity, *in situ* characterisation of nanomaterials in systems beyond the cellular level are indirect and require post-exposure tissue processing and analysis.

3.2.3 What to measure?

It is often the case that the property or metric of interest cannot be measured directly (or completely) because of limitations in existing analytical or detection methods. It is also frequently the case that no single technique provides adequate information to completely characterise and support the quality control, exposure, toxicity and risk assessments for any given material.

To determine what should be measured when evaluating the risks from nanoparticles, it is important to first consider which particle attributes are likely to influence the impacts.

From an aerosol exposure assessment perspective, measuring particle mass concentration in the workplace and environment is the most frequently employed approach. However, such conventional pump-based filter sampling is not the best solution for exposure assessment of nanoparticles as the mass concentration is not necessarily well-suited to the toxicity assessment of inhaled nanoparticles and the existing devices used for monitoring are designed for PM₁₀, PM_{2.5} and PM₁ size fractions and do not give specific information about particle concentrations below 1 µm aerodynamic diameter. Other indicators are emerging for characterising nanoparticle aerosols, including particle number and surface area, in addition to mass concentration, as well as metrics relating to particle shape. Instrumentation is available or being developed for these metrics and is described in later sections of this chapter and elsewhere in the literature (e.g. Kaluza *et al.* 2009; BSI PD 6699-1, Tantra and Cumpson, 2007).

From a hazard assessment perspective, physico-chemical characterisation features in the screening strategy for the hazard identification of engineered nanomaterials published in 2005 by the expert working group convened by the International Life Sciences Institute Research Foundation/Risk Science Institute (Oberdorster *et al.* 2005). The physico-chemical properties considered to be important in understanding the toxic effects of test materials include particle size and size distribution, agglomeration state, shape, crystal structure, chemical composition, surface area, surface chemistry, surface charge, and porosity.

The need for enhanced efforts in characterisation featured in a subsequent workshop convened by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), in November 2005, to develop testing strategies to establish the safety of nanomaterials. It was concluded that although many physico-chemical factors can influence toxicity, including nanoparticle composition, its dissolution, surface area and characteristics, size, size distribution, and shape that largely determine the functional, toxicological and environmental impact of nanomaterials (Warheit *et al.* 2007).

The Testing Programme of OECD Working Party on Manufactured Nanomaterials (OECD 2008) specifies a non-prioritised list of physico-chemical properties and material characterisation endpoints within phase one of the programme. These are: agglomeration/aggregation, water solubility, crystalline phase, dustiness, crystallite size, representative TEM picture(s), particle size distribution, specific surface area, zeta potential (surface charge), surface chemistry (where appropriate), photocatalytic activity, pour density, porosity, octanol-water partition coefficient (where relevant), redox potential and radical formation potential.

Exhaustive lists of ideal physico-chemical properties to characterise are often suggested. The statement of a hypothesis is often required to identify and prioritise to a set of relevant and realistic characteristics. Warheit (2008) acknowledges that recommendations of ideal physico-chemical characteristics become “laundry lists” without adequate prioritisation.

Other workshops and publications considering nanomaterial characterisation (Aitken *et al.* 2008; Stone *et al.* 2009) have developed from the early proposed strategies and sought to prioritise the physico-chemical characteristics considered most relevant to toxicology and environmental studies, on the basis of current knowledge.

The most recent strategic development is that of the Minimum Information for Nanomaterials Characterisation Initiative (<http://characterizationmatters.org>, accessed 16th October 2009) which seeks to develop and sustain a “community from the bottom up that is interested in supporting effective nanomaterial characterisation for toxicology studies”. The highest priority physico-chemical properties recommended under the MinChar Initiative are particle size / size distribution, agglomeration / aggregation, purity, surface area, surface chemistry, composition, shape, solubility, stability and surface charge.

From the perspective of exposure measurement, Maynard and Aitken (2007) suggested a possible approach to simplify the task of identifying and prioritising measurement requirements, by considering separately particle attributes potentially associated with biological response following inhalation, and measurable quantities that are related to these attributes. They acknowledge that this is not a new idea, but given the complexity of engineered nanomaterials, it is valuable to return to first principles and systematically work through how this approach may enable the development of a viable framework within which to reach a consensus, develop and compare nanoparticle characterisation. The principal advantage is that this approach enables the inherent complexity of characterising engineered nanomaterials to be reduced to a smaller, more manageable set of measurement challenges. It also enables a measurement framework to be developed that has applicability beyond current passive engineered nanomaterials, and onto second and even third generation engineered nanomaterials.

In a review of published dose–response data on acute lung inflammation in rats and mice after instillation of titanium dioxide particles or six types of carbon nanoparticles, Wittmaack *et al.* (2007) explored a number of dose metrics and concluded that the physico-chemical

characterisation of nanoparticles and the methods to determine surface toxicity had to be improved significantly before the appropriate dose metric for lung inflammation could be identified safely.

Maynard and Aitken (2007) proposed a comprehensive, but not necessarily conclusive, set of attributes potentially associated with mechanisms leading to nanostructured particle toxicity following inhalation. Many of these draw from the discussion of key characteristics suggested by Oberdorster *et al.* (2005). Particle size does not appear explicitly, but rather is implicitly assumed to be relevant to each particle class and attribute suggested. Maynard and Aitken made a subjective judgment of the relevance of four physical metrics - particle number, length, surface area and mass concentration for each of their proposed particle class-attribute combinations. These assessments are made assuming that something is known about the size, the size range and/or the size distribution of the particles being monitored. For each particle class/attribute combination, the relevance of a given exposure metric has been evaluated as none, low, medium or high, depending on whether the attribute is thought to be applicable to the given particle class.

It is important to note that all characterisation methods are subject to a variety of limitations and are required to be used by trained persons with sufficient skills to interpret the data and identify spurious outputs. The use of multiple techniques may often be needed to construct a more complete characterisation of the material, but also brings the potential complexity of interpreting dissimilar data for the same characteristic, gathered using instruments based on completely different physical principles.

3.3 ATTRIBUTE-FOCUSED OVERVIEW OF CHARACTERISATION TECHNIQUES

A range of techniques have been adapted or developed for the characterisation of nanomaterials, including microscopic, spectroscopic, spectrometric and chromatographic techniques. Overviews of these instrumental techniques are available in, for example, Ruzer and Harley (2005), Vincent (1995) and Zhang *et al.* (2009). The selection of an appropriate technique depends on the type of material, the required characterisation and the resolution/quality of the data needed.

A number of reviews have been published, covering the principal techniques being applied to the characterisation of nanomaterials. Scott (2003) addresses the basis of characterisation in the context of material manufacture. Examples of recent technological advances include photonic, ultrasonic and electrosonic measurement of particle size, tomographic determination of concentration and multiphase flow, optical trapping and atomic force microscopy techniques for measuring inter-particle forces, and in-line imaging techniques combined with image analysis to measure shape factors. Examples are also provided on how some of these techniques have been implemented in on-line and in-line applications in industry.

Ju-Nam and Lead (2008) highlight previously published reviews and provide a brief account of the large number of parameters which must be measured, including size and size distribution, specific surface area, shape and chemistry.

A special issue of nine reviews and articles in *Microscopy Research and Technique* (Mao 2004) addresses the role microscopy has in characterising nanostructural features and probing chemical or physical processes. The techniques described include transmission electron microscopy (TEM), scanning electron microscopy (SEM), scanning probe microscopy (SPM), atomic force microscopy (AFM) and scanning near-field optical microscopy (SNOM).

Two comprehensive reviews, providing tabulated information on a range of techniques, are those of Hasselov *et al.* (2008) and Tiede *et al.* (2008).

Hasselov *et al.* (2008) discuss methodological aspects in relation to the fields of nanometrology, particle size analysis and analytical chemistry. Differences in both the type of size measures (length, radius, aspect ratio, etc.), and the type of average or distributions

afforded by the specific measures are compared. The strengths of single particle methods, such as electron microscopy and atomic force microscopy, with respect to imaging, shape determinations and application to particle process studies are discussed, together with their limitations in terms of counting statistics and sample preparation. Methods based on the measurement of particle populations are discussed in terms of their quantitative analyses, but the necessity of knowing their limitations in size range and concentration range is also considered. The advantage of combining complementary methods is highlighted and a series of tables depict concisely which techniques are applicable to characterising a range of physico-chemical properties.

Tiede *et al.* (2008) provide a comprehensive review of the different analytical techniques available for the detection as well as physical and chemical characterisation in product formulations, environmental matrices and food materials. As limited work has been done to date on the detection and characterisation of engineered nanoparticles in food, they acknowledge that the review draws heavily on studies reporting characterisation of nanoparticles in raw products and environmental matrices where much more information is available. Possible future directions of nanoparticle analysis and characterisation in biological, environmental or food samples are identified and areas of further research are recommended.

Most recently, Rao (2009) uses case studies of gold, rhenium trioxide and gallium nitride nanocrystals; zinc oxide, nickel, and cobalt nanowires; inorganic and carbon nanotubes; and two-dimensional graphene, in the course of reviewing the application of electron microscopy and scanning probe microscopes, in addition to standard techniques such as X-ray and neutron diffraction, X-ray scattering, and various spectroscopies.

A summary of the techniques applicable to the aforementioned key physico-chemical properties for nanoparticles, adapted from Zuin *et al.* (2007) and enhanced by consideration of additional literature, is provided in Table 3.1. An overview of the principal techniques applied to the characterisation of the key nanomaterial physico-chemical properties, is presented below.

Table 3.1 Overview of techniques for the characterisation of key physico-chemical properties of nanoparticles (adapted from Zuin *et al.* 2007)

Technique / Instrument	Nanoparticle attribute										
	Mass	Number	Size distribution	Shape	Aggregation state	Surface area	Chemical composition	Purity	Surface chemistry	Surface charge	Crystal structure
Scanning Electron Microscopy		✓ A B bm	✓ A B bm	✓✓ A B bm	✓✓ A B bm						✓ A
Transmission Electron Microscopy		✓ A B bm	✓ A B bm	✓✓ A B bm	✓✓ A B bm						✓ A
Scanning Probe Microscopy (AFM, STM)		✓ A B bm	✓ A B bm	✓✓ A B bm	✓✓ A B bm						
Tapered Element Oscillating Microbalance (TEOM)	✓✓ A										
Differential Interference Contrast Microscopy Confocal Laser Scanning Microscopy Fluorescence Microscopy			bm	bm	bm						
Differential Mobility Analyser Condensation Particle Counter		✓✓ A	A								
Dynamic Light Scattering			✓✓ A B								
Scanning Mobility Particle Sizer Electrical Low Pressure Impactors	✓ A	✓✓ A	✓✓ A			✓ A					
Epiphaniometer (diffusion charging)						✓ A					
BET Adsorption Measurement			✓			✓✓					
Zeta Potential Analysis										✓✓ B	
Thermogravimetric Analysis Differential Scanning Calorimetry							✓	✓	✓		✓
Flow Field Flow Fractionation			✓✓ B bm		✓ B bm		✓ B bm		✓ B bm		
X-ray Diffraction							✓				✓✓ A
Auger Electron Spectroscopy							✓✓		✓		
X-ray Photoelectron Spectroscopy							✓	✓	✓✓		
FT-IR Spectroscopy							✓	✓	✓		✓
UV-vis Spectroscopy			✓				✓ B	✓	✓✓		
Raman Spectroscopy							✓	✓	✓		✓
NMR Spectroscopy					✓✓		✓✓		✓✓		✓
Electron Spin Resonance							✓ B bm		✓ bm		✓ bm
Aerosol TOF-MS		✓ A	✓ A				✓ A	A			
Secondary Ion Mass Spectrometry							✓✓ bm				
ICP-MS Atomic Absorption / Optical Emission Spectroscopy							✓✓ A B bm				
High Performance Liquid Chromatography Gel Permeation Chromatography			✓ B bm				✓ bm		✓ bm		

Highly applicable (✓✓) or applicable (✓) for characterising **as-supplied** nanoparticles; applicable for characterising **administered** nanoparticles as a suspension in aerosol (A) or in biological fluid (B); applicable for **after-administration** characterisation in biological matrices (bm).

3.3.1 Number concentration

The principal technique used to provide a direct measurement of particle number concentration in air is based on single particle light scattering (see, for example, Ruzer and Harley (2005) and Vincent (1995)). The most widely used type of instrument for detecting and counting nanoparticle aerosols is the Condensation Particle Counter (CPC). CPCs operate by condensing vapour onto the sampled particles, growing them to a size range which can be detected optically. In this way particle number concentration is measured but not particle size. The detection range typically goes from ~3 nm to ~1000 nm, depending on the instrument.

Off-line estimates of particle number can be obtained from image processing with microscopy techniques, discussed later, including SEM or TEM. Such image processing may also provide information on size, shape, structure, and in some cases, compositional information from particles.

Instruments are available which provide information on size-selective particle number concentration. The most commonly used instrument of this type is the Scanning Mobility Particle Sizer (SMPS), which is effectively an instrument combining a Differential Mobility Analyser with a CPC. Devices of this type are capable of measuring aerosol size distribution from approximately ~3-800 nm. A disadvantage of the SMPS is that it is relatively slow and requires a scanning approach to measure different size intervals in series (taking several minutes). The Fast Mobility Particle Sizer (FMPS) partially resolves this issue by allowing measurements to be made with a time resolution of one second or less. However, the FMPS is typically less sensitive than the SMPS at low particle concentrations.

Size-selective analysis of number concentration can also be achieved using an Electrical Low Pressure Impactor (ELPI), covering a size range of 3-50 nm, and giving an online measurement of particle number concentration, by calculation from the particle charge and active surface area. This instrument allows size fractions of particles to be collected for subsequent analysis.

3.3.2 Size

There are many available techniques for detecting and accurately characterising the size, the aggregation state, and the stability of nanoparticles in liquid media. Powers *et al.* (2007) identified guidelines for selecting and conducting these measurements, which are available from a variety of sources, including national and international standards organisations including the International Standards Organisation (ISO) and American Society for Testing and Materials (ASTM). The key to meaningful determination of the primary size distribution of a sample is a well-dispersed system and measurement of a sufficient number of particles to achieve statistical reliability. The most commonly employed techniques are based on dynamic light scattering (DLS), microscopy (e.g. SEM, TEM), and Brunauer-Emmett-Teller (BET) adsorption isotherm (also used for surface area measurement). Other more specialised and less frequently used techniques include x-ray and neutron diffraction techniques, differential mobility analysis and time-of-flight mass spectrometry. Field flow fractionation (FFF) is a chromatographic technique which can in principle separate nanoparticles in complex mixtures with no regard to their chemical composition according to different mobility induced by a force (electric, thermal, gravitational, or flow, depending on the instrumental configuration) orthogonal to a main laminar flow. This non-destructive technique also allows the collection of separated fractions of the sample for further characterisation. Field flow fractionation can be used and coupled online with multi-angle light scattering (MALS) and UV-Vis spectrophotometry to measure respectively the size and concentration of nanoparticles. For some specific nanoparticles, such as gold, ultraviolet-visible spectroscopy (UV-Vis) spectra can also give very accurate information on the size distribution and their aggregation.

For nanoparticles in aqueous media, DLS is one of the most commonly employed techniques, providing characterisation information on size and size distribution, of particular benefit to toxicity assessment as it measures size in solutions that resemble more to the exposure conditions. However, the technique may be of limited use when particles are difficult to maintain in a dispersed state. The size obtained by DLS is usually greater than that measured

by microscopy or BET techniques. DLS can provide an indication of the particle suspension stability with respect to time and medium. Dhawan *et al.* (2009) highlighted a recent study revealing the utility of DLS in studying the dependence of state of dispersion, exposure medium, presence of serum, time between sample preparation and exposure etc. on *in vitro* toxicity assessment of nanoparticles (Murdock *et al.* 2008). In addition, it provides the value of zeta potential in different solutions and under different conditions of pH and ionic strength. Dhawan concluded that DLS methodology is less time-consuming and cost-effective for giving an ensemble measurement of particles and provides better statistics. The major limitation of these DLS techniques is that they cannot discriminate agglomerates of nanoparticle from individual, larger particles.

For dry powders, differential mobility analysis can be used for dispersed material, or the BET adsorption isotherm can provide an estimated average size based on a nonporous spherical model. The latter method has the added advantage of providing a direct measurement of specific surface area and micro- or meso-porosity, both of which are key properties of interest.

Microscopy is one of the most powerful techniques and is often relied on exclusively to provide information regarding size, shape, and morphology. For nanoparticles, electron microscopy (most typically SEM and TEM) is normally required to capture images with the necessary resolution, and it is the only technique that provides reliable information regarding shape at this scale.

TEM is a method for producing magnified images or diffraction patterns by passing an electron beam through a solid sample in a high vacuum environment. TEM typically requires samples to be less than 100 to 200 nm thick if internal details are required. Thicker samples may be viewed with higher energies. The resolution is typically in the range of 0.5-3 nm, with the technique capable of imaging lattice planes and individual row of atoms with resolutions better than 0.2 nm. Some methods use additional detectors, such as an energy dispersive detector or electron energy loss spectrometer, that allow the composition of the sample to be determined with spatial resolutions below 10 nm.

Conventionally, electron microscopies analyse the sample in dry solid form and not in suspensions. Drying samples under vacuum may alter the size and shape of the particles being characterised. With SEM, where samples are not electrically conducting, plasma sputter-coating the surface-adhered particles with a layer of a conducting material (usually gold, a few nanometers thick) is often required. This process may modify the sample being characterised. The analysis of particles in solution has been advanced through the development of Environmental Scanning Electron Microscopy. This offers the potential for dispersed samples prepared for exposure experiments to be characterised as well as limiting the need to dry samples which may influence the observed size distribution.

The quality of the images to be analysed is of critical importance and it should also be noted that electron microscopy normally provides only two-dimensional images, so care must be taken to avoid bias introduced by orientation effects. High-resolution microscopy may be subject to artefacts caused by sample preparation or special analysis conditions. For example, TEM, which requires high vacuum and thin sample sections to enable the electron beam to penetrate through the sample. When applied to the characterisation of nanoparticles in tissue, sample preservation, fixation, and staining require skill to preserve detail and avoid introduction of artefacts.

3.3.3 Shape

Nanoparticles can exhibit various shape and structures, such as spheres, needles, tubes, rods, platelets, etc. Shape is important as it is the variation of the hydrodynamic radius between spherical particles and oblong ones (larger for the latter) with the same mass, which triggers a variation in their mobility and diffusion in both gas and liquid phases. The second effect is that the shape influences the deposition and adsorption kinetics in biological media.

The shape of nanoparticles is usually determined by electron microscopy, which includes many qualitative and semi-quantitative techniques to investigate the morphology (size and shape) and

also the aggregation state. Other microscopy techniques are also available for the determination of size and shape, as well as the aggregation state, such as scanning probe microscopy (SPM). SPM includes both atomic force microscopy and scanning tunnelling microscopy (STM), which are all based, with some minor modifications, on a scanning probe (called the tip), which is moved across a substrate where particles have been deposited. SPM techniques allow individual nanoparticles and aggregates to be profiled in three dimensions, while SEM and TEM can measure only two dimensions. Similarly to SEM and TEM, AFM data can provide quantitative information about the size distribution with a software-based image processing, even if limited to a small sample surface. An advantage over TEM is that both liquids and solids can be analysed and the images can be measured in all environments. STM images give directly the three-dimensional morphology of complex samples such as CNT and can resolve simultaneously both their atomic structure and the electronic density. As for TEM and SEM, the deposition process is mainly responsible for the overall result, and many fluctuations can occur in the obtained size distribution.

3.3.4 Aggregation state

Nanoparticle aggregation can occur in solution, powder form, and gas phase, depending on the size, chemical composition and surface charge. Aggregation can also depend on the production, storage and handling conditions. For example, carbon nanotubes can be present as complex aggregates of ropes and bundles even just after their synthesis by gas-phase reaction. The aggregation affects the stability of nanoparticle dispersions prepared for toxicological experiments.

Powers *et al.* (2006) highlights that the state of dispersion is typically estimated using comparative particle size measurements. This requires a reliable method of measuring the baseline primary particle size distribution or fully dispersed size distribution of the material. Shaking, sonication, and/or surfactants are commonly used to disperse nanoparticles in solution. These are acceptable tools to probe as-received powders, but they may damage cells and interfere with toxicity testing if used in living systems. Dispersion of dry aerosol nanoparticles can be problematic, and it is often not possible to achieve a fully re-dispersed dry aerosol once a dry nanoparticle material has aggregated. By comparing changes in particle size distribution to that of an ideal dispersion, a qualitative assessment or proxy measure of the degree of agglomeration can be made. One such method, the average agglomeration number (AAN), is derived from the ratio of the volume based median particle size to the average equivalent spherical volume derived from BET gas adsorption (Hackley and Ferraris 2001).

3.3.5 Surface area

As previously highlighted, surface area is recognised as an important nanoparticle property from a toxicological perspective. Reduction in size to the nanoscale level causes a steady increase of the surface/volume ratio, and therefore a greater percentage of atoms to be displayed on the surface rather than in the inner bulk lattice, with an increased potential for biological interaction.

Measurement of surface area by gas adsorption is a high vacuum method and requires a clean, dry sample of the nanomaterial. Nitrogen is the most common adsorbate, although many other gases such as argon, carbon dioxide, or krypton are also used. The BET technique involves measuring the amount of adsorbate released on vaporisation. The BET surface represents the surface area that is freely accessible to gases. The primary particle diameter (assumed as equivalent sphere diameter) is subsequently calculated from already available specific surface area and density of particles. Although this method provides measurement of two parameters simultaneously, i.e. size as well as surface area, the drawback of this procedure is in the assumption of a mono-dispersed spherical system which reports only an average size and does not provide the size distribution.

One instrument which has been successfully used to measure aerosol surface area directly is the Epiphaniometer. In this device the aerosol is passed through a charging chamber where lead isotopes created from a decaying actinium source attached to the particle surfaces. The particles are transported through a capillary to a collecting filter, where the amount of

radioactivity measured is proportional to the particle surface area. While the epiphaniometer has been used successfully for monitoring environmental aerosols (Shi *et al.* 2001), it has not been used widely in assessing aerosol exposure, possibly due to its use of a radioactive source, and its complexity of use.

Emerging methods such as diffusion charging have begun to provide a more viable approach to measuring aerosol surface area in situ. Recently developed nanoparticle surface area monitors measure the surface area of particles (reported as $\text{mm}^2 \text{cm}^{-3}$) deposited in the lung. Sampled particles are charged, collected in an electrically isolated filter and the charge rate measured. These new devices are an important addition to the range of instruments available to characterise nanoparticles. The implications of a number of issues, however, remain to be considered including the effect of initial aerosol charge, the composition of the material, presence of aggregates and the effect of particle shape. The advantages and disadvantages of measuring deposited particle surface area, rather than aerosol surface area, also need to be considered further.

3.3.6 Chemical composition

The chemical composition, in terms of elemental composition and chemical structure, is an intrinsic property of all materials and it is consequently an important parameter influencing the behaviour of nanoparticles. Nanoparticles can have very different chemical compositions, from completely inorganic, e.g. metals (iron, nickel, zinc, titanium, gold, silver, palladium, iridium, and platinum), and metal oxides (titanium oxide, zinc oxide, silica, iron oxide, etc.), to entirely organic (fullerenes, CNT, nanopolymers, biomolecules). Chemical purity is an important chemical parameter to be taken into consideration, since some nanoparticles (e.g. CNT) may contain metal impurities, such as Fe, Ni, Co, which could interfere with toxicity assessments.

Electron spectroscopy for chemical analysis (ESCA), X-ray photoelectron spectroscopy (XPS) and secondary ion mass spectroscopy (SIMS), in particular, have been extensively used for characterising the chemical composition of nanoparticles. In addition, several spectroscopic techniques including inductively coupled plasma (ICP) techniques, X-ray diffraction (XRD), nuclear magnetic resonance (NMR), UV-Vis and fluorescence, can be used to provide specific chemical composition information.

XPS is a non-destructive technique well suited for the investigation of surface elemental composition of nanomaterials and is currently the most widely used surface-analysis technique. It provides detailed qualitative information about chemical elements on the surface and their chemical environment, such as coating type and coverage extent, binding states and oxidation states. XPS requires vacuum conditions to maintain the surface free from contamination, so it cannot be directly applied to liquid samples. It is usually applied to the surface characterisation of metallic nanoparticles, such as aluminum, nickel, and gold, CNT, and core-shell structures.

Differential scanning calorimetry (DSC) can give qualitative and quantitative information about physical and chemical changes that involve endothermic and exothermic processes, while thermogravimetric analysis (TGA) can provide information regarding the presence of volatile contaminants or labile components in the nanoparticle measuring changes in weight of a sample with increasing temperature.

Combining an inductively-coupled plasma with the selectivity and sensitivity of optical emission spectrophotometry (OES) or mass spectrometry (MS), provide well-suited techniques for detailed analysis of the main components, as well as trace impurities. The fundamental prerequisite is a complete dissolution of the sample prior to analysis. It cannot provide information about the chemical structure of the investigated material, and it offers a relatively low sensitivity with regard to some lighter elements. Elemental analysis is in principle more appropriate for organic nanoparticles, but the high carbon content and the relatively high thermal stability of some nanoparticles, such as fullerenes and carbon nanotubes, could be in excess of the instrument's oxidation capabilities.

The aerosol time-of-flight mass spectrometry (ATOF-MS), now a commercially available technique, allows detailed chemical analysis of nanoparticles in aerosol form, also subdividing

the analysed particles into size, with results similar to those attainable by FFF coupled with TOF-MS.

Electron paramagnetic resonance (EPR) and electron spin resonance (ESR) spectroscopies are versatile, nondestructive, analytical qualitative and quantitative techniques that can provide very valuable structural information of investigated material, such as defects in crystals and magnetic properties, but it is mainly applied for the determination of paramagnetic elements and free radicals. They have been applied to the characterisation of metallic nanoparticles such as gold, palladium, nickel, iron, zinc oxide, and titanium dioxide. ESR can also be applied to the characterisation of magnetic nanoparticles, such as Fe₃O₄.

Fourier transform infrared spectroscopy (FTIR) and Raman Spectroscopy (RS) can provide qualitative information about organic structures from analysis of the analyte's vibrational bond energies. FTIR is widely used for the characterisation of fullerene and carbon nanotubes, metals and metal oxide nanoparticles such as gold and zinc oxide. The Raman Spectroscopy is a fast and non-destructive method to investigate the phase changes (amorphous or crystalline), the size variations, and the lattice stress. Structural information and interfacial characteristics of CNT can be determined.

An emerging technique for high throughput single nanoparticle analysis of surface-enhanced resonant Raman scattering (SERRS) tags uses flow spectroscopy capable of analysing hundreds of nanoparticles per second. By measuring Rayleigh and Raman scattering from thousands of individual tags, tag preparations can be characterised based on their brightness and uniformity. Early publications suggest that the rapid analysis of individual nanoparticles using high spectral resolution flow spectroscopy will be useful in many areas of nano-engineering (Sebba *et al.* 2009).

NMR spectroscopy is a non-destructive technique used to investigate the surface and bulk features of chemicals, the electronic structure and the surrounding electronic environment of specific elements, so allowing the accurate identification of individual atom positions in a given chemical structure. The technique can be used for organic and inorganic nanoparticles, either in solid state or as a dispersion in solution. NMR is also being used increasingly to image non-invasively the bio-distribution of nanomaterials *in vivo*. For example, Faraj *et al.* (2009) using a combination of helium-3 and proton magnetic resonance imaging in a rat model to evaluate the bio-distribution and biological impact of raw SWCNT and super-purified SWCNT.

Auger electron spectroscopy (AES) can be used to examine elemental compositions of surfaces, providing compositional information for nanoparticles such as carbon nanotubes and metal oxides (e.g. TiO₂).

TEM can also provide additional crystallographic information, such as the surface atoms arrangement and defects at atomic scale. If the energy dispersive spectrometry (EDS) option is applied, detailed information on the chemical composition of the sample surface can also be obtained.

3.3.7 Surface Charge

Nanoparticles, when dispersed in liquid media, may carry an electric charge on their surface. This charge can depend upon the particle nature and the surrounding medium. Their size and surface charge are major factors affecting the dispersion of nanoparticles. Size and charge can also influence the adsorption of ions, contaminants, and biomolecules, and the way cells react when exposed to them.

The surface charges of nanoparticle are approximated through zeta potential measurements. The zeta potential is a function of the surface charge of the particle, adsorbed species on its surface, and the composition and ionic strength of the surrounding solution. Zeta potential measurements are normally performed in pure water with a small amount (1–10 mM) of monovalent background electrolyte. A titration is used to find the isoelectric point (IEP), defined as the pH where the zeta potential is zero. Typically, the IEP of a material under controlled conditions should be reported in addition to the zeta potential (sign and magnitude) under

anticipated physiological conditions (pH and ionic strength). Zeta potentials of nanoparticles are typically measured by light-scattering electrophoresis or electroacoustophoresis methods. Potentiometric titrations can also be used to acquire surface charge information. In particular, the pKa values of particle surface functional groups can be determined along with information on surface charge density.

3.3.8 Crystal structure

Many materials with the same chemical composition can have different lattice structures, and exhibit different physico-chemical properties. Several structural investigations on inorganic nanoparticles indicate that also the crystal lattice type may have an important role on the overall structure of nanoparticles, because of the very high portion of surface atoms with respect to the bulk lattice. The size reduction may create discontinuous crystal planes that increase the number of structural defects, as well as disrupt the electronic configuration of the material, with possible toxicological consequences.

XRD is used to investigate the surface atomic structure (e.g., crystal structure, lattice defects and charge distribution) of nanoparticles.

TEM can provide additional crystallographic information, such as the surface atoms arrangement and defects at atomic scale. If the energy dispersive spectrometry (EDS) option is applied, detailed information on the chemical composition of the sample surface can be obtained.

3.4 SUMMARY

Effort is being made towards improving the characterisation basis for toxicological studies, such as identifying the key physico-chemical characteristics of nanoparticles and how they can be measured. It is important to emphasise that multiple techniques should be used wherever possible to develop a more complete understanding of particle characteristics. This is particularly important with respect to particle sizing and dispersion.

The review of Powers *et al.* (2006), amongst others, concluded that a consensus may be emerging about the importance of characterisation. The body of literature published since confirms that there is now a consensus that thorough and accurate particle characterisation is an essential part of assessing the potential toxicity of nanoparticles in biological systems. Appropriate and common characterisation of test materials is important to ensure that results are reproducible, and also to provide the basis for understanding the properties of nanoparticles that determine their biological effects. Some of the key parameters influencing the biological activity of nanoparticles remain unknown or to be fully understood at this point. Hence, the characterisation of test materials should be as comprehensive as possible and broad in scope. A study conducted with material that has not been characterised with respect to a property later found to be critical for toxicity will ultimately be of little value.

Complete characterisation of test materials is time consuming, expensive, complex and may never be fully available. To some extent, the characterisation required depends on the objectives of the study. However, there are a number of fundamental properties that researchers in the field generally agree must be addressed. This subset forms the basis of a minimum set of characteristics that should be measured for test materials used in toxicity studies. In addition to composition, these include size and shape, state of dispersion, surface area, and surface chemistry.

The practicalities of implementing solutions to the characterisation challenges highlighted are emerging, albeit slowly. An invaluable means of progressing the science of nanoparticle characterisation is the communication of practice. This could include the publication of protocols (for example, as is being undertaken as part of the NanoImpactNet project – <http://www.nanoimpactnet.eu/>, accessed 16th October 2009) and continued recognition of the importance of robust characterisation data being reported alongside hazard and exposure assessments in peer-reviewed journal articles.

3.5 REFERENCES

Aitken, R.A., Hankin, S.M., Tran, C.L., Donaldson, K., Stone, V., Cumpson, P., Johnstone, J., Chaudhry, Q., Cash, S. and Garrod J. 2008, "A Multidisciplinary Approach to the Identification of Reference Materials for Engineered Nanoparticle Toxicology", *Nanotoxicology*, vol. 2, no. 2, pp. 71-78.

Borm, P., Klaessig, F.C., Landry, T.D., Moudgil, B., Pauluhn, J., Thomas, K., Trottier, R. and Wood, S. 2006. "Research Strategies for Safety Evaluation of Nanomaterials. Part V: Role of Dissolution in Biological Fate and Effects of Nanoscale Particles". *Toxicological Sciences*, vol. 90, no. 1, pp. 23-32.

BSI PD 6699-1:2007, *Nanotechnologies – Part 1: Good Practice Guide for Specifying Manufactured Nanomaterials*, British Standards Institute, UK.

Dhawan, A., Sharma, V. and Parmar, D. 2009, "Nanomaterials: A Challenge for Toxicologists", *Nanotoxicology*, vol. 3, no. 1, pp. 1-9.

Faraj, A.A., Cieslar, K., Lacroix, G., Gaillard, S., Canet-Soulas, E. and Cremillieux, Y. 2009, "In Vivo Imaging of Carbon Nanotube Biodistribution using Magnetic Resonance Imaging", *Nano Letters*, vol. 9, no. 3, pp. 1023-1027.

Hackley, V.A. and Ferraris, C.F. 2001, *Recommended Practice Guide: The Use of Nomenclature in Dispersion Science and Technology*, National Institute of Standards, USA. Accessed at: http://www.nist.gov/public_affairs/practiceguides/SP960-3.pdf (16th October 2009).

Hasselov, M., Readman, J.W., Ranville, J.F. and Tiede K. 2008, "Nanoparticle Analysis and Characterization Methodologies in Environmental Risk Assessment of Engineered Nanoparticles", *Ecotoxicology*, vol. 17, pp. 344-361.

Ju-Nam, Y. and Lead, J.R. 2008, "Manufactured Nanoparticles: An Overview of their Chemistry, Interactions and Potential Environmental Implications", *Science of the Total Environment*, vol. 400, pp. 396-414.

Kaluza, S., Balderhaar, J.K, Orthen, B., Honnert, B., Jankowska, E., Pietrowski, P., Rosell, M.G., Tanarro, C., Tejedor, J., Zugasti, A. 2009, *Exposure to Nanoparticles*, European Agency for Safety and Health at Work. Accessed at: http://osha.europa.eu/en/publications/literature_reviews/workplace_exposure_to_nanoparticles (16th October 2009).

Mao, C. 2004, "Introduction: Nanomaterials Characterization Using Microscopy", *Microscopy Research and Technique*, vol. 64, pp. 345-346.

Maynard, A.D. and Aitken, R.J. 2007, "Assessing Exposure to Airborne Nanomaterials: Current Abilities and Future Requirements", *Nanotoxicology*, vol. 1, no. 1, pp. 26-41.

Murdock, R.C., Braydich-Stolle, L., Schrand, A.M., Schlager, J.J., Hussain, S.M. 2008, "Characterization of Nanomaterials Dispersion in Solution Prior to In Vitro Exposure Using Dynamic Light Scattering Technique", *Toxicological Sciences*, vol. 101, no. 2, pp. 239-253.

Oberdorster, G., Maynard, A., Donaldson, K., Castranova, V., Fitzpatrick, J., Ausman, K., Carter, J., Karn, B., Kreyling, W., Lai, D., Olin, S., Monteiro-Riviere, N., Warheit, D., Yang, H. 2005, "Principles for Characterizing the Potential Human Health Effects from Exposure to Nanomaterials: Elements of a Screening Strategy", *Particle Fibre Toxicology*, doi:10.1186/1743-8977-2-82:8.

OECD 2008. *List of Manufactured Nanomaterials and List of Endpoints for Phase One of The OECD Testing Programme*. ENV/JM/MONO(2008)13. Accessed at:

[http://www.olis.oecd.org/olis/2008doc.nsf/LinkTo/NT000034C6/\\$FILE/JT03248749.PDF](http://www.olis.oecd.org/olis/2008doc.nsf/LinkTo/NT000034C6/$FILE/JT03248749.PDF) (16th October 2009).

Powers, K.W., Brown, S.C., Krishna, V.B., Wasdo, S.C., Moudgil, B.M. and Roberts S.M. 2006, "Research Strategies for Safety Evaluation of Nanomaterials. Part VI. Characterization of Nanoscale Particles for Toxicological Evaluation", *Toxicological Sciences*, vol. 90, no. 2, pp. 296-303.

Powers, K.W., Palazuelos, M., Moudgil, B.M., Roberts, S.M. 2007, "Characterization of the Size, Shape, and State of Dispersion of Nanoparticles for Toxicological Studies", *Nanotoxicology*, vol. 1, no. 1, pp. 42-51.

Rao, C.N.R., and Biswas K. 2009, "Characterization of Nanomaterials by Physical Methods", *Annual Review of Analytical Chemistry*, vol. 2, no. 21, pp. 21.1-21.28.

Ruzer, L and Harley, N. (Eds) 2005, *Aerosols Handbook – Measurement, Dosimetry and Health Effects*, 1st edn, CRC Press, Boca Raton.

Shi, J.P., Harrison, R.M. and Evans D. 2001, "Comparison of ambient particle surface area measurement by ephaniometer and SMPS/APS", *Atmospheric Environment*, vol. 35, pp. 6193-6200.

Scott, D.M. 2003, "Characterizing Particle Characterization", *Particle and Particle Systems Characterization*, vol. 20, pp. 305-310.

Sebba, D.S., Watson, D.A. and Nolan, J.P. 2009, "High Throughput Single Nanoparticle Spectroscopy", *Nano*, vol. 3, no. 6, pp. 1477-1484.

Stone, V., Nowack, B., Baun, A., van den Brink, N., von der Kammer, F., Dusinska, M., Handy, R., Hankin, S., Hasselov, M., Joner, E. and Fernandes T. 2009, "Nanomaterials for Environmental Studies: Classification, Reference Materials Issues, and Strategies for Physico-chemical Characterisation", *Science of the Total Environment* (manuscript submitted).

Tantra, R. and Cumpson, P. 2007, "The Detection of Airborne Carbon Nanotubes in Relation to Toxicology and Workplace Safety", *Nanotoxicology*, vol. 1, no. 4, pp. 251-265.

Teeguarden, J.G., Hinderliter, P.M., Orr, G., Thrall, B.D., Pounds, J.G. 2007, "Particokinetics in vitro: Dosimetry Considerations for In Vitro Nanoparticles Toxicity Assessments", *Toxicological Sciences*, vol. 95, no.2, pp. 300-312.

Tiede, K., Boxall, A.B.A., Tear, S.P., Lewis, J., David, H. and Hasselov M. 2008, "Detection and Characterization of Engineered Nanoparticles in Food and the Environment", *Food Additives and Contaminants*, vol. 25, no. 7, pp. 795-821.

Vincent, J.H. 1995, *Aerosol Science for Industrial Hygienists*, Elsevier Science Ltd, London.

Warheit, D.B., Borm, P.J.A., Hennes, C., Lademann, J. 2007, "Testing Strategies to Establish the Safety of Nanomaterials: Conclusions of an ECETOC Workshop", *Inhalation Toxicology*, vol. 19, pp. 631-643.

Warheit, D.B. 2008, "How Meaningful are the Results of Nanotoxicity Studies in the Absence of Adequate Material Characterization?", *Toxicological Sciences*, vol 101, no. 2, pp. 183-185.

Wittmaack, K. 2007, "In Search of the Most Relevant Parameter for Quantifying Lung Inflammatory Response to Nanoparticle Exposure: Particle Number, Surface Area, or What?", *Environmental Health Perspectives*, vol. 115, no. 2, pp. 187-194.

Zhang, S., Li, L., Kumar, A. 2009, *Materials Characterization Techniques*, CRC Press, Boca Raton.

Engineered Nanoparticles: Review of Health and Environmental Safety

Zuin, S., Pojana, G., Marcomini, A. 2007, "Effect-Oriented Physicochemical Characterization of Nanomaterials" in *Nanotoxicology: Characterization, Dosing and Health Effects*, eds. N.A. Monteiro-Riviere and C.L. Tran, 1st Ed, Informa Healthcare, New York, pp. 19-57.

4 EXPOSURE

4.1 INTRODUCTION

4.1.1 Exposure

The risk of health effects which may arise in an individual or a population as a result of exposure to a chemical agent, is generally considered to be a function of the intrinsic harmfulness of the chemical (its toxicity) and the dose (amount) which accumulates in the specific biological area of interest. In an occupational context it is unusual to be able to quantify the dose, specifically in the case of insoluble particulates. In order to quantify and manage the risks, it is usual to use exposure as a proxy for dose. Terminology has recently been adopted by the International Society of Exposure Analysis (ISEA), in which exposure is defined as “the contact between an agent and a target,” that is, the human as the target and a contact at an exposure surface over an exposure period (Zartarian *et al.* 2005). Knowledge and control of exposure is critical in risk assessment and management.

Critical questions in relation to exposure are how much, how long and how many people exposed. Thus, exposure is usually measured (quantified or assessed) in terms of its intensity (concentration) and duration (or frequency). Control of exposure (to zero) effectively removes the risks from the toxic agent. Without exposure there is no risk.

The main routes by which workers can be exposed to particles are inhalation, ingestion and dermal penetration.

Inhalation is considered to be the primary route by which particles suspended in air can enter the bodies of workers. Once inhaled, particles will deposit in all regions of the respiratory tract. The location and extent to which particles deposit is dependent on the particle size (ICRP 1994).

Ingestion exposure to particles in general, may arise from hand to mouth contact, by sucking or licking a contaminated surface or by eating contaminated food. It may also be caused by swallowing mucous which contain deposited particles cleared from the lungs. Occupational ingestion exposure has received little interest thus far.

Dermal exposure is of increasing concern in workplace scenarios (e.g. Brouwer *et al.* 2005). Workers may be exposed via the skin by handling or touching materials or surfaces coated with nanomaterials. One of the challenges of managing the risks arising from dermal exposures is that protective equipment, such as gloves, which are designed to prevent exposure can in themselves act as reservoirs of contaminant, exacerbating the exposure.

4.1.2 Plausible Exposures relating to nanoparticles

There are multiple scenarios through which humans could become exposed to engineered nanomaterials including occupational, environmental and consumer exposure scenarios. These are illustrated in Figure 4.1 which is taken from the report of the Royal Society/ Royal Academy of Engineering (RS/RAEng 2004).

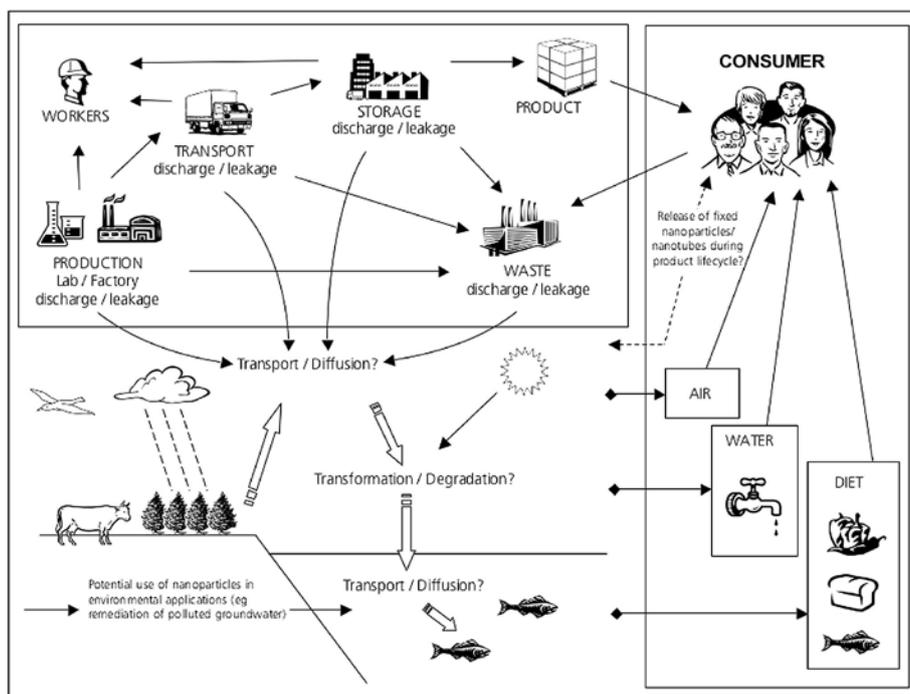


Figure 4.1: Plausible exposure routes for nanoscale materials based on current and potential future applications (extracted from RS/RAEng 2004)

In an occupational setting, exposure to nanomaterials can occur for workers at all phases of the material life cycle. During the development of a new material, it is probable that the material will be produced under tightly controlled conditions, typically in small quantities. Accidental releases, for example due to spills, are also a possibility. This suggests that relatively few people are likely to be exposed at this stage. Once the material moves into commercial production, exposures can potentially occur during synthesis of the material or in downstream activities such as recovery, packaging, transport, and storage. In these circumstances, the quantities of materials being handled will typically be much larger. Depending on the specific properties of the new material, it may be incorporated subsequently in a range of other products or may be used in other processes as a feed-stock material. Aitken *et al.* (2006) identified a range of applications for various classes on nanoparticles. These are shown in Table 4.1.

Manufacture or use of these products may give rise to scenarios with the potential for exposure to occur. Such products may include composite materials. These may be subsequently re-engineered or reprocessed e.g., by cutting, sawing or finishing giving rise to possible exposures. Finally, we could consider end of life scenarios where the material is disposed of, perhaps by incineration or some other process such as shredding or grinding.

Hence, for a single nanoparticle type, there are a multiple exposure scenarios which may or may not occur depending on the details of manufacture, use and disposal of that material. Throughout these scenarios, the population exposed, the levels of exposure, the duration of exposure, and the nature of the material to which people are exposed, are all different.

Table 4.1: Current applications of the four classes of nanoparticles (adapted from Aitken *et al.* 2006)

Applications	Materials		
	Fullerenes (C ₆₀ , C ₇₀ , C ₈₀ , derivatised)	Carbon nanotubes (SWCNT and MWCNT)	Metals and metal oxides
Hydrogen storage	X	X	
Environmental remediation			X
Catalysis			X
Drug delivery	X	X	X
Medical imaging	X	X	X
Photovoltaics	X	X	X
Textiles		X	
Therapeutics	X	X	
Reinforced composites		X	
Electronics and electronic devices	X	X	X
Optics and optical devices	X		X
Coatings and pigments	X	X	X
Cosmetics			X
Ceramics applications			
Anti-oxidants			
Lubrication	X		X
Sensors and sensing devices	X	X	X
Absorbents			X
Energetics and energetic materials		X	X
Magnetics and magnetic devices			X
Water purification and filtration media		X	X
Air emissions reduction			X
Natural and green products			
Quantum computing			X
Masonry and building materials		X	
Photonics and photonic devices	X		X
Surfactants			

4.1.3 Exposure metrics

In early studies to assess the health effects of inhaled particles, dust (particle) samples were collected by drawing air through a filter or other media and then analysing this off-line to define estimates of exposure, usually expressed as a concentration in air. For example, in early studies in the coal industry, samples collected were analysed by counting particles deposited on the filter under a light microscope (Walton and Vincent 1998). This resulted in an estimate of exposure in terms of particle *number concentration*, expressed as number of particles per cc (cm^3) or per m^3 of air. Epidemiology studies in that industry later showed a good correlation between pneumoconiosis and *mass concentration*, expressed typically as mg m^{-3} . Assessment in terms of mass was less demanding and more accurate than manual counting under a microscope and so was the preferred choice. Subsequently, Workplace Exposure Limits (WEL), based on mass concentrations, have become the norm for regulating exposure for most hazardous chemicals or particles (HSE 2005). One exception to this is in the measurement of fibres, where based on understanding of the mechanisms of harm, exposure measures and limit values are expressed as fibre number concentrations.

There are three main metrics, all of which could have some utility in measuring exposure to nanoparticles. These are: i) mass concentration (units mg m^{-3}); ii) number concentration (units per m^{-3}) and; iii) surface area concentration units ($\text{m}^2 \text{m}^{-3}$). A case may be made for the use of any of these metrics under certain circumstances. There is a sustained view based on the toxicology literature, that possible health effects arising from exposure to nanoparticles may be better correlated with surface area, rather than with mass concentration. This is based on studies which have examined inflammation in both *in vitro* and *in vivo* systems (See Section 6).

Particle number is also widely suggested as an appropriate metric. This is based on reported associations between particulate air pollution and exacerbations of illness in people with respiratory disease and rises in the numbers of deaths from cardiovascular and respiratory disease among older people (Seaton *et al.* 1995).

In their structured review of this issue, Maynard and Aitken (2007) were unable to find sufficient evidence to select one of these metrics in preference to others and so recommended that, where possible, all three should be measured.

4.1.4 Overview of our findings

In general, there is a paucity of published data concerning exposure to nanoparticles. Various types of studies have been used to in an attempt to provide the maximum information available.

There are a few studies which have speculated on possible or plausible exposure scenarios. In addition to the overview provided by the Royal Society report, more detailed reviews, particularly of the potential for occupational exposure have been developed (Aitken *et al.* 2004, 2006). There are also a number of inventory studies which have tried to identify and categorise uses of nanoparticles (e.g. BAUA 2008). While these studies are useful in mapping the exposure landscape, they do not in themselves lead to numerical estimates of exposure

Of more utility are modelling studies. Two such studies have been identified which provide useful information relating to environmental and consumer exposure (Mueller *et al.* 2008; Boxall *et al.* 2007).

For the materials of interest we have identified only 11 studies which have reported measured exposure data. These are shown in Table 4.2. All of these are in the occupational setting; no studies have reported consumer exposures or exposures in the environment. All but one of the studies identified have reported only inhalation exposure; one study reported dermal exposure and no studies reported ingestion exposure. Most of the studies were carried in university settings, however, some industrial settings were also found.

Metrics assessed are number concentration (including fibre number concentration), size differentiated number concentration, mass concentration and size differentiated mass concentration. Surface area concentration was only measured directly in one study. A range of

methods were used. Almost all studies used more than one measurement method. Methods for measurement of exposure have been described by several authors (Maynard and Aitken 2007; ISO 2007) and an overview has been provided in Chapter 3.

Table 4.2: Exposure assessment studies identified containing relevant exposure data

Year	Lead Author	CNT	Fullerenes	Metals	Metal oxide
2004	Maynard	SWCNT			
2006	Methner	CNF			
2008	Han	MWCNT			
2008	Bello	MWCNT			
2009	Bello	MWCNT			
2008	Fujitani		mixed		
2008	Yaganeh	mixed	mixed		
2007	Hsu				TiO ₂
2008	Demou			metal	metal oxide
2009	Peters				LiTiO _x
2008	Tsai			Ag	Al ₂ O ₃

4.2 CARBON FULLERENES

Only two studies were identified with relevant exposure data for mixed fullerenes. These are shown in Table 4.3.

Table 4.3: Relevant fullerene studies identified

Setting		Activity						Reference
Production	Laboratory	Synthesis	Recovery/ bagging	Cleaning	Handling/ processing	Deliberate agitation	Sawing Composite	
#			E	E		E		Fujitani <i>et al.</i> 2008
#		0	E	E				Yaganeh <i>et al.</i> 2008

- setting examined

E – elevated exposures found

0 – assessed but no elevated exposures found.

Fujitani *et al.* (2008) measured the physico-chemical properties, number concentrations and number size distributions of aerosols in a fullerene factory in Japan. At this facility, mixed fullerene was extracted by solvent from soot generated by the combustion of hydrocarbon – oxygen mixtures. The mixed fullerenes produced included C₆₀, C₇₀ and other higher fullerenes. The fullerenes are produced as particles which are aggregates or agglomerated of the primary fullerenes. The diameter of the mixed fullerene particles was stated as about 20 µm.

The factory has a production capacity of 40000 kg yr⁻¹. Fullerenes are produced in a closed system, intended to minimise the potential for exposure during production. After the drying process, the fullerene is transported to and kept in a storage tank until it is removed and bagged. A vacuum cleaner is used in the bagging operation. After a certain amount of fullerene is bagged, it is weighed in the same room.

Results were presented as number and volume distributions, including size differentiated times series, enabling comparison of results for a series of activities: non-work, bagging, use of a vacuum cleaner and moderate agitation. For the time series, the results were grouped into four size bands: 10-50 nm, 50-100 nm, 100-2000 nm and > 2000 nm. The source, workers, and measuring instruments were all within a 1.5 m range. Measurements were carried out in only one day in the facility, and on a second day outside the facility to determine environmental conditions

Data extracted from the paper (and therefore of limited accuracy) is shown in Table 4.4. Note that concentration changes measured did not occur immediately at the activity event, and so the numbers reported here are the highest levels to which concentration rose after the event.

Table 4.4: Data extracted from Fujitani *et al.* (2008)

Size range (nm)	Number concentration / cm ⁻³				Calculated volume concentration / nm ³ cm ⁻³ (x10 ⁹)			
	10:50	50:100	100:2000	>2000	10:50	50:100	100:2000	>2000
start measuring indoor air during non-work	10000	7000	3000	0	0.1	2	10	10
start recovery and bagging	15000	7000	3000	0	0.2	2	10	50
end bagging all workers leave room	10000	7000	3000	0	0.1	2	10	10
start vacuuming	15000	7000	3000	0	0.3	2	10	30
start moderate agitation	10000	7000	3000	0	0.1	2	10	1000
start extreme agitation	10000	7000	3000	0	0.1	2	20	5000
start measuring outdoor air	25000	10000	5000	0	0.8	4	10	10

Yeganeh *et al.* (2008) characterised airborne particle concentrations during the production of carbonaceous nanomaterials, “such as fullerenes and carbon nanotubes”, in a commercial nanotechnology facility. They measured fine particle mass concentrations (PM2.5), sub-µm size distributions, and photo-ionisation potential (an indicator of the particles' carbonaceous content), at three locations inside the facility: inside the fume hood where nanomaterials were produced, just outside the fume hood, and in the background. Three activities were assessed, “arc reaction”, “sweeping” (which is actually recovery) and “vacuuming”. Comparison with outdoor measurements was also made.

Average PM2.5 and particle number concentrations were not significantly different inside the facility compared with outdoors. However, large, short-term increases in PM2.5 and particle number concentrations were associated with physical handling of nanomaterials and *other production activities* (“including drilling and cutting of graphite and metal”). In many cases, an increase in the number of sub-100 nm particles accounted for the majority of the increase in total number concentrations.

In the work area, during periods of arc reaction, approximate number concentrations (14-673 nm) of 30 – 50000 cm⁻³ were observed. One peak of 600000 cm⁻³ attributed to other production activities. One smaller peak (80000 cm⁻³) on the second day of monitoring was attributed to sweeping. PM2.5 mass concentrations ranged from 50 to 125 µg m⁻³ but did to seem to vary with activity. Based on the measurements in this study, the engineering controls at the facility appear to be effective at limiting exposure to nanomaterials.

In summary, only two studies were identified as relevant to occupational exposure to fullerenes, both describing production activities. A range of methods and measurement metrics (particle number and mass concentration) have been used in an attempt to quantify exposure. Both studies showed some evidence of elevated exposures.

4.3 CARBON NANOTUBES

Table 4.5 provides an overview of the relevant studies considered in this section.

Table 4.5. Relevant CNT studies identified

Material	Setting		Activity						Reference
	Production	Laboratory	Synthesis	Recovery/ bagging	Cleaning	Handling/ processing	Deliberate agitation	Sawing composite	
SWCNT	#	#		E	E	E	E		Maynard <i>et al.</i> 2004
CNF		#				E		E	Maynard <i>et al.</i> 2006
MWCNT		#				F			Han <i>et al.</i> 2008
MWCNT		#	0	0		0			Bello <i>et al.</i> 2008
MWCNT		#						E	Bello <i>et al.</i> 2009

- setting examined
 E – elevated exposures found
 F – exposure to “fibres” found
 0 – assessed but no elevated exposures found

Maynard *et al.* (2004) was the first study to report exposures during production and handling of CNT. Maynard carried out a field study in which airborne and dermal exposure to SWCNT were investigated while recovering (handling) unrefined material as part of the production process. Measurements of unprocessed airborne nanotube exposures were conducted at four plants where SWCNT material was removed from production vessels and handled prior to processing. Two production techniques for were investigated: laser ablation and high-pressure carbon monoxide (HiPCO). This field work was complemented by a laboratory based study to evaluate the physical nature of the aerosol formed from SWCNT material during mechanical agitation.

Instruments used to detect these particles included CPC and SMPS (described in Chapter 3). Filter samples for analysis by electron microscopy were also collected. The filter samples were taken over the time period the workers spent in the enclosure, which was typically about half an hour.

Estimates of nanotube concentrations ranged from 0.7 µg m⁻³ in the ablation facility to 53 µg m⁻³ in the HiPCO process. SEM analysis of filter samples indicated that many of the particles appeared to be compact, rather than having the open, low-density structure more generally associated with unprocessed SWCNT. Some open structures were observed, but these included some large (non-respirable) clumps.

For dermal exposure, estimates of the SWCNT material on the individual gloves ranged from 217 µg to 6020 µg, with most of the material appearing on the parts of the gloves in direct contact with surfaces (i.e. inner surfaces of fingers and palms). Although the use of gloves and PPE will minimise dermal exposure during handling of this material, the possibility for large clumps to become airborne and remain so for long periods may lead to dermal exposures in less well protected regions.

Methner *et al.* (2006, 2007) reported the “Identification and characterisation of potential sources of worker exposure to carbon nanofibres during polymer composite laboratory operations”. The goal of this study was to examine various operations involved in the handling or processing of

carbon nanofibre (CNF) materials, and to determine whether emission of these materials occurred for these operations.

Measurements made with real-time instruments capable of sizing and determining airborne particle concentrations indicated that most processes did not release substantial quantities of CNF when compared to background particle measurements. However, the potential for release of engineered nanomaterials does exist during some processes. Transferring of CNFs prior to weighing and mixing with solvent resulted in a small increase in the airborne concentration (number and mass) of particle sizes greater than approximately 500 nm (as indicated by the ELPI), suggesting some release of aggregated nanofibre material. Operation of the wet saw resulted in a small increase in the airborne number concentration of particles larger than 400 nm (as indicated by ELPI), along with a corresponding increase in both the mass concentration measured by the aerosol photometer and total carbon. "A few" fibre bundles of varying diameters and lengths were also reported. Some fibres observed on the TEM grids had diameters clearly larger than 100 nm. The majority of fibres observed appeared as loosely bundled agglomerates rather than as single fibres, which the authors considered to be general agreement with real-time aerosol instrument data. There was no attempt to quantify the concentration of these bundled objects.

Han *et al.* (2008) monitored the possible exposure to MWCNT release in a research laboratory. To estimate the potential exposure of researchers and evaluate the improvement of the workplace environment after the implementation of protective control measures, personal and area monitoring were conducted in an MWCNT research facility where the researchers handled unrefined materials. Measurements were made in the post production recovery of MWCNT (laboratory A), processing (laboratory B) and in a blending activity, part of a composite formulation process (laboratory C).

In this paper, mass concentrations of up to 0.43 mg m^{-3} were reported during blending before exposure control was implemented. Following implementation of exposure control (hood and ventilation), the maximum measured concentration (mass) reduced to 0.04 mg m^{-3} . Even without exposure control, mass concentrations during production were not detected and during weighing were described as low (0.1 mg m^{-3}). Data are summarised below in Table 4.6 which is reproduced from the paper.

The key finding of this study, however, was the identification and quantification of high aspect ratio aerosols, using approaches analogous to the WHO fibre counting method (WHO 1998). This is the first study which has attempted to quantify release using methodology which is consistent with that recommended by WHO for other fibres. Han adapted the method to count fibres using a TEM. It is not clear from the paper, the extent to which all of the counting rules relevant to WHO have been applied. (WHO counting rules include the definition of a fibre in terms of minimum length and aspect ratio, and rules for touching fibres, and for fibres attached to particles). For example, many of the images in the paper show clumped or overlapping fibres. No comment is made in the paper as to how such aggregates were dealt with.

The fibres were only found in the blending scenario and were found on both personal and area samples at approximately the same level. The reported number concentration was very high (193.6 and $172.9 \text{ fibre ml}^{-1}$). These are three orders of magnitude greater than typical asbestos workplace exposure limits of $0.1 \text{ fibre ml}^{-1}$. Enclosure of the blending activity reduced the fibre count by four orders of magnitude, indicating effective control of fibre release. Interestingly, following the installation of engineering measures, in one area of Laboratory B, where weighing was carried out, one sample showed a fibre concentration of $1.99 \text{ fibre ml}^{-1}$.

It is important to note however that all of the fibres counted were shorter than what would be considered a fibre under WHO rules. The reported maximum length of the fibres observed was 1500 nm, which would be too small to be considered a fibre under the WHO criteria, where the minimum size of a fibre is 5000 nm, i.e., if these sample were counted under strict WHO rules, the fibre count would be zero.

Table 4.6: Total Particle Concentration in CNT Laboratory Before and After Installation of Engineering Equipment (mg m⁻³) (Data reproduced from Han *et al.* 2008)

Lab	Sample	Operation	Before	After
A	Personal	Thermal CVD	Not detected	Not detected
	Area	Thermal CVD	Not detected	0.0388
	Personal	Al/CNT ball milling	Not detected	Not measured
B	Area	Weighing	0.1133	Not detected
	Area	Weighing/ spraying	0.0366	Not measured
	Personal	CNT solution spraying	0.1930	0.0309
C	Personal	Blending	0.3317 (open blender)	Not detected (inside encapsulation)
	Area	Blending	0.4345 (open blender)	Not detected (inside encapsulation)
	Area	Blending	0.2090	Not detected

Bello *et al.* (2008) reported exposure to nanoparticles during CVD growth of vertically-aligned mats of CNT and during subsequent handling and recovery. The work was carried out in a university research laboratory. “Forests” of vertically-aligned CNT were grown by atmospheric pressure CVD at 750°C using a horizontal quartz tube furnace at atmospheric pressure. Measurements were made during synthesis and recovery. No increases in the total particle number concentration at any particle size range as compared to background were observed during each cycle of the furnace operation. Nanoscale particles of 10 and 100 nm, prominent in the FMPS size distribution output were observed in the TEM images and were attributed to indoor air background. Larger carbonaceous particles (confirmed by EDS, no images shown) up to 1 µm were also seen, especially during the opening of the furnace. TEM analysis did not reveal the presence of CNT in the laboratory air. No evidence of individual or bundles of CNT were found. The personal filter (evaluated with FE-SEM) contained particulate matter of various typical sizes but “no apparent nanoscale fibres or fibrous bundles”.

Overall the study found no detectable quantity of CNT, bundles or similar-sized carbonaceous particles released to the occupational environment during CVD growth of the CNT forest on substrates in the tube furnace, or during subsequent handling and delamination of the forest. Note though, that in this study, the sample duration was only a few minutes. No indication was given concerning the quantities handled and there was no reported attempt to quantify, in terms of fibre number.

In Bello *et al.* (2009), exposure to nanoscale particles and fibres during machining of hybrid advanced composites containing carbon nanotubes (as produced in Bello *et al.* 2008) was examined. This study investigated airborne exposures to nanoscale particles and fibres generated during dry and wet abrasive machining of two three-phase advanced composite systems containing CNT, µm-diameter continuous fibres (carbon or alumina), and thermoset polymer matrices. Measurements were made of airborne concentrations at sources and in the breathing zone of workers. This is the only study which has reported surface area concentrations. The key results of this study are reproduced in Tables 4.7 and 4.8.

Significant exposures to nanoscale particles (compared to background) were observed during dry cutting of all composites. Breathing zone exposures were generally, but not always, lower than the source exposures. The particle size distribution of the aerosol was similar for most composites. The elemental composition of these particles matched the expected composition of processed composites reasonably well. Although the particle number concentration was dominated by the nanoscale and fine fractions, 71–89% of the total surface area was dominated by the respirable (1–10 µm) aerosol fraction.

Table 4.7: Airborne concentration at source (adapted from Bello *et al.* 2009)

Composite	AM (SEM) of total number concentration (# cm ⁻³)		AM surface area (µm ² cm ⁻³) by size range (its fraction of the total surface area)			
	FMPS	APS	$d_p \leq 0.1 \mu\text{m}$	$0.1 < d_p \leq 1.0 \mu\text{m}$	$1 < d_p \leq 10 \mu\text{m}$	$10 < d_p \leq 20 \mu\text{m}$
Background	4.82E + 3 (0.63E + 4)	11.4 (0.08)	286 (0.26)	340 (0.29)	642 (0.44)	10 (0.00)
Wet cutting of base-carbon	9.4E + 4 (0.28E + 4)	47.6 (2.0)	12580 (0.11)	99373 (0.85)	4077 (0.04)	68 (0.00)
Dry cutting						
Base-alumina	1.48E + 5 (3.7E + 4)	135.2 (4.8)	6367 (0.10)	4429 (0.06)	65551 (0.83)	863 (0.01)
CNT-alumina	0.38E + 5 (0.13E + 4)	285.3 (19.1)	1118 (0.01)	17046 (0.14)	214506 (0.84)	2281 (0.01)
Base-carbon	2.83E + 5 (9.5E + 4)	1003.8 (33.5)	6914 (0.02)	35056 (0.09)	411140 (0.89)	3944 (0.01)
CNT-carbon	2.94E + 5 (1.7E + 4)	867.1 (52.2)	7809 (0.04)	40898 (0.25)	339971 (0.71)	4386 (0.01)

d_p , Aerodynamic diameter; AM, arithmetic mean; SEM, standard error of mean; FMPS, fast mobility particle sizer instrument; APS, aerodynamic particle sizer instrument.

Table 4.8 Airborne concentrations in breathing zone (adapted from Bello *et al.* 2009)

Composite	AM (SEM) of total number concentration (# cm ⁻³)		AM surface area (µm ² cm ⁻³) by size range (its fraction of the total surface area)			
	FMPS	APS	$d_p \leq 0.1 \mu\text{m}$	$0.1 < d_p \leq 1.0 \mu\text{m}$	$1 < d_p \leq 10 \mu\text{m}$	$10 < d_p \leq 20 \mu\text{m}$
Base-alumina	0.88E + 5 (0.51E + 4)	72.2 (4.6)	3102 (0.13)	4053 (0.16)	24768 (0.71)	44 (0.00)
CNT-alumina	0.28E + 5 (0.69E + 3)	62.2 (4.6)	711 (0.04)	10517 (0.29)	33374 (0.67)	54 (0.00)
Base-carbon	3.19E + 5 (1.12E + 4)	777.5 (26.1)	7311 (0.03)	32487 (0.12)	270961 (0.85)	1769 (0.00)
CNT-carbon	1.53E + 5 (0.77E + 4)	215.7 (11.5)	4150 (0.05)	30917 (0.34)	79899 (0.61)	544 (0.00)

d_p , Aerodynamic diameter; AM, arithmetic mean; SEM, standard error of mean; FMPS, fast mobility particle sizer instrument; APS, aerodynamic particle sizer instrument.

Sub-µm and respirable fibres were generated from dry cutting of all composites. No obvious differences were found in the behaviour of base- and CNT-composites with regards to their tendency to generate respirable fibres and particles. Breathing zone concentrations of respirable fibres (~0.2 fibres cm⁻³) were observed, and were approximately an order of magnitude lower than source concentrations. The authors also reported that sub µm long, thin (5–20 nm in diameter) needle-like fibres were observed for all composites. The authors reported that although they could not be quantified, these fibers were “common”. The authors suggest that based on the fibre morphology and elemental composition that these fibres may be originating from two distinct processes: fracturing of advanced fibers along their axis or perpendicular to it, and fracturing of the CNT composite at the interface.

Wet cutting in all but one test (where there was a broken guard), reduced exposures to background levels. In one test, during which the guard around the rotary wheel was visibly damaged, wet cutting generated significant airborne exposures compared to background. Although airborne particulate matter was clearly emitted during this test (as confirmed by deposition of black aerosol on surfaces) and the size distribution was distinct from dry cutting it is unclear how much of that aerosol was water.

CNT, either individual or in bundles, were not observed despite “extensive” electron microscopy of collected samples.

In summary, for CNT a range of methods and measurement metrics (number mass and surface area concentrations) have been used in an attempt to quantify exposure. Most studies showed some evidence of elevated levels. Only the Han study clearly demonstrated the release of fibres or “fibre-like” aerosols. This study was also the only one to attempt quantification of the exposure using methods similar to those used currently for other fibres such as asbestos. Most exposure seemed to be associated with absent, defective or disabled control systems.

4.4 METALS

Two studies were relevant to exposure to metals. These are shown in Table 4.9.

Table 4.9: Relevant metal studies identified

Material	Setting		Activity						Reference
	Production	Laboratory	Synthesis	Recovery /bagging	Cleaning	Handling/ processing	Deliberate agitation	Sawing composite	
Metal	#		E						Demou <i>et al.</i> 2008
Silver		#				E			Tasai <i>et al.</i> 2008

- setting examined
 E – elevated exposures found
 0 – assessed but no elevated exposures found

Demou *et al.* (2008) reported a study which was carried out at a pilot scale “nanostructured particle” gas phase production facility. The facility produced metal-based nanoparticles embedded in a larger porous oxide matrix. The study could therefore be considered under metal or metal oxide as no additional characterisation was provided. The study objectives were to: i) identify and quantify the sources of nanostructured particle emissions in the facility; ii) characterise the emitted particles in terms of airborne concentration and size; and iii) identify, quantify and propose measures for the mitigation of the potential exposure relative to task. The study particularly looked to assess the distribution of contaminants in the workplace.

A condensation particle counter, DustTrak and scanning mobility particle sizer were used to quantify real-time size, mass and number, over a 25 day period. Temporal and spatial analysis of particle concentrations and sizes was performed during production, maintenance and handling. Number-based particle retention of breathing mask filters used under real-time production and exposure conditions in the workplace was quantified. The results demonstrate elevated number concentrations during production, which can be an order of magnitude higher than background levels.

Average concentrations for the sub- µm particles during production were 59100 particles cm⁻³ and 0.188 mg m⁻³. The study demonstrates real-time worker exposure during gas-phase nanoparticle manufacturing and indicates clear differences between periods where the reactor was operational and other periods. Assessment by particle number was more sensitive than mass concentration measurements.

Tsai *et al.* (2008) assessed airborne nanoparticle exposures associated with the manual handling of alumina and silver nanoparticles in fume hoods (as almost all of the data referred to alumina nanoparticles and so it is discussed in detail in Section 4.5). The silver particles appeared to have a primary size of less than 100 nm but were highly aggregated into particles

of several μm . For handling 15 g of silver, a peak size of 100-200 nm and a peak count of 7000 particles cm^{-3} was reported.

In summary, only two relevant studies were identified as relevant to occupational exposure to metals, one concerned with manufacture and one with powder handling. A range of methods and measurement metrics (particle number and mass concentration) have been used in an attempt to quantify exposure. Both studies showed some evidence of elevated exposures

4.5 METAL OXIDES

Four studies were identified which were relevant to metal-oxide exposures. These are shown in Table 4.10.

Table 4.10: Relevant metal oxide studies identified

Material	Setting		Activity						Reference
	Production	Laboratory	Synthesis	Recovery/ bagging	Cleaning	Handling/ processing	Deliberate agitation	Sawing composite	
TiO ₂		#				E			Hsu <i>et al.</i> 2007
metal oxide	#		E						Demou <i>et al.</i> 2008
LiTiO _x	#		0			E			Peters <i>et al.</i> 2009
Al ₂ O ₃		#				E			Tsai <i>et al.</i> 2008

- setting examined

E – elevated exposures found

0 – assessed but no elevated exposures found

As described above, Tsai *et al.* (2008), assessed airborne nanoparticle exposures associated with the manual handling of nano-alumina and nano-silver in fume hoods. The nanoalumina is described in this section. Handling experiments were performed to measure airborne particle concentration whilst handling nanoparticles in three fume hoods located in different buildings, under a range of operating conditions. The aluminium oxide (Al₂O₃) nanoparticles used had a reported density of 3600 kg m⁻³ and primary particle size ranging from 27 to 56 nm; when dried, these particles formed agglomerates with a nominal size of 200 nm. The quantities handled were 100 g and 15 g.

Airborne concentrations of nanoparticles were measured using the FMPS spectrometer (Model 3091, TSI) in the range from 5.6 to 560 nm. Normalised particle number concentrations were calculated in each size channel, based on the average concentration during each measuring time period. The background concentration measured before each experiment was used as the baseline for subtraction from the source concentration. Particle number concentration was measured at the breathing zone.

Particle number concentrations were reported as size distributions, and aggregate counts were not provided. In a conventional fume hood, particle number concentration measured at the breathing zone increased significantly during the 100 g handling. Peaks at 10 nm and 200 nm were reported which varied according to activity, summarised below in Table 4.11.

Table 4.11: Results from Tsai *et al.* (2008)

Handling	Particle number concentration (particles cm ⁻³)	
	10 nm	200 nm
100g Transfer	16000	2000
100g Transfer	4000	2000
100g Pouring	16000	12000
100g Pouring	5000	12000
15g Transfer	0	500
15g Pouring	0	500

The size of the 200 nm peak related to the setting of the fume hood to “high, mid, low sash”. The numbers shown relate to “low sash”. The 10 nm peak was largely independent of setting. Integrating these numbers would imply *total* number concentrations in the order of greater than 200000 particles cm⁻³. Similar patterns but much smaller numbers were reported when handling 15 g quantities as shown in Table 4.11.

Elsewhere the authors report total number concentrations for the different types of hoods which are much lower. They report an increase as a function of time and different performance for different fume hoods. For the conventional type, the maximum reported concentration was 2000 particles cm⁻³, two orders lower than the above calculated numbers. The maximum concentration measured was for the “bypass” type hood, where a concentration of 13000 particles cm⁻³ was measured. We have been unable to resolve the difference between these two sets of measurements.

Peters *et al.* (2009) used two methods to distinguish airborne engineered nanomaterials from other airborne particles in a facility that produces nano-structured lithium titanate metal oxide powder. The first method involved off-line analysis of filter samples collected with conventional respirable samplers at each of seven locations (six near production processes and one outdoors). Throughout most of the facility and outdoors, respirable mass concentrations were low (< 0.050 mg m⁻³) and were attributed to particles other than the nanomaterial (assessed at < 10% by mass titanium as determined with inductively coupled plasma atomic emission spectrometry). However, in a single area with extensive material handling, mass concentrations were greatest (0.118 mg m⁻³) and contained up to 39% ± 11% lithium titanium, indicating the presence of airborne nanomaterial. Analysis of the filter samples collected in this area by transmission electron microscope and scanning electron microscope revealed that the airborne nanomaterial was associated only with spherical aggregates (clusters of fused 10-80 nm nanoparticles) that were larger than 200 nm. This analysis also showed that nanoparticles in this area were the smallest particles of a larger distribution of sub-micrometer chain agglomerates likely from welding in an adjacent area of the facility. The second method used two hand-held, direct-reading, battery-operated instruments to obtain a time series of very fine particle number (< 300 nm), respirable mass, and total mass concentration, which were then related to activities within the area of extensive material handling. This activity-based monitoring showed that very fine particle number concentrations (< 300 nm) had no apparent correlation to worker activities, but that sharp peaks in the respirable and total mass concentration coincided with loading a hopper and replacing nanomaterial collection bags. These findings were consistent with those from the filter-based method in that they demonstrate airborne nanoparticles in this facility to be dominated by “incidental” sources (e. g. welding or grinding), and that the airborne “engineered” product is predominately composed of particles larger than several hundred nm.

Hsu *et al.* (2007) investigated nanoparticle emission of TiO₂ nanoparticles coated on different substrates including wood, polymer, and tile. Emissions were evaluated in a simulation box and measured with an SMPS. The coating process for the substrate followed the instructions given by the supply company. The simulation process was intended to mimic weathering and human contact. In the simulation box, a UV light, fan, and rubber knife were used to simulate the sunlight, wind, and human contacting conditions. Overall, the emission rate was extremely low. Among the three selected substrates, the coated tiles were found to have the highest particle

emission (reported as 22 particles cm^{-3} at 55 nm) due to nanopowder separation during the simulation process. The results show that, under the conditions of UV lamps, a fan and scraping motion, particle number concentration or average emission rate decreased significantly after 60 and 90 minutes for TiO_2 /polymer and TiO_2 /wood, respectively. However, the emission rate continued to increase after 2 hours of testing for TiO_2 /tile. The authors concluded that nanoparticle emission evaluation is necessary for products with nanopowder coating.

In summary, four studies were identified as relevant to occupational exposure to metal oxides. One of these is Demou et al which has already been discussed fully under the “metals” section. Of the three remaining, one was a powder handling activity, one a manufacturing activity and one a surface treatment simulation. Both the powder handling and manufacturing studies showed evidence of elevated exposures. The surface treatment study demonstrated relate of particles but at very low levels.

4.6 EFFECTIVENESS OF EXPOSURE CONTROL

In occupational settings exposure control is most often achieved through engineering control (e.g. by enclosure, or ventilation processes) or through the use of respiratory protective devices such as facemasks.

For air velocities prevailing in workplaces, airborne nanoparticles can be considered as having no inertia. They will therefore behave in a similar way to a gas and if not fully enclosed will diffuse rapidly and will remain airborne for a considerable time. Because of their high diffusion velocity, these particles will readily find leakage paths in systems in which the containment is not complete. Engineering control systems designed for use to control nanoparticles such as enclosures, local ventilation or general ventilation therefore should be of similar quality and specification to that which is normally used for gases rather than for particulate challenges. Like all such systems however, effective performance is highly dependent on appropriate use and maintenance.

Exposure control for nanoparticles is likely not to be highly dependent on the composition of material since the primary process of both collection and dispersion is diffusion, and this is largely a function of physical size. Because of this, trying to differentiate by particle type in this section is not likely to be helpful.

Several of the studies discussed above have considered the effectiveness of the control systems operating. In general, these have been shown to be broadly effective, with exposure only occurring where the systems are not used properly, are defective or have been deliberately disabled (e.g. Han *et al.* 2008).

Filtration theory indicates that commercial respirator filters should be efficient collectors of particles in the nm size range due to capture of particles by diffusion (e.g. Aitken *et al.* 2004). In fact conventional filtration theory suggests that the most penetrating particle size for respirator filters is likely to be of the order of 300 - 500 nm, which represents a minimum between the mechanisms of diffusion (greater for smaller particles) and impaction (greater for larger particles). For this reason, methods for commercial approval of respirator filters routinely use a challenge aerosol in this size range. However, it has been suggested that this model may break down at particle sizes on a few nm due to a process called thermal rebound (Wang and Casper 1991).

Rengasamy *et al.* (2008) describe a study investigating the filtration performance of NIOSH-approved N95 and P100 filtering facepiece respirators against six different monodisperse silver aerosol particles in the range of 4-30 nm diameter. They constructed a particle test system which could deliver and measure penetration of monodisperse silver particles. Five models of N95 and two models of P100 filtering facepiece respirators were challenged with monodisperse silver aerosol particles of 4, 8, 12, 16, 20, and 30 nm at 85 l min^{-1} flow rate and percentage penetrations were measured. Consistent with single-fibre filtration theory, N95 and P100 respirators challenged with silver monodisperse particles showed a decrease in percentage penetration with a decrease in particle diameter down to 4 nm. Penetrations less than 1 particle per 30 minutes for 4-8 nm particles for one P100 respirator model, and 4-12 nm particles for the

other P100 model, were observed. Small, but significant, differences were seen in the results obtained between silver nanoparticle and NaCl particles (the usual test aerosol for commercial testing). The authors state that the filtration data for 4-30 nm monodisperse particles supports previous studies that indicate NIOSH-approved air-purifying respirators provide expected levels of filtration protection against nanoparticles. Although this study uses silver nanoparticles, the results obtained should also be applicable to other metals, metal oxides and fullerenes since the primary parameter which governs collection is physical size.

Whilst this demonstrates the effective filtration provided by devices of this type, it does not deal with leakage around the facepiece which is likely to be the primary issue in relation to respirator performance. No studies have as yet dealt with this for nanoparticles.

For filters of the type which may be used in air cleaning, a recent publication from the University of Minnesota (Pui *et al.* 2008) reports on the effectiveness of simple in-car filtration systems. The paper characterises the reduction of nanoparticle number concentrations when using the recirculation mode of the ventilation system in two types of cars. In simulated heavy-traffic situations, where particle number concentrations can be greater than 50000 particles cm⁻³ for particle diameters less than 50 nm, the authors determined that setting the car's air ventilation systems in recirculation mode can decrease the number concentrations to background levels of less than 4000 particles cm⁻³ in approximately 3 minutes.

In summary, exposure control for nanoparticles is likely not to be highly dependent on the composition of material since the primary process of both collection and dispersion is diffusion in that this is largely a function of physical size. Most of the exposure studies have shown that exposure only occurs where the exposure control systems are defective or have been deliberately deactivated. In a general sense the methods used appear to work, provided they have been properly maintained. One exception to this is the Demou study where exposure was observed. Respirator and other filters appear to be effective at collecting nanoparticles, as theory predicts.

4.7 ENVIRONMENTAL EXPOSURE

It is clearly possible that fugitive emission from processes in which nanomaterials are produced, could potentially lead to increased air concentrations of these nanomaterials. Wide environmental exposure, as well as specific to workers or consumers, could occur as a result of these releases. In this section we consider the possibility of human exposure through release to the environment.

In their 2009 review, SCENIHR evaluated the knowledge base on the release of nanomaterials into the environment and the subsequent exposure of humans from that route. In their view, there was no adequate information available on this topic (SCENIHR 2009), as indicated by the following text:

“Examples of exposure routes for nanomaterials via the environment are inhalation by humans, and other air-breathing species, and uptake by aquatic organisms from water or sediments. Assessment of exposure concentrations of dispersed nanomaterials requires detailed insight into the processes that act on these materials in the environment. However, currently available knowledge of these processes is insufficient to allow quantitative predictions of the environmental fate of nanomaterials.”

This section aims to assess and identify what evidence there is in regards to environmental exposure to specific groups of nanomaterials.

4.7.1 Fullerenes

Boxall *et al.* (2007) provided an early estimate of uses of specific nanomaterials in a variety of consumer products in the UK. These authors made a variety of assumptions in terms of market penetration and use, which resulted in a range of exposure scenarios in different media (water, soil, air). As displayed in Table 4.12, average predicted environmental concentration values of fullerenes proposed by Boxall *et al.* (2007) for aquatic and soil systems were 0.31 µg l⁻¹ and

13.2 µg kg⁻¹, respectively. In contrast, no specific data could be proposed for PEC of fullerene in air.

Table 4.12: Average PECs for selected nanoparticles (extracted from Boxall et al. 2007)

Material	Compartment		
	Water (µg l ⁻¹)	Soil (µg kg ⁻¹)	Air (mg m ⁻³)
Ag	0.010	0.43	-
AlO ₃	0.0002	0.01	-
Au	0.14	5.99	-
CeO ₂	< 0.0001	< 0.01	6 x 10 ⁻⁷
Fullerenes	0.31	13.2	-
SiO ₂	0.0007	0.03	-
TiO ₂	24.5	1030	7
ZnO	76	3194	-

4.7.2 Carbon nanotubes

Mueller and Nowack (2008) used a life-cycle perspective to model the quantities of specific nanomaterials released into the environment. Three types of nanomaterials were studied: nanoparticulate silver, nanoparticulate TiO₂ and CNT. The quantification was based on a substance flow analysis from products to air, soil, and water in Switzerland.

The model inputs used were: estimated worldwide production volume, allocation of the production volume to product categories, particle release from products, and flow coefficients within the environmental compartments. The PEC were then compared to the predicted no effect concentrations (PNEC) derived from the literature to estimate a possible risk. The expected concentrations of the three nanoparticles in the different environmental compartments vary widely, caused by the different life cycles of the nanoparticle containing products. The calculated PEC values for CNT are shown below in Table 4.13.

The authors note that the lack of information concerning nanomaterial usage in consumer products is a limitation to their work. In order to address the uncertainty of data in this work, two scenarios were modelled: a realistic exposure scenario based on the most reliable data and a high exposure scenario including the worst-case assumptions. A future scenario was not conducted, as the predictions for the development of the production volumes of the nanoparticle are too vague. As the authors state, it seems to be likely that the production volumes of all three substances will increase significantly in the coming years. These conclusions are also relevant to the metals, and metal oxides PEC calculations described below.

Table 4.13: Calculated PEC values for CNT (Mueller and Nowack, 2008)

Compartment	Realistic exposure	High exposure
air (µg m ⁻³)	0.0015	0.0023
water (µg l ⁻¹)	0.0005	0.0008
soil (µg kg ⁻¹)	0.01	0.02

4.7.3 Metals

As described above, Mueller and Nowack (2008) used a life-cycle perspective to model the quantities of engineered nanoparticles released into the environment including Ag nanoparticles. The quantification was based on a substance flow analysis from products to air,

soil, and water in Switzerland. For silver, Mueller and Nowack (2008) calculate the following PECs, displayed in Table 4.14.

Table 4.14: Calculated PECs for silver (Mueller and Nowack, 2008)

Compartment	Realistic exposure	High exposure
air ($\mu\text{g m}^{-3}$)	0.0017	0.0044
water ($\mu\text{g l}^{-1}$)	0.03	0.08
soil ($\mu\text{g kg}^{-1}$)	0.02	0.1

In contrast and as previously mentioned, Boxall *et al.* (2007) provided an early estimate of uses of specific nanomaterials in a variety of consumer products in the UK. As shown earlier in Table 4.12, the average PECs for silver proposed by Boxall *et al.* (2007) for aquatic systems and terrestrial systems were 0.010 and 0.43 $\mu\text{g kg}^{-1}$. In addition, the average predicted environmental concentrations for gold in aquatic and terrestrial systems were 0.14 $\mu\text{g l}^{-1}$ and 5.99 $\mu\text{g kg}^{-1}$, respectively. No specific data was proposed for PECs of these metals in air.

4.7.4 Metal oxides

For TiO_2 , Mueller and Nowack (2008) calculated the following PECs, displayed in Table 4.15.

Table 4.15: Calculated PECs for TiO_2 (Mueller and Nowack, 2008)

Compartment	Realistic exposure	High exposure
air ($\mu\text{g m}^{-3}$)	0.0015	0.042
water ($\mu\text{g l}^{-1}$)	0.7	16
soil ($\mu\text{g kg}^{-1}$)	0.4	4.8

Again in contrast and as previously shown in Table 4.12, Boxall *et al.* (2007) provided average PECs for TiO_2 for aquatic systems and terrestrial systems of 24.5 $\mu\text{g l}^{-1}$ and 1030 $\mu\text{g kg}^{-1}$. In addition, the average PECs for AlO_3 , CeO_2 , SiO_2 and ZnO were provided for aquatic and terrestrial systems. With the exception of CeO_2 , PECs were not proposed for these metals in air.

However, an application which could lead to increased exposure of the general population is the use of metal oxides as a fuel additive. Boxall *et al.* (2007) did estimate the potential concentrations of cerium-oxide (CeO_2) in air, based on assumptions of the quantity of cerium-oxide present in fuel, the uptake of fuel and by using dispersion models developed and validated and employed by the Highways Agency. In their assessment, using a mix of traffic type with traffic flowing at 40 km hr^{-1} and at 1000 vehicles day^{-1} , they estimated a cerium-oxide concentration at a distance of 5 meters from the road of 0.0006 $\mu\text{g m}^{-3}$. In relation to occupational exposure this is very low. Boxall *et al.* (2007) estimate, however, did not take into account standing traffic in congested city centre streets.

This same application was assessed by Park *et al.* (2008) based on the introduction of the product ENVIROX to the London and Newcastle Stagecoach bus fleets in 2003 and 2005. Their study uses two models developed under contract with the European Commission (TRENDS and COPERT) which take account of traffic mix and growth (vehicle type, diesel/petrol) to calculate PM emissions for a baseline scenario and a “trap application” scenario. Atmospheric emissions of CeO_2 were calculated up to the year 2020 for 15 member states assuming a 5 ppm concentration in the diesel fuel. Air concentrations were calculated using the EPA HIWAY2 model (USEPA 1980). Full details are provided in the paper. The main emission results provided by Park *et al.* (2008) are shown in Table 4.16.

Table 4.16: Emission results from Park *et al.* (2008)

	Worst case	Best case
Urban (g person⁻¹ yr⁻¹)	2.9	0.2
Rural (g km⁻² yr⁻¹)	116.8	6.6
Highway (g km⁻¹ yr⁻¹)	560.6	295.5

Based on the HIWAY 2 model, concentrations in a “street canyon” were estimated to be between 5 and 25 ng m⁻³. The authors point out that these figures are likely to be overestimates since the model assumes that the CeO₂ does not fall out or get washed out of the atmosphere.

4.8 CONSUMERS’ EXPOSURE TO NANOMATERIALS

Many of the applications suggested or realised for nanomaterials indicate the possibility of human and environmental exposure. The Woodrow Wilson database cites many examples including personal care, skin products, food additives, cleaning and sealing products, paints and other products. However, until now there have been few attempts to quantify these exposures.

4.8.1 Fullerenes

The use of fullerenes has been reported in skin care products (Boxall *et al.* 2007). Examples include Sircuit OMG Serum and Zelens Fullerene C-60 Day Cream (<http://www.nanotechproject.org/inventories/consumer/>; accessed 15th October 2009). The use of fullerenes in drug delivery has also been reported. These applications may lead to dermal exposure, exposure by injection and exposure via aquatic media.

4.8.2 Carbon nanotubes

Mueller and Nowack (2008) indicated that possible applications of CNT which could result in exposures to humans included plastics, sporting equipment, electronic equipment, building materials and specialist paints. The use of CNT in textiles has also been reported. The main exposures would result from mechanical processing or abrasion of these products and from disposal/recycling.

4.8.3 Metals

Mueller and Nowack (2008) indicated that possible applications of silver which could result in exposures to humans include textiles, cosmetics, sprays, cleaning agents, metal product, plastics and paint. The use of nanosilver as a food supplement has also been widely reported (<http://www.nanotechproject.org/inventories/consumer/>; accessed 15th October 2009). Other metals are likely to have a similar range of applications. These applications lead to potential exposures by inhalation, dermal uptake and by ingestion. These exposures have not yet been quantified to any extent.

4.8.4 Metal oxides

Mueller and Nowack (2008) also indicated that possible applications of TiO₂ could result in exposures to humans via applications including cosmetics, sprays, cleaning agents, metal product, plastics and paint. The use of nanosilver as a food supplement has also been widely reported (<http://www.nanotechproject.org/inventories/consumer/>; accessed 15th October 2009). Other metals oxides are likely to have a similar range of applications. These applications lead to potential exposures by inhalation, dermal uptake and by ingestion.

Some work has been done to quantify exposure to metal oxide nanoparticles in sunscreens. In a review carried out on the behalf of DEFRA, Boxall *et al.* (2007) used a simple modelling approach to estimate possible exposure by inhalation and dermal exposure from spray-on sunscreen. Based on information obtained about the quantity of nanomaterials present in the sunscreen, the suppliers instructions regarding usage of the product and making an estimate of

the fugitive spray emission being 10% of the total spray (indicating that 90% of the material actually deposits on the surface of the skin), they estimated that an inhalation exposure concentration of 3.5 mg m⁻³ was plausible. In an occupational context this would be a non-trivial exposure concentration, albeit that the duration of exposure to this concentration will be very short.

In an earlier part of their report they also estimated approximately 3 g might be applied daily, which would give a skin coverage of about 1 mg cm⁻² of skin. Of this mass dose, approximately 5% is the nanoparticle ingredient. Although very few materials have an occupational exposure limit, this again would be a non-trivial exposure.

4.9 CONCLUSIONS AND RECOMMENDATIONS

In general, there is a paucity of published information and data concerning exposure to nanomaterials. Various types of studies have been used in an attempt to provide the maximum information available.

There are a few studies which have indicated possible or plausible exposure scenarios. While these studies are useful in mapping the exposure landscape, they do not in themselves lead to clear numerical estimates of exposure. Exposures are clearly plausible in occupational, consumer and environmental settings throughout the lifecycle of materials and products.

We have also considered modelling studies and have identified two (Boxall *et al.* 2007; Mueller and Nowack 2008) which provide useful information relating to environmental and consumer exposure.

For the materials of interest we have identified only 11 studies which have reported measured exposure data. All of them were carried out in the occupational setting – no studies have reported consumer exposures or exposures in the environment. All but one of the studies have reported only inhalation exposure – one study reported dermal exposure and no studies reported ingestion exposure. Most of the studies were carried out in research settings, however, some industrial settings were also found. A wide range of instruments and approaches were used and exposures were reported in terms of number, mass and surface area concentrations/doses, as totals and differentiated as a function of size. Most studies showed some evidence of elevated exposures although these were often associated with ineffective or deliberately disabled control systems. Studies which have assessed the effectiveness of respiratory filters have shown that, as theory predicts, they are efficient collectors of nanomaterials.

More information and data on occupational, consumer and environmental exposure are urgently needed to support effective risk assessment and characterisation.

4.10 REFERENCES

- Aitken, R.J., Chaudhry, M.Q., Boxall, A.B. and Hull, M. 2006, "Manufacture and use of nanomaterials: current status in the UK and global trends", *Occupational medicine (Oxford, England)*, vol. 56, no. 5, pp. 300-306.
- Aitken, R.J., Creely, K.S. and Tran, C.L. 2004, *Nanoparticles: An Occupational Hygiene Review*, HSE Books, Sudsbury, UK.
- BAUA. 2009, *Exposure to nanomaterials in Germany*. Accessed at: http://www.baua.de/nn_49456/en/Topics-from-A-to-Z/Hazardous-Substances/Nanotechnology/pdf/survey.pdf (16th October 2009).
- Bello, D., Hart, A.J., Ahn, K., Hallock, M., Yamamoto, N., Garcia, E.J., Ellenbecker, M.J. and Wardle, B.L. 2008, "Particle exposure levels during CVD growth and subsequent handling of vertically-aligned carbon nanotube films", *Carbon*, vol. 46, no. 6, pp. 974-977.
- Bello, D., Wardle, B., Yamamoto, N., Guzman deVilloria, R., Garcia, E., Hart, A., Ahn, K., Ellenbecker, M. and Hallock, M. 2009, "Exposure to nanoscale particles and fibers during machining of hybrid advanced composites containing carbon nanotubes", *Journal of Nanoparticle Research*, vol. 11, no. 1, pp. 231-249.
- Brouwer, D.H., Gijsbers, J.H. and Lurvink, M.W. 2004, "Personal exposure to ultrafine particles in the workplace: exploring sampling techniques and strategies", *The Annals of Occupational Hygiene*, vol. 48, no. 5, pp. 439-453.
- Demou, E., Peter, P. and Hellweg, S. 2008, "Exposure to Manufactured Nanostructured Particles in an Industrial Pilot Plant", *Ann.Occup.Hyg.*, vol. 52, no. 8, pp. 695-706.
- Fujitani, Y., Kobayashi, T., Arashidani, K., Kunugita, N. and Suemura, K. 2008, "Measurement of the physical properties of aerosols in a fullerene factory for inhalation exposure assessment", *Journal of occupational and environmental hygiene*, vol. 5, no. 6, pp. 380-389.
- Han, J.H., Lee, E.J., Lee, J.H., So, K.P., Lee, Y.H., Bae, G.N., Lee, S.B., Ji, J.H., Cho, M.H. and Yu, I.J. 2008, "Monitoring multiwalled carbon nanotube exposure in carbon nanotube research facility", *Inhalation toxicology*, vol. 20, no. 8, pp. 741-749.
- Hsu, L.Y. and Chein, H.M. 2007, "Evaluation of nanoparticle emission for TiO₂ nanopowder coating materials", *Journal of Nanoparticle Research*, vol. 9, no. 1, pp. 157-163.
- International Commission on Radiological Protection (ICRP). 1994, *Human respiratory tract model for radiological protection*.
- Maynard, A.D. and Aitken, R.J. 2007, "Assessing exposure to airborne nanomaterials: Current abilities and future requirements", *Nanotoxicology*, vol. 1, no. 1, pp. 26-41.
- Maynard, A.D., Baron, P.A., Foley, M., Shvedova, A.A., Kisin, E.R. and Castranova, V. 2004, "Exposure to carbon nanotube material: aerosol release during the handling of unrefined single-walled carbon nanotube material", *Journal of toxicology and environmental health. Part A*, vol. 67, no. 1, pp. 87-107.
- Methner, M.M., Birch, M.E., Evans, D. and Hoover, M.D. 2006, *NIOSH Health Hazard Evaluation Report*, University of Dayton Research Institute, Ohio.
- Methner, M.M., Birch, M.E., Evans, D.E., Ku, B.K., Crouch, K. and Hoover, M.D. 2007, "Identification and characterization of potential sources of worker exposure to carbon nanofibers during polymer composite laboratory operations", *Journal of Occupational and Environmental Hygiene*, vol. 4, no. 12, pp. D125-30.

Engineered Nanoparticles: Review of Health and Environmental Safety

Mueller, N.C. and Nowack, B. 2008, "Exposure modeling of engineered nanoparticles in the environment", *Environmental science and technology*, vol. 42, no. 12, pp. 4447-4453.

Peters, T.M., Elzey, S., Johnson, R., Park, H., Grassian, V.H., Maher, T. and O'Shaughnessy, P. 2009, "Airborne Monitoring to Distinguish Engineered Nanomaterials from Incidental Particles for Environmental Health and Safety", *Journal of Occupational and Environmental Hygiene*, vol. 6, no. 2, pp. 73-81.

The Royal Society and The Royal Academy of Engineering (RS/Eng). 2004, *Nanoscience and nanotechnologies: opportunities and uncertainties*, The Royal Society, London.

Tsai, S., Ada, E., Isaacs, J.A. and Ellenbecker, M.J. 2009, "Airborne nanoparticle exposures associated with the manual handling of nanoalumina and nanosilver in fume hoods", *Journal of Nanoparticle Research*, vol. 11, pp. 147-161.

U.S. Environmental Protection Agency. 1980, *User's guide for HIWAY-2, A highway air pollution model*, EPA-600/8-80-018. Research Triangle Park, NC: U.S. EPA. Accessed at: <http://www.epa.gov/AMD/files.html>.

Walton, W.H. and Vincent, J.H. 1998, "Aerosol instrumentation in occupational hygiene: An historical perspective", *Aerosol Science and Technology*, vol. 28, pp. 417.

Yeganeh, B., Kull, C.M., Hull, M.S. and Marr, L.C. 2008, "Characterization of airborne particles during production of carbonaceous nanomaterials", *Environmental science and technology*, vol. 42, no. 12, pp. 4600-4606.

Zartarian, V., Bahadori, T. and McKone, T. 2005, "Adoption of an official ISEA glossary", *J. Expos. Anal. Environ. Epidemiol.*, vol. 15, pp. 1-5.

5 ENVIRONMENTAL FATE AND BEHAVIOUR

5.1 INTRODUCTION

As the use of nanomaterials increases across a wide range of sectors, environmental release is an inevitable consequence. Nanomaterial introduction into the environment may occur both intentionally and non-intentionally throughout the life-cycle of nanomaterial production, use and disposal. For example, wastewater treatment streams, landfill leachates and waste incineration products are all likely to contain nanomaterials from nano-enabled products that reach the environment following either disposal at the end of their useful life, accidental spills during production or later transport of nanomaterials, or release from wear and tear of products containing nanomaterials. In addition, intentional release into the environment must be considered, such as via the use of nano-zero valent iron in the remediation of groundwater polluted with chemicals such as chlorinated solvents (Nowack and Bucheli 2007).

Models and frameworks to describe the fate and distribution of nanomaterials are slowly being developed, some of which incorporate classical knowledge of colloid science (SCENIHR 2009) and others which apply principles used for chemical fate modelling (Mueller and Nowack 2008). Traditional predictions of fate and transport are based on inherent properties such as phase transfer properties (e.g. boiling point, vapour pressure, partition coefficients), reactivity (e.g. photo-reactivity and hydrolysis) and biological degradation behaviour (Mackay and Hendry 2009). A major knowledge gap hampering the development of such model-based approaches is our current lack of understanding of the novel physico-chemical properties exhibited by many nanomaterials and the effect these have on particle behaviour. In addition, it is most likely that those nanomaterials released into the environment will also exist as modified forms of their primary counterpart (SCENIHR 2009). Model-based approaches must be developed using empirical data as they become available, to predict nanomaterial fate and behaviour. On this basis, those models developed to date are still extremely primitive and are not sufficient to develop an understanding of nanomaterial fate and transport, and it is likely to be some time before a robust approach is available.

In order to develop a full understanding of the potential risks posed by nanomaterials, further examination of their environmental transport and fate within air, soil and water bodies is necessary. Although the current lack of quantitative exposure data hampers subsequent prediction of the environmental fate and thus concentration, the knowledge-base in this area continues to grow and develop rapidly, and much consideration has been given to extrapolating evidence from laboratory studies and from knowledge obtained with industrial chemicals. This chapter outlines some of the general principles of environmental fate and behaviour, and provides a summary of key knowledge to date.

Recently, several key reviews examining environmental fate and behaviour of nanomaterials have been published, including Klaine *et al.* (2008) and Nowack (2009). Knowledge from colloid science has also been used to provide useful additional information on the potential fate and behaviour of nanomaterials (Lead and Wilkinson 2006).

Behaviour of nanomaterials in the environment is dependent on type, form and physico-chemical characteristics of the nanomaterial in question, as well as those of the receiving environment (Chen *et al.* 2008; Chen and Elimelech 2008; Saleh *et al.* 2008b). Nanomaterial transport and distribution are influenced by a number of factors, such as Brownian diffusion, inertia effects, gravitational influences, thermal influences, pH, ionisation, and presence/absence of Natural Organic Matter (NOM). These interactions ultimately affect the processes the nanomaterial consequently undergoes in its transport and subsequent fate. Table 5.1 summarises some of the processes nanomaterials may undergo on entry into the environment.

Table 5.1: Summary of potential environmental processes affecting nanomaterials

Process	Description
Dissolution	Process whereby a solid nanomaterial dissolves into a solvent to yield a solution
Settling / Sedimentation	Process whereby nanomaterials in suspension/solution to settle out of the fluid in which they are entrained
Speciation	Association of a nanomaterial with other molecular or ionic dissolved chemical substances
Association with biotic/abiotic suspended particulate material	Processes whereby nanomaterials interact with other materials in the environment around them e.g. via adherence, sorption etc.
Transformation	Process whereby a nanomaterial undergoes either a biological or chemical transformation
Agglomeration / Disagglomeration	The process by which nanomaterials come together or spread apart within their existing environment
Complete mineralisation	The conversion of a carbon-containing nanomaterial to an inorganic state via biotic and abiotic decomposition
Diffusion	The net transport of nanomaterials from a region of higher concentration to one of lower concentration by random molecular motion
Deposition	The settling of nanomaterials from within a solution, suspension mixture or vapour, e.g. from an aerosolised form into water
Re-suspension	The renewed suspension of insoluble nanomaterials after they have been precipitated, e.g. from on a surface into gas or from sediment into water

Knowledge to date indicates that in many cases rather than remaining intact, nanomaterials will tend to aggregate, agglomerate or become associated with other dissolved, colloidal or particulate matter present in the environment (SCHENIR 2009). However, the novel physico-chemical characteristics which make nanomaterials desirable also present a challenge for determining how they interact with the environment, how, when and where they are distributed, and in what form they ultimately end (Darlington *et al.* 2009).

To date, for the nanomaterials considered within this review, there has only been one peer reviewed publication providing information on concentrations or amounts within environmental compartments such as soils or run-off (Kaegi *et al.* 2008). Much of the current knowledge therefore lies within estimates of quantities of nanomaterials based on predicted use and calculated exposure scenarios (Boxall *et al.* 2007; Mueller and Nowack 2008), although these are based on simplified assumptions and scenarios of use and environmental transfer across media. Nevertheless, methods for measuring nanomaterials in specific environmental matrices are being developed for various materials (Christian *et al.* 2008; Hassellöv *et al.* 2008; Tiede *et al.* 2008). Most recently, Kaegi *et al.* (2008) published what may be the first refereed scientific paper providing quantitative information on transport of nanomaterials in the environment, tracing leached synthetic TiO₂ nanoparticles from paint on house façades into receiving water bodies.

Appropriate metrics for measuring engineered nanomaterials in the environment are still subject to much discussion, and in particular those pertaining to exposure concentrations, or dose, are

considered to be of high importance. Differences in behaviour across different physical and chemical species of the same nanoparticulate material must also be considered. In addition, their tendency to aggregate/agglomerate, adsorb to NOM and, in the case of wastewater treatment, potentially associate with the solid phase, must be taken in consideration, as all of these processes could lead to environmental 'hot spots' where concentrations of nanoparticles are particularly high.

Current knowledge indicates that nanomaterials are most likely to exist associated with sediments and soils in the environment (Baalousha *et al.* 2008; Klaine *et al.* 2008). However, the free dispersed forms of the material are of particular importance. In order to estimate possible presence of free nanomaterials within the environment, knowledge on release scenarios must be improved, as well as knowledge on exposure scenarios in which they may be found and the various processes which act on nanomaterials in the environment. Until such a time, assessment of exposure concentrations of dispersed nanomaterials remains a difficult task (SCENIHR 2009).

5.2 ENVIRONMENTAL FATE AND BEHAVIOUR OF NANOMATERIALS IN AIR

Aerosol science is a well established and documented scientific field. However, there exist some major issues to be addressed in validation of definite hypotheses on behaviour, transport and fate of air-borne nanomaterials. For example, development of methods for accurate sampling or tracking of nanomaterials within the air compartment, elucidation of the effect of differing particle morphologies (of both nanomaterials and their agglomerates), and overcoming the issue of differentiating airborne manufactured nanomaterials from background levels in the atmosphere. As an illustration of the latter issue, investigations by Murr and colleagues (Murr *et al.* 2004; Murr and Guerrero 2006) suggest that CNT can originate from general combustion processes, and as such can be found in many ambient locations.

In addition, as nanomaterials may range from 1-100nm in size, the processes governing their transport are very diverse. Mädler and Friedlander (2007) illustrate that whilst transport of small nanoparticles (circa 1 nm) may have similarities to molecular transport, as the size of the nanoparticle increases, momentum exchange with the gas molecules and nanoparticle inertia alters and extends into the regime where corrections to the gas continuum model can be used.

To date, much of the available data have been gathered in the context of worker exposure so that any risks can be assessed and required management procedures implemented (SCENIHR 2009). This has provided preliminary information and data in relation to the determination and characterisation of nanomaterial distribution assessment of potential exposure, and effectiveness of personal protective equipment (Aitken *et al.* 2009). Most of these studies relate to only one or two types of nanomaterials, and it is unclear whether it will be possible to extrapolate the results to other types. No quantitative or qualitative measurement of manufactured nanomaterials in ambient air outwith the occupational environment have been reported. Simplified modelling work has been undertaken to predict nanomaterial distribution and concentrations in the environment (Boxall *et al.* 2007; Muller and Nowack 2008). However, whilst these models are useful tools, they are very simple, and under development and do not include sufficient detail to allow the drawing of conclusions on environmental transport and behaviour of nanomaterials.

Aitken *et al.* (2004) provide a discussion of processes governing nanoparticulate behaviour in air with a particular focus on the workplace. Key processes governing nanomaterial behaviour include diffusion, agglomeration, and potential re-suspension of aerosol from deposited nanomaterials. The same authors report that in traditional aerosol science, the key process governing aerosol behaviour is particle size, whilst inertial, gravitational and diffusional forces are also important. As particle size decreases, diffusional forces become increasingly important and nanoscale particles are thus likely to behave in a manner more akin to a gas or vapour (ICRP 1994).

Particle diffusion (governed by Brownian motion) is a process whereby particles suspended in gas are constantly and randomly bombarded from all sides by molecules of the gas, causing them to move in a seemingly random fashion. Particle diffusion coefficient (rate) is inversely

proportional to particle diameter. Particles with a high diffusion coefficient (such as those on the nanoscale) therefore have high mobility and will mix rapidly in aerosol systems. Upon release into the environment, atmospheric diffusion will lead nanomaterials to migrate rapidly from a high concentration to a lower one, and thus resulting in rapid dispersion and potential for particles to travel a great distance from source (Aitken *et al.* 2004).

Agglomeration is the process by which particles 'coagulate' or 'stick together' to form larger bodies (although these may still be on the nanoscale). It is in essence a by-product of the multiple collisions particles undergo during diffusion. Agglomeration rate is governed primarily by particle mobility and number concentration, both of which increase as particle size decreases (Aitken *et al.* 2004). Thus, 'aerosolised' nanoparticles tend to agglomerate rapidly, even at a low mass concentration. Kasper (2008) highlights that aged nanoparticle aerosols are also not recognisable by a specific particle size range, and tend to populate a size range almost indistinguishable from ubiquitous background aerosol as determined by simple size distribution measurements. This means that tracking and measurement of nanoparticle concentrations within air is very difficult, unless measurements are taken close to a point source emission.

Deposition is a process by which nanoparticles may be removed from the atmosphere, for example onto soil, water or plants (Aitken *et al.* 2004). Deposition of particles is dependent on the gravitational settling velocity, a characteristic which is proportional to the diameter of the particle. Nanoparticles in air will therefore deposit at a much slower rate than larger particles, and contribute to the potential for nanoparticles to be transported over great distances within the environment before being deposited.

Potential re-suspension of nanoparticles from deposits into the atmosphere is a complex concept, influenced by a number of factors including particle size, shape, charge and the energy applied to the potential re-suspension situation. Particles are held together in agglomerates (and to the surface on which they are deposited) by short-range attractions called Van der Waals forces (Aitken *et al.* 2004). It is thus questionable as to how likely re-suspension would be, once particles are deposited, a view supported by the work of Maynard *et al.* (2004) which indicated re-suspension of CNT into aerosol was not easily achieved.

In summary, the current lack of data in relation to environmental fate and behaviour of nanomaterials represents a major gap in developing a holistic view on the fate and transport of nanomaterials within the environment. However, much work is underway, and it is likely that in the coming years more relevant scientific information will emerge on which to develop further understanding of the area (Aitken et al. 2009; SCENIHR 2009).

At the current time, most of our knowledge stems from aerosol science, which provides preliminary information from which to develop further understanding of nanoparticle fate and transport within air. To date, very little work has been undertaken in other environmental media and as such it is difficult to assess further the environmental fate of nanomaterials. There is, therefore, much need for systematic studies on different types of nanomaterials using a range of physico-chemical parameters (e.g. size, shape, form, surface area) to generate data and to support development of reliable models. In addition, predictive modelling of emission scenarios and subsequent transport pathways (both for indoor and outdoor air) will play an important role in furthering understanding in this area. In concordance with this, the development of more sophisticated predictive models is currently underway, especially for the workplace setting. The development of valid real-time methods by which to selectively detect and quantify specific aerosolized nanoparticles is also crucial, as is the characterisation of sources and release mechanisms for nanomaterials into the environment, according to source strength and nanomaterial physico-chemical characteristics (Wu et al. 2008). A final consideration which must be taken into account is the choice of suitable metrological strategies for monitoring transport and fate of nanomaterials within aerosols; such a strategy must be made carefully, as it is predicted that size of these materials may increase rapidly via agglomeration.

5.3 ENVIRONMENTAL FATE AND BEHAVIOUR OF NANOMATERIALS IN WATER

There exists a body of knowledge on dispersal, accumulation, breakdown, and persistence of conventional chemicals in the environment. However, relatively little is known about the potential fate and behaviour of nanomaterials in aquatic environments. Development of a greater understanding of the mechanisms underlying nanomaterial fate and transport, and the influences which govern these, is imperative in order to assess risks associated with current and future environmental exposure to free nanomaterials (Aitken *et al.* 2009).

It is generally expected that dispersed nanomaterials within water will behave according to the well-described and understood phenomena which govern colloid science (Jones 2002; Lyklema 2005). Colloidal suspensions of nanomaterials are generally expected to be unstable; for example, upon collision, particles may approach each other close enough for weakly attractive Van der Waals forces to become dominant over repulsive electrostatic forces and steric hindrance (SCENIHR 2009). Consequently, particles may adhere to each other and then settle due to gravity (Baalousha *et al.* 2008; Ju-Nam and Lead 2008; Saleh *et al.* 2008b). In accordance with this, it has been shown that suspensions of dispersed nanomaterials may only be stable under narrow ranges of well-defined environmental conditions (Baalousha *et al.* 2008).

Natural waters may contain other suspended or dissolved materials, both colloidal and solid (including natural nanomaterials). Thus, engineered nanomaterials introduced into water bodies usually can, and are indeed expected to adhere to such materials (SCENIHR 2009).

In accordance with the principles of colloid science (under natural conditions), nanomaterial fate and transport may therefore be influenced by factors such as pH, ionic strength and presence of natural organic matter upon release into water (Lead and Wilkinson 2006). Within sea water, with high pH and ionic strength, electric double layers of colloid particles are much smaller than in freshwater, allowing for closer inter-particle approach, a process which usually leads to increased aggregation. In addition, the intrinsic properties and characteristics of the materials, including their specific chemistry, will influence their fate and behaviour. The surface properties of the nanomaterials are very important for their aggregation behaviour, and thus for their mobility in aquatic and terrestrial systems, and as such for their interaction with and general bioavailability to organisms.

Consequently, depending on their type, nanomaterials may undergo a number of processes, including partitioning into sediment and suspended particulate material, biological degradation and abiotic degradation (Boxall *et al.* 2007; Fortner *et al.* 2005).

Boxall *et al.* (2007) and Mueller and Nowack (2008) have provided estimates of nanomaterials present in surface waters and other media based on predicted use and calculated exposure scenarios. The availability of such models will be valuable in providing quantitative estimates of environmental concentrations (Aitken *et al.* 2009). Gao *et al.* (2009) recently published predictions on dispersion and toxicity of fullerenes, nano silver and nano copper in natural river water samples, but model approaches alone are not enough to make informed risk assessments and the development of monitoring methods for environmental samples is crucial. While techniques for identification and quantification of nanomaterials in various environmental matrices are being developed, it remains a challenging task to attempt monitoring in real-world environmental samples, although progress is slowly being made (Christian *et al.* 2008, Hassellöv *et al.* 2008, Tiede *et al.* 2008).

In the following section, factors of importance for fate and behaviour in the aquatic environment will be briefly reviewed for the four groups of nanomaterials relevant for the review.

5.3.1 Fullerenes

Given the sparse solubility of fullerenes in water there is much research undertaken into their behaviour in aqueous suspensions/solutions, their functionalisation and derivatives, all of which

have unusual properties and characteristics (Wudl 2002). Fortner *et al.* (2007) described the reaction of water stable fullerene aggregates with ozone in water. Their findings showed that the presence of dissolved ozone in the aqueous phase led to fullerene aggregate dissolution, concurrent with formation of water-soluble fullerene oxide species, and thus highlighting the importance of considering the aqueous reactivity of fullerene-based nanomaterials in assessing their transport and fate.

Compared to the synthetic aqueous media used in most laboratory studies, the environmental behaviour of fullerenes may be significantly influenced by the presence of NOM. This has been shown by several studies, including Xie *et al.* (2008) who examined the effects of aquatic NOM on the physico-chemical properties of aqueous C₆₀ nanoparticles. The authors carried out a thorough characterisation of the C₆₀ for particle size, morphology, and electrophoretic mobility in the absence and presence of two model NOM components, Suwannee River humic acid and fulvic acid. They reported that NOM caused disaggregation of C₆₀ crystals and aggregates, thus leading to significant alterations in particle size and morphology, and thus potential alterations in the transport and fate of the C₆₀ nanoparticles in the aquatic environment. Another issue to consider is how to quantify the fullerene aggregates in water. Chen *et al.* (2008) investigated methods for quantifying stable aqueous C₆₀ aggregates of approximately 60 to 70 nm in diameter in water. Of the three methods investigated, aqueous C₆₀ concentrations as low as 300 ng l⁻¹ in water were quantified using solid-phase extraction separation method. The other methods (evaporation of sample to dryness and extraction using 20% NaCl into toluene) were less successful in complex water matrices.

In summary, fullerenes are of low solubility in water; much research to date has focussed on their behaviour in aqueous suspension/solution, their functionalisation and derivatives (Wudl, 2002). The presence of NOM within the aquatic environment may significantly influence transport and behaviour of fullerenes (Xie et al. 2008). Fullerenes may interact with other materials/chemicals within water bodies such as ozone (Fortner et al. 2007).

5.3.2 Carbon nanotubes

In their 2008 review, Kennedy *et al.* (2008) discussed important factors affecting the partitioning and toxicity of nanotubes in the aquatic environment. The group considered that whilst the hydrophobicity and van der Waals interactions between nanotubes suggest aggregation and sedimentation in aquatic systems, engineered surface modifications may alter their environmental fate. Column stability and settling experiments carried out by the group indicated that un-functionalised or coated MWCNT settled more rapidly than functionalised MWCNT, although this finding would be dependent on functionalisation type. In addition, the group observed that stabilisation of MWCNT was especially notable in the presence of NOM. Their results highlight the need to avoid classifying all CNT into one category in terms of their predicted environmental fate and transport.

Hyung *et al.* (2007) considered how NOM present in the aquatic environment may affect CNT fate and transport. These authors investigated how the aqueous stability of MWCNT was altered by the presence of NOM. Those MWCNT tested were seen to be readily dispersed forming a aqueous suspension in the presence of humic acids, which remained stable for over 1 month. Their findings indicate that through this association, dispersal of carbon based nanomaterials in the natural aqueous environment may occur to a greater extent than expected.

Further to Hyung *et al.* (2007), Hyung and Kim (2008) investigated the effect of NOM characteristics and water chemistry (such as pH and ionic strength) on adsorption of NOM to MWCNT. They reported that NOM adsorption to MWCNT varied greatly dependant on the type of NOM, and in direct proportion to the aromatic hydrocarbon content of NOM. In addition, water chemical parameters greatly altered adsorption, which increased as pH decreased and ionic strength increased. Consideration of both the type of NOM present in any aquatic environment into which CNT may be transported, and the effect that NOM may have on CNT dispersion and stability, are therefore clearly important factors to take into account in the development of knowledge of CNT fate and transport.

The influence of other materials (e.g. mineral and other content) within the aquatic environment on MWCNT has also been investigated. In their work on the stability of MWCNT suspensions, Han *et al.* (2008) reported that the presence of the clay minerals, montmorillonite and kaolinite, resulted in deposition of some types of surfactant-facilitated MWCNT suspensions. These authors postulated that this effect could be due to either removal of surfactant from solution and MWCNT surface by the clay minerals, or bridging between the clay mineral and the MWCNT via surfactant. This is therefore also an important consideration to take into account in relation to MWCNT transport and behaviour within aquatic bodies.

In summary, the work published to date on the fate and behaviour of CNT in the environment, focuses on MWCNT. Functionalisation and stabilisation of MWCNT affect their fate and behaviour in water bodies (Kennedy et al. 2008). To date, NOM has been found to have an effect on fate and transport of the MWCNT types tested (Hyung et al. 2007, Hyung and Kim, 2008, Kennedy et al. 2008). The characteristics of the NOM present and water quality parameters have been reported to affect MWCNT fate and behaviour in the environment (Hyung and Kim, 2008). Mineral content was found to have an effect on the dispersion and fate of MWCNT (Han et al. 2008).

5.3.3 Metals

The fate and behaviour of metal nanoparticles in aqueous suspension are expected to be dependent on the metals' chemical identity and therefore no general conclusion can be made. Note that this would also be an impossible task for their bulk counterpart as e.g. the fate and behaviour of mercury, cadmium, or iron will be very different. In the following, a few studies dealing with metal nanoparticles will be mentioned in relation to important parameters shown to affect their behaviour in aqueous media.

Diegoli *et al.* (2008) investigated the interaction of manufactured gold nanoparticles and NOM, by testing the effect of humic acid on citrate- and acrylate-stabilised gold nanoparticles. The group found that at high ionic strengths, the gold nanoparticles underwent extensive aggregation. Humic acid was found to enhance gold nanoparticle stability at extremes of pH – probably by substituting or coating the original stabiliser on the particles. As with CNT, the presence of NOM is therefore most likely to be an influencing factor on the behaviour of metal nanomaterials within the environment.

In considering the agglomeration behaviour of metal nanomaterials in water, Schrick *et al.* (2004) demonstrated that unsupported iron nanoparticles within water rapidly agglomerate. However, association of the iron nanoparticles with delivery vehicles such as anionic hydrophilic carbon, and poly(acrylic acid) (PAA), led to the formation of colloidal suspensions which settled very slowly (hours to days) in water.

A recently published paper by Gao *et al.* (2009) also considers dispersion and agglomeration of nanomaterials in water bodies. The study provides predictions on dispersion and toxicity of manufactured fullerenes, nano-silver and nano-copper in natural river water samples (from the Suwannee River Basin) and deionised water samples, in order to determine the effect of NOM concentration and solution ionic strength. The dispersion of all three nanoparticles tested varied significantly with solution chemistry: NOM, pH and concentrations of various electrolytes all had significant effects on the agglomeration pattern of all three nanoparticles tested. It was observed that Ag nanoparticles had a considerably altered agglomeration pattern from Cu nanoparticles and C₆₀, a difference potentially stemming from Ag nanoparticle's lower hydrophobicity. In contrast, presence of NOM (as with the Suwannee River water samples) brought about steric repulsion forces as the NOM adsorbed quickly onto the surface of the nanomaterial and stabilised the suspension, so much so that even a small NOM concentration was enough to prevent aggregation.

The effect of dispersant type, pH and concentration of nanomaterial has been investigated for nanoparticulate copper by Li *et al.* (2007). Using zeta-potential, absorbancy and sedimentation photographs, these authors showed that zeta-potential related well to absorbancy. In addition, they showed that the higher the absolute value of the zeta potential, the better the Cu nanoparticles were dispersed and the greater their stability in liquid.

In a study which focussed specifically on nanoparticulate silver within water bodies, Benn and Westerhoff (2008) investigated nanoparticulate silver released from commercial clothing (socks) into water, and its fate in wastewater treatment plants. Using microscopic analysis, physical separation and ion-selective analysis, they found silver particles between 100-500 nm in diameter of both colloidal and ionic forms leached from 6 sock samples tested. As the leaching varied between sock type tested, they postulated that the manufacturing process may control the extent to which silver was released. In addition, the researchers used the data gathered to build a basic model to predict how well a typical wastewater treatment facility could treat a high concentration of influent silver.

In summary, as with CNT, NOM has been demonstrated to have an effect on metal nanoparticle fate and transport (Diegoli et al. 2008). Agglomeration patterns of metal nanoparticle within water vary greatly due to differences in their different physico-chemical properties. NOM, pH and concentrations of various electrolytes may all have significant effects on the agglomeration pattern of metal nanoparticles (Gao et al. 2009). Association of nanomaterials with delivery vehicles (e.g. iron nanoparticles) can lead to formation of steady colloidal suspensions (Schrack et al. 2004). Zeta-potential has been demonstrated to be related to absorbency for nanoparticulate copper (Li et al. 2007)

5.3.4 Metal Oxides

As it is the case for nanoparticulate metals, the fate and behaviour of metal oxide nanoparticles in aqueous solution/suspension is expected to be very dependent on the individual metal oxide's chemical identity. It is therefore difficult to draw any general conclusions about the environmental fate and behaviour of nanoparticulate metal oxides. However, the following section provides a summary of relevant findings to date that may contribute to a general understanding of the phenomena involved in predicting the fate and behaviour of metal oxide nanoparticles in the environment.

As an initial consideration, it is important to note that the first paper which offered quantitative information on transport of nanoparticles into the environment deals with nanoparticulate metal oxides. As described previously, the paper, authored by Kaegi *et al.* (2008), traced leached synthetic TiO₂ nanoparticles from paint on house façades into receiving water bodies; TiO₂ nanoparticles (in the size range of 20-300 nm) were found in concentrations in the range of a few mg l⁻¹ in a small stream. In an investigation of the influence of agglomeration and surfactants on the clearing efficiency of cerium oxide nanoparticles in a model wastewater treatment plant, Limbach *et al.* (2008) reported that whilst the majority of nanoparticles could be captured via adhesion to sludge, a significant proportion was recovered in the water effluent. In particular, up to 6% of the cerium oxide nanoparticles added were identified in the plant exit stream. These authors highlighted that clearance could also be significantly altered by surface charge and addition of dispersion stabilising surfactant. Further investigation into the agglomeration of those nanoparticles found in wastewater streams revealed they carried a high stabilisation against clearance, due to adsorption onto the bacteria from the sludge.

In our attempt to assess the fate and behaviour of nanomaterial metal oxides it is important to understand what external and internal (to the material) parameters may affect their stability. This has been investigated in several studies. Zhang *et al.* (2008) studied the stability of various metal oxide nanoparticles in water. The group examined characteristics, dispersion, and stability of commercially available TiO₂ (two sizes), Fe₂O₃, ZnO, NiO, and silica nanoparticles in water as well as their removal by water treatment processes. Their results found that all nanoparticles, apart from silica, showed rapid aggregation in tap water due to electric double layer compression. Silica remained stable in tap water due to its low Hamaker constant.

Ghosh *et al.* (2008) investigated whether the colloid-like behaviour of aluminium oxide nanoparticles was altered as a function of pH and the presence of natural organic matter (in the form of various humic acid types). The study found that surface charge of the nanoparticles decreased with addition of humic acids, with increasing aggregation shown as the pH of the suspension approached zero charge (i.e. where van der Waals attraction forces dominate over electrostatic repulsion). At acid pH in the presence of humic acid, nanoparticles showed strong

aggregation whereas at alkaline pHs colloidal stability was enhanced. Ghosh *et al.* (2008) also observed that the hydrophobic state of the humic acid molecules had a strong influence on aggregation of nanoparticles, something which they speculated may be due to their conformational behaviour in a particular solution condition.

Further investigation of both NOM and environmental conditions was undertaken by Baalousha *et al.* (2008), who investigated interactions between manufactured iron oxide nanoparticles (~7 nm in diameter) and humic acid, under varying environmental conditions. These authors reported that with increasing pH and humic acid concentrations, the nanoparticles were seen to undergo increasing aggregation. Additionally, they observed that humic acid presence altered the type of aggregate formed: open, porous aggregates were formed in the absence of humic acid, whilst compact ones were formed in its presence.

Recently Baalousha (2009) published a further examination of the influence of particle concentration, pH and NOM on iron oxide aggregation and disaggregation. Increasing nanoparticle concentration was shown to concurrently increase aggregation, particularly at a pH close to the point of zero charge. However, a high concentration of nanoparticles in the presence of a high humic acid concentration increased disaggregation of iron oxide nanoparticles, an effect which was not seen at lower nanoparticle concentrations or without humic acid. Baalousha (2009) also highlighted the role of NOM in disaggregation of iron oxide nanoparticle aggregation, reporting that small iron oxide nanoparticle aggregates (of about 170nm) with a surface coating of humic acid were seen during the disaggregation process, an observation which indicates that nanoparticles may mimic natural colloidal behaviour.

In summary, absorption of other materials present within water bodies (such as bacteria) has been shown to affect transport of metal oxide nanoparticles (Limbach et al. 2008). Metal oxide nanoparticle clearance within aquatic systems has been shown to be significantly altered by surface charge and addition of dispersion stabilising surfactant (Limbach et al. 2008). Although removal of metal oxide nanoparticles in standard wastewater treatment systems is high up to 6% per weight was found within the output streams from model waste water systems (Limbach et al. 2008). Several types of metal oxide nanoparticles have been shown to undergo rapid aggregation in tap water as a result of electric double layer compression (Zhang et al. 2008). As for metal nanoparticles and CNT, NOM has been found to have an effect on fate and transport of metal oxide nanoparticles (Ghosh et al. 2008). The characteristics of the NOM present and of the surrounding aquatic environment (e.g. pH level) have also been reported to affect metal oxide transport and behaviour (Ghosh et al. 2008; Baalousha 2009).

5.3.5 Summary

A number of important considerations for nanomaterial fate and transport within the aquatic environment have been highlighted to date. One consistent finding is that most nanomaterials interact with NOM, and that this influences the fate and transport of nanomaterials in water and may also be of significance for their biological effects. Absorption of other materials present within the aquatic environment has also been shown to have an impact on nanomaterial transport. Nevertheless, the lack of actual measured data in relation to the environmental fate and behaviour of nanomaterials in water represents a major gap in developing realistic prediction of fate and transport of nanomaterials in the aquatic environment. Most of our current knowledge stems from colloid science, which provides preliminary information, but there is a need for systematic studies on different types of nanomaterials within aquatic bodies using a range of physico-chemical parameters (e.g. size, shape, form, surface area) to generate data and to support development of reliable models. Predictive modelling of emission scenarios and subsequent transport pathways will also play an important role in furthering understanding in this area.

5.4 ENVIRONMENTAL FATE AND BEHAVIOUR OF NANOMATERIALS IN SOIL AND SEDIMENT

As is described within the fate and transport in the water sub-section, nanomaterials, if not degraded or dissolved and depending on the receiving environment and material, will tend to aggregate/agglomerate and eventually settle onto the substrate (SCENIHR 2009; Baalousha *et al.* 2008; Ju-Nam and Lead 2008; Saleh *et al.* 2008a). Within soil and sedimentary systems it is expected that nanomaterials will adhere to solids. As such, a fundamental understanding of the transport properties of these materials in sediment and soil compartments is necessary not only to assess their potential environmental impact but also to develop nanomaterials specifically aimed at soil related applications such as remediation of contaminants (Leocanet and Wiesner 2004).

5.4.1 Fullerenes

The mobility of nanomaterials in porous media has been investigated by Leocanet *et al.* (2004), who studied eight nanomaterials in a test system resembling a sandy groundwater aquifer. These included silica (2 sizes), anatase TiO₂, fullerol (hydroxylated C₆₀), clusters of C₆₀ referred to as *n*-C₆₀, and single-wall carbon nanotubes. Of those tested, fullerene-based nanomaterials which had been functionalised to facilitate dispersal in water exhibited the highest mobility, colloidal aggregates of C₆₀ were amongst the least mobile and SWCNT were between the two. The authors suggested that the same hydrophobicity that prevents fullerene dispersal in water is also anticipated to lead to relatively limited mobility in aquatic environments. However, as fullerene species may become more hydrophilic through manufactured or environmental modification (e.g. functionalisation) this principle cannot be applied across the board.

The effects of flow velocity on fullerene transport and deposition was also investigated by Leocanet and Wiesner (2004). Using three varieties of fullerene nanoparticles in porous media, their study compared fullerene nanoparticles to the colloid theory which states that 'particle deposition in porous media is a sequence of particle transport to the immobile surface or "collector" followed by attachment'. These authors reported that fullerene nanoparticles appear to share unique transport properties in porous media, which span a range of fullerene preparations.

The influence of electrolyte species and concentration on aggregation and transport of nanoscale fullerene nanoparticles in water-saturated quartz sands has been described by Wang *et al.* (2008). They found that as NaCl electrolyte concentration increased from 1-100 mM, there was little change in particle diameter measured. However, when the same experiment was repeated using CaCl₂ electrolyte, the C₆₀ particle diameter increased over 7 fold. This effect was attributed to agglomeration of C₆₀ nanoparticles, due to a net attractive force between particles and suppression of the electrical double layer. In relation to ionic strength, the group found that at low strengths (~3 mM), C₆₀ nanoparticles were readily transported through 40- to 50- mesh quartz sand. However, heightened ionic strength led to retention of C₆₀ nanoparticles in sand column, regardless of electrolyte species present. Their findings clearly indicate that C₆₀ nanoparticle transport and retention in water-saturated sand is strongly dependant on electrolyte conditions.

In consideration of the applicability of currently accepted transport and deposition models to nanoscale fullerenes, Li *et al.* (2008a) explored transport and deposition of nanoscale C₆₀ aggregates within water-saturated porous media. By comparing experimental column measurements to models based on clean-bed filtration theory, the group were able to show clearly that pre-existing models are not directly applicable to predict nanoparticle transport and fate. Li *et al.* (2008b) reported that the sorption capacity of C₆₀ nanoparticles to soil was strongly dependant on the soil's organic content; and that increased sorption reduced bioavailability.

Wang *et al.* (2008) also conducted experimental and mathematical modelling studies to investigate the transport and retention of nanoscale C₆₀ aggregates in water-saturated porous media (glass beads and Ottawa sand). They observed that up to 77% of the mass of C₆₀ introduced was retained in columns filled with Ottawa sand. Furthermore, they found that it was

possible to accurately simulate the retention profile and the effluent concentrations when the numerical model accounted for the C_{60} attachment kinetics and a limiting retention capacity of the solid phase.

In summary, fullerene nanoparticles appear to share unique transport properties in porous media, which span a range of fullerene preparations (Leocanet and Wiesner 2004). Fullerene-based nanoparticles functionalised to facilitate dispersal in water exhibit greater mobility in porous media than colloidal aggregates of C_{60} or SWCNT (Leocanet et al. 2004). The hydrophobicity which prevents fullerene dispersal in water is anticipated to lead to relatively limited mobility in aquatic and sedimentary environments (Leocanet et al. 2004). C_{60} nanoparticle transport and retention in water-saturated sand is strongly dependant on electrolyte conditions (Wang et al. 2008). Pre-existing clean-bed filtration theory models are not directly applicable to predict nanoparticle transport and fate (Li et al. 2008a). Finally, sorption capacity of C_{60} nanoparticles to soil is strongly dependant on the soil's organic content; and increased sorption reduces C_{60} bioavailability (Li et al. 2008b).

5.4.2 Carbon nanotubes

Only one study into the transport of single-walled carbon nanotubes in soil was identified. Jaisi *et al.* (2008) investigated the transport and deposition of carboxyl-functionalised SWCNT in quartz sand over a range of solution chemistries. The group reported that increasing solution ionic strength or addition of calcium ions resulted in increased SWCNT deposition (filtration), a finding consistent with conventional colloid deposition theories and confirming the importance of CNT physico-chemical characteristics in their transport. The group proposed that SWCNT shape and structure, particularly the very large aspect ratio and its highly bundled (aggregated) state in aqueous solutions, contribute considerably to straining (trapping of particles in pores that are too small to allow passage) in flow through porous media. In addition, the group showed that deposited SWCNT could be mobilised from quartz sand upon introduction of low ionic strength solution (KCl).

In summary, CNT shape and structure (in particular their high aspect ratio) contribute considerably to trapping in flow through porous media (Jaisi et al. 2008). The addition of low ionic strength solution can mobilise deposited SWCNT in quartz sand (Jaisi et al. 2008).

5.4.3 Metals

A recently published study by Darlington *et al.* (2009) investigated characteristics affecting the fate and transport of aluminium nanoparticles through a soil column system containing white quartz sand and soil (collected from an area of Florida USA known to have routine use of devices containing Al nanoparticles). The type of solution and surface functionalisation had a marked effect on the charge, stability and agglomeration state of the Al nanoparticles, which in turn altered transport through the soil matrix. In particular, it was found that electrostatically induced binding effects of the Al nanoparticles to the soil matrix were greater in positively charged than negatively charged particles, and that salts found within the environment greatly impacted on Al nanoparticle transport, causing agglomeration and potentially reducing bioavailability.

Doshi *et al.* (2008) also investigated the transport of nanoparticulate aluminium. Their study examined transport of two sizes of aluminium nanoparticles through sand columns with either aluminium oxide, or carboxylate ligand coatings (Alex and L-Alex respectively). As pH controls solubility and electrostatic interactions between the Al nanoparticles and porous media, pH was found to be a major determinant of Al nanoparticle transport. In addition, surface characteristics of the particles were also shown to be of importance.

Saleh *et al.* (2008a) investigated the effect of ionic strength and composition on mobility of surface-modified nanoscale zero-valent iron through porous material. The group tested a number of bare, polymer- and surfactant- modified zero-valent iron nanoparticles in water-saturated sand columns at low particle concentrations (where filtration theory is applicable). They concluded that electrosteric stabilisation of nanoparticles provides the best resistance to

altered transport caused by changing electrolyte conditions likely to be encountered in groundwater aquifers.

An investigation into transport of iron nanoparticle through various types of porous media by Yang *et al.* (2007) found that PAA modified iron nanoparticle slurry was able to travel through silica sand easily, but not through loamy sand. The authors attributed the finding to the different characteristics of the two soil types.

Schrack *et al.* (2004) found nanoparticle diffusion to be the dominant filtration mechanism of transport of zero-valent iron nanoparticle through soil and sand packed columns. Their study, which was in response to an observation that anionic, hydrophilic carbon (Fe/C) and poly-acrylic acid (Fe/PAA) supported nanoparticles settle very slowly in water, found that in clay-rich soils, the sticking coefficient of zero-valent iron nanoparticles associated with PAA was greater than that of Fe/C particles, whilst unsupported Fe nanoparticles agglomerated quickly in water and were thus efficiently filtered by all soils tested except clay (where clay-rich platelets acted as an anionic support material).

In summary, the type of solution and surface functionalisation has a marked effect on the charge, stability and agglomeration state of the Al nanoparticles, which in turn alters transport through the soil matrix. (Darlington et al. 2009). Electrostatically induced binding effects of Al nanoparticles to soil matrices have been shown to be greater in positively charged than negatively charged particles (Darlington et al. 2009). Salts found within the environment have been shown to greatly impact on Al nanoparticle transport, causing agglomeration and potentially reducing bioavailability (Darlington et al. 2009). As pH controls solubility and electrostatic interactions between nano Al and porous media, it has been found to be a major determinant of Al transport (Doshi et al. 2008). Surface characteristics of Al nanoparticles have been shown to be of importance in their transport (Doshi et al. 2008). PAA modified iron nanoparticle slurry is able to travel through silica sand easily, but not through loamy sand, most likely due to the different characteristics of the matrix of the two soil types (Yang et al. 2007). Finally, nanoparticle diffusion was found to be the dominant mechanism of transport through soil and sand packed columns for zero-valent iron nanoparticles (Schrack et al. 2004).

5.4.4 Metal oxides

Leocanet and Wiesner (2004) investigated the effects of flow velocity on transport and deposition of metal oxide nanoparticles in porous media against the colloid theory that 'particle deposition in porous media is a sequence of particle transport to the immobile surface or "collector" followed by attachment'. Anatase (TiO₂) and silica (SiO₂) nanoparticles were found to pass through the test system as a function of velocity with a constant attachment efficiency factor.

In summary, anatase (TiO₂) and silica (SiO₂) nanoparticles have been shown to pass through a porous media test system as a function of velocity with a constant attachment efficiency factor (Leocanet and Wiesner 2004).

5.4.5 Summary

As with transport and fate of nanomaterials in water systems, there is a general lack of data in relation to the environmental fate and behaviour of nanomaterials in soil and sediment. It may even be stated that the paucity in data relevant for soil and sediment behaviour of NM is so pronounced, particularly for metal oxides and carbon nanotubes, that no general conclusions can be drawn. In addition, much of the research conducted to date is not really comparable due to, for example, the use of nanomaterials with different functionalisation, different experimental approaches and different levels of attention to characterisation of the nanomaterials studied. The current lack of analytical tools for the detection and quantification of nanomaterials in soil matrices is also a problem.

From the literature identified as relevant to the transport of nanomaterials in soil and sediment, there seem to be many studies addressing various aspects in regards to remediation of contaminated media. These can offer some information on transport and, due to the use of

nanoparticles for remediation purposes, there is a strong incentive to gain knowledge on transport properties of selected nanomaterials. The EMERGNANO report (Aitken *et al.* 2009) states that much work is ongoing to assess transport of NMs in soil, from which valuable output should be generated. However, the study's authors also note that of the work ongoing there is much overlap and as such the scope of outcomes will be lesser than had the research effort as a whole been more coordinated.

5.5 MODELLING APPROACHES

It is clearly conceivable that fugitive emission from processes in which nanomaterials are produced, could potentially lead to increased air concentration of these nanomaterials. As well as environmental exposure in these circumstances, it is possible that the general public, as well as the environment, would become exposed.

Some attempts at modelling environmental exposure have been carried out, most notably by Boxall *et al.* (2007) and by Mueller and Nowack (2008), although these were very preliminary studies, and as previously noted are not sufficiently developed to form a sound basis for accurate predictive modelling. Nonetheless their contribution to our developing understanding of the field is worth noting and their further improvement will undoubtedly lead to models of environmental exposure which are both practical and reliable in the future.

As described previously, Mueller and Nowack (2008) estimated the quantities of engineered nanoparticles released into the environment throughout their expected product life-cycles. Three types of nanoparticles were included: silver, TiO₂, and carbon nanotubes. The quantification was based on a substance flow analysis from products to air, soil, and water in Switzerland. The model inputs selected were: estimated worldwide production volume, allocation of the production volume to product categories, particle release from products, and flow coefficients within the environmental compartments. Predicted environmental concentrations (PEC) calculated using this model were then compared to the predicted no effect concentrations (PNEC) derived from the literature to estimate a possible risk. The expected concentrations of the three nanoparticles in the different environmental compartments were found to vary widely, the basis of this being differences in life cycles between the nanomaterial containing products.

In this study, full dispersion of nanomaterials was considered within the different media and transfer across media was considered to follow a specific pattern. The authors note that the lack of information concerning nanomaterial usage in consumer products is a limitation to their work. For Ag and TiO₂ the range between the estimations for worldwide production is wide. In order to address the uncertainty of data, two scenarios were modelled: a realistic exposure scenario based on the most reliable data and a high exposure scenario including the worst-case assumptions. A future scenario (i.e. future predicted usage pattern) was not conducted, as the predictions for the development of the production volumes of the nanomaterials are too vague. It seems to be realistic, though, that the production volumes of all three materials will increase significantly in the coming years.

5.6 CONCLUSIONS AND RECOMMENDATIONS

The general paucity of data in the area of environmental fate and behaviour to date represents a major obstacle in developing a holistic view of the fate and transport of nanomaterials within the environment and therefore of environmental exposure. It may even be stated that the paucity of data relevant for soil and sediment behaviour of nanomaterials is so pronounced, particularly for metal oxides and carbon nanotubes, that no general conclusions can be drawn. Much work is underway, and it is likely that in the coming years more relevant scientific information will emerge on which to develop further understanding of the area (Aitken *et al.* 2009; SCENIHR 2009).

Most of our current knowledge of transport of nanomaterials within air, soil and water compartments remains rooted in aerosol and colloid science. This background provides preliminary information from which to develop further understanding of nanoparticles' fate and transport. There is therefore a need for systematic studies to be undertaken on different types

of nanomaterials using a range of physico-chemical parameters (e.g. size, shape, form, surface area) to generate data which will support development of reliable and truly relevant models.

A recurring problem with the research conducted to date is its lack of comparability due to, for example, the use of nanomaterials with different functionalisation, different experimental approaches, different levels of attention to characterisation of the nanomaterials used, and variation in timescale of studies conducted. Although it is well known that different types of nanomaterials, with different functionalisations will have different fates and behaviour in the environment, it is also important that consistent data become available via rigorous and comparable experimental approaches. As the body of work in the area expands, such variation in approaches may result in a lack of comparability of data generated by different research groups. The EMERGNANO report (Aitken *et al.* 2009) states that much work is ongoing to assess transport of nanomaterials in the environment, from which it is expected valuable output should be generated. However, the study's authors also note that there is much overlap in the work being undertaken by different groups (an observation perhaps symptomatic of a wider-scale lack of coordination in research effort between funding bodies and government). As such the scope of outcomes is likely to be lesser than had the research effort as a whole been more coordinated.

Development of valid real-time methods by which to selectively detect and quantify specific nanomaterials within air, water and soil systems has been identified as crucial. Likewise, characterisation of sources and release mechanisms for nanomaterials into the environment, according to amounts being released and nanomaterial physico-chemical characteristics is a key task to address (Wu *et al.* 2008), as is development of metrological strategies for monitoring transport and fate of nanomaterials within different environmental compartments. This must be done carefully should, as is predicted, the nanomaterials alter in size rapidly via processes such as agglomeration.

Those models of nanomaterial fate, transport and exposure available to date are still extremely primitive and thus not sufficient alone for making accurate predictions. However, there is little doubt that such models will play an important role in furthering understanding in this area. Thus for nanomaterials in air, water and soil, modelling of emission scenarios and subsequent transport pathways is considered key. In concordance with this, work to develop more sophisticated predictive models is underway for a number of settings.

Most applications of engineered nanomaterials require functionalisation of their surface (e.g. to ensure successful incorporation into the product for which they are intended). Examination of the literature clearly shows that functionalisation of nanomaterials also has an important effect on their subsequent transport and fate. Considerable further work is therefore required to develop a full understanding of the effect different functionalisations have on the transport and fate of nanomaterials in air, water and soil systems.

In addition, many of those studies examining nanomaterials in soil and water environments highlight the importance of interactions between nanomaterials and other components present within that environmental compartment. For example, one consistent finding was that most nanomaterials interact with NOM in the aquatic environment, which will influence the fate and transport of nanoparticles and may also influence the nanomaterials' biological effects. Absorption of or to other materials present within the aquatic or sedimentary environment has also been shown to have an impact on nanoparticle transport.

A final observation stems from the literature identified as being specifically relevant to the transport of nanoparticles in soil and sediment, which focus on remediation of contaminated media. Results from these studies can offer some information on transport and, due to the use of engineered nanoparticles for remediation purposes, there is a strong incentive to gain knowledge on transport properties of selected nanomaterials which should lead to valuable contributions to the field.

5.7 REFERENCES

Aitken, R.J., Creely, K.S. and Tran C.L. 2004, *Nanoparticles: an occupational hygiene review*, HSE Books, Sudbury, UK.

Aitken, R.J., Hankin, S.M., Ross, B., Tran, C.L., Stone, V., Fernandes, T.F., Donaldson, K., Duffin, R., Chaudhry, Q., Wilkins, T.A., Wilkins, S.A., Levy, L.S., Rocks, S.A. and Maynard, A. 2009, *EMERGNANO: A review of completed and near completed environment, health and safety research on nanomaterials and nanotechnology*, Report on DEFRA project CB0409.

Baalousha, M., Manciualea, A., Cumberland, S., Kendall, K. and Lead, J.R. 2008, "Aggregation and surface properties of iron nanoparticles: influence of pH and natural organic matter", *Environmental Toxicology and Chemistry*, vol 27, pp.1875-82.

Baalousha, M. 2009, "Aggregation and disaggregation of iron oxide nanoparticles: Influence of particle concentration, pH and natural organic matter", *Science of the Total Environment*, vol. 407, no. 6, pp. 2093-2101.

Benn, T.M. and Westerhoff, P. 2008, „Nanoparticle silver released into water from commercially available sock fabrics”, *Environmental Science and Technology*, vol. 42, no. 11, pp. 4133-4139.

Boxall, A.B., Chaudhry, Q., Sinclair, C., Jones, A., Aitken, R., Jefferson, B., *et al.* 2007, *Current and future predicted environmental exposure to engineered nanoparticles*. DEFRA Report; 2007.

Chen, Z., Westerhoff, P. and Herckes, P. 2008, "Quantification of C60 fullerene concentrations in water", *Environ Toxicol Chem*, vol. 27, pp. 1852-9.

Chen, K.L. and Elimelech, M. 2008, "Interaction of fullerene (C60) nanoparticles with humic acid and alginate coated silica surfaces: measurements, mechanisms, and environmental implications", *Environmental Science and Technology*, vol. 42, pp. 7607-14.

Christian, P., Von der Krammer, F., Baalousha, M. and Hofmann, T. 2008, "Nanoparticles: structure, properties, preparation and behaviour in environmental media", *Ecotoxicology* vol. 17, pp. 326-43.

Darlington, T.K., Neigh, A.M., Spencer, M.T., Nguyen, O.T. and Oldenburg, S.J. 2009, "Nanoparticle characteristics affecting environmental fate and transport through soil", *Environmental Toxicology and Chemistry*, vol. 28, no. 6, pp. 1191–1199.

Diegoli, S., Manciualea, A.L., Begum, S., Jones, I.P., Lead, J.R. and Preece, J.A. 2008, "Interaction between manufactured gold nanoparticles and naturally occurring organic macromolecules", *Science of the Total Environment*, vol. 402, no. 1, pp. 51-61.

Doshi, R., Braidia, W., Christodoulatos, C., Wazne, M. and O'Connor, G. 2008, "Nano-aluminium: Transport through sand columns and environmental effects on plants and soil communities", *Environmental Research*, vol. 106, no. 3, pp. 296-303.

Fortner, J.D., Kim, D., Boyd, A.M., Falkner, J.C., Moran, S., Colvin, V.L., Hughes, J.B. and Kim, J. 2007, "Reaction of Water-Stable C60 Aggregates with Ozone", *Environmental Science and Technology*, vol. 41, no. 21, pp. 7497-7502.

Fortner, J.D., Lyon, D.Y., Sayes, C.M., Boyd, A.M., Falkner, J.C., Hotze, E.M., *et al.* 2005, "C60 in water: nanocrystal formation and microbial response", *Environmental Science and Technology*, vol. 39, pp. 4307-16.

Gao, J., Youn, S., Hovsepian, A., Vernica, L.L., Wang, Y., Bitton, G. and Bonzongo, J.J. 2009, "Dispersion and Toxicity of Selected Manufactured Nanomaterials in Natural River Water Samples: Effects of Water Chemical Composition", *Environmental Science and Technology*, vol. 43, no. 9, pp. 3322-3328.

Ghosh, S., Mashayekhi, H., Pan, B., Bhowmik, P. and Xing, B. 2008, "Colloidal Behaviour of Aluminium Oxide Nanoparticles As Affected by pH and Natural Organic Matter", *Langmuir*, vol. 24, no. 21, pp. 12385-12391.

Han, Z., Zhang, F., Lin, D. and Xing, B. 2008, "Clay Minerals Affect the Stability of Surfactant-Facilitated Carbon Nanotube Suspensions", *Environmental Science and Technology*, vol. 42, no. 18, pp. 6869-6875.

Hassellöv, M., Readman, J.W., Ranville, J.F. and Tiedje, K. 2008, "Nanoparticle analysis and characterization methodologies in environmental risk assessment of engineered nanoparticles", *Ecotoxicology*, vol. 17, pp. 344-61.

Hyung, H., Fortner, J.D., Hughes, J.B. and Kim, J.H. 2007, "Natural organic matter stabilizes carbon nanotubes in the aqueous phase", *Environmental Science and Technology*, vol. 41, pp. 179-84.

Hyung, H. and Kim, J.H. 2008, "Natural Organic Matter (NOM) Adsorption to Multi-Walled Carbon Nanotubes: Effect of NOM Characteristics and Water Quality Parameters", *Environmental Science and Technology*, vol. 42, no. 12, pp. 4416-4421.

ICRP. 1994, "Human Respiratory Tract Model for Radiological Protection", ICRP Publication 66, *Annals of the ICRP*, vol. 24, pp. 1-3.

Jaisi, D.P., Saleh, N.B., Blake, R.E. and Elimelech, M. 2008, "Transport of Single-Walled Carbon Nanotubes in Porous Media: Filtration Mechanisms and Reversibility", *Environmental Science and Technology*, vol. 42, no. 22, pp. 8317-8323.

Jones, R.A. 2002, *Soft condensed matter*. Oxford University Press, New York.

Ju-Nam, Y. and Lead, J.R. 2008, "Manufactured nanoparticles: An overview of their chemistry, interactions and potential environmental implications", *Science of the Total Environment*, vol. 400, pp. 396-414.

Kaegi, R., Ulrich, A., Sinnet, B., Vonbank, R., Wichser, A., Zuleeg, S., Simmler, H., Brunner, S., Vonmont, H., Burkhardt, M. and Boller, M., 2008. "Synthetic TiO₂ nanoparticle emission from exterior facades into the aquatic environment", *Environmental Pollution*, vol. 156, pp. 233-239.

Kasper, G. 2008, "Life cycle of airborne nanoparticles and its implications for filtration and personal protection devices", *Nanosafe 2008 Conference*, November 5-7, Grenoble, France.

Kennedy, A.J., Hull, M.S., Steevens, J.A., Dontsova, K.M., Chappell, M.A., Gunter, J.C., *et al.* 2008, "Factors influencing the partitioning and toxicity of nanotubes in the aquatic environment", *Environmental Toxicology and Chemistry*, vol. 27, pp. 1932-41.

Klaine, S.J., Alvarez, P.J.J., Batley, G.E., Fernandes, T.F., Handy, R.D., Lyon, D.Y., Mahendra, S., McLaughlin, M.J. and Lead, J.R. 2008, "Nanomaterials in the environment: behaviour, fate, bioavailability, and effects", *Environmental Toxicology and Chemistry*, vol. 27, pp. 1825-1851.

Lead, J.R. and Wilkinson, K.J. 2006, "Natural aquatic colloids: current knowledge and future trends", *Environmental Chemistry*, vol. 3, pp. 159-171.

Lecoanet, H.F., Bottero, J-Y., and Wiesner, M.R. 2004, "Laboratory Assessment of the Mobility of Nanomaterials in Porous Media", *Environmental Science and Technology*, vol. 38, no. 19, pp. 5164-5169.

Lecoanet, H.F. and Wiesner, M.R. 2004, "Velocity effects on fullerene and oxide nanoparticle deposition in porous media", *Environmental Science and Technology*, vol. 38, no. 16, pp. 4377-82.

Li, X., Zhu, D. and Wang, X. 2007, "Evaluation on dispersion behaviour of the aqueous copper nano-suspensions", *Journal of Colloid and Interface Science*, vol. 310, no. 2, pp. 456-463.

Li, Y., Wang, Y., Pennell, K.D., and Abriola, L.M. 2008a, "Investigation of the transport and deposition of fullerene (C60) nanoparticles in quartz sands under varying flow conditions", *Environmental Science and Technology*, vol. 42, no. 19, pp. 7174-80.

Li, D., Lyon, D.Y., Li, Q., Alvarez, P.J. 2008b, "Effect of soil sorption and aquatic natural organic matter on the antibacterial activity of a fullerene water suspension", *Environmental Toxicology and Chemistry*, vol. 27, no. 9, pp. 1888–1894

Limbach, L.K., Bereiter, R., Müller, E., Krebs, R., Gälli, R., Stark, W.J. 2008, "Removal of Oxide Nanoparticles in a Model Wastewater Treatment Plant: Influence of Agglomeration and Surfactants on Clearing Efficiency", *Environmental Science and Technology*, vol. 42, no. 15, pp. 5828-5833

Lyklema, J. 2005, "Pair Interactions" in *Fundamentals of interface and colloid science, Volume IV, Particulate colloids*, eds. Lyklema, J. Elsevier Academic Press, Amsterdam.

Mackay, C.s E. and Henry, K.M. 2009, "Environmental Fate and Transport", in *Nanotechnology and the Environment*, eds. Sellers *et al.* CRC Press, Taylor and Francis Group, LLC., USA.

Mädler, L. and Friedlander, S.K. 2007, "Transport of Nanoparticles in Gases: Overview and Recent Advances", *Aerosol and Air Quality Research*, vol. 7, no. 3, pp. 304-342.

Maynard, A.D., Baron, P.A., Foley, M., Shvedova, A.A., Kisin, E.R. and Castranova, V. 2004, "Exposure to carbon nanotube material: aerosol release during the handling of unrefined single-walled carbon nanotube material", *Journal of Toxicology and Environmental Health, Part A: Current Issues*, vol. 67, pp. 87-107.

Mueller, N. and Nowack, B. 2008, "Exposure modelling of engineered nanoparticles in the environment", *Environmental Science and Technology*, vol. 42, pp. 4447-53.

Murr, L.E., Bang, J.J., Esquivel, E.V., Guerrero, P.A. and Lopez, D.A. 2004, "Carbon nanotubes, nanocrystal forms, and complex nanoparticle aggregates in common fuel-gas combustion sources and the ambient air". *Journal of Nanoparticle Research*, vol. 6, pp. 241-51.

Murr, L.E. and Guerrero, P.A. 2006 "Carbon nanotubes in wood soot", *Atmospheric Science Letters*, vol. 7, pp. 93-5.

Nowak, B. and Bucheli, T.D. 2007, "Occurrence, behaviour and effects of nanoparticles in the environment", *Environmental Pollution*, vol. 150, pp. 5-22.

Nowack, B. 2009 "The behaviour and effects of nanoparticles in the environment", *Environmental Pollution*, vol. 157, no. 4, pp. 1063-1064.

Saleh, N., Kim, H-J., Phenrat, T., Matyjaszewski, K., Tilton, R.D. and Lowry, G.V. 2008a, "Ionic Strength and Composition Affect the Mobility of Surface-Modified Fe0 Nanoparticles in Water-Saturated Sand Columns", *Environmental Science and Technology*, vol. 42, no. 9, pp. 3349-3355

Saleh, N.B., Pfefferle, L.D. and Elimelech, M. 2008b, "Aggregation kinetics of multiwalled carbon nanotubes in aquatic systems: measurements and environmental implications", *Environmental Science and Technology*, vol. 42, pp. 7963-9.

SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks). 2009, *Risk assessment of products of nanotechnologies*, European Commission.

Engineered Nanoparticles: Review of Health and Environmental Safety

Schrack, B., Hydutsky, B.W., Blough, J.L. and Mallouk, T.E. 2004, "Delivery Vehicles for Zerovalent Metal Nanoparticles in Soil and Groundwater", *Chemistry of Materials*, vol. 16, no. 11, pp. 2187-2193

Tiede, K., Boxall, A.B.A, Tear, S.P. *et al.* 2008, "Detection and characterization of engineered nanoparticles in food and the environment", *Food Additives and Contaminants*, vol. 25, no. 7, pp. 795-821.

Wang, Y., Li, Y. and Pennell, K.D. 2008, "Influence of electrolyte species and concentration on the aggregation and transport of fullerene nanoparticles in quartz sands", *Environmental Toxicology and Chemistry*, vol. 27, no. 9, pp. 1860–1867.

Wu Q *et al.* 2008, *The Behaviour of Aerosols Released to Ambient Air from Nanoparticle Manufacturing - A Pre-normative Study*, NANOTRANSPORT project publishable final activity report, EU project NMP4-CT-2006-033371. Accessed at: http://research.dnv.com/NANOTRANSPORT/NANOTRANSPORTdownload/A-NANOTRANSPORT-publisable_f.doc (16th October 2009)

Wudl, F. 2002. "Fullerene materials", *Journal of Materials Chemistry*, vol. 12, pp. 1959-1963.

Xie, B., Zhihua, X., Wenhua, G. and Oilin, L. 2008, "Impact of Natural Organic Matter on the Physicochemical Properties of Aqueous C60 Nanoparticles", *Environmental Science and Technology*, vol. 42, no. 8, pp. 853-2859.

Yang, G.C.C, Tua, H. and Hunga, C. 2007, "Stability of nano-iron slurries and their transport in the subsurface environment", *Separation and Purification Technology*, vol. 58, no. 1, pp. 166-172.

Zhang, Y., Chen, Y., Westerhoff, P., Hristovski, K. and Crittenden, J.C. 2008, "Stability of commercial metal oxide nanoparticles in water", *Water Research*, vol. 42, no. 8-9, pp. 2204-2212.

6 HUMAN TOXICITY

6.1 INTRODUCTION

The physico-chemical characteristics of the nanomaterial 'types' investigated in the ENRHES review vary greatly, in terms of their composition, morphology, and size, to name a few. It is therefore of interest to evaluate the toxic potential of the nanomaterials under investigation, and to identify whether the same underlying mechanisms drive each of their toxicities, and therefore to determine whether any generalisations can be made regarding nanomaterials as a whole. In addition, the review process aims to reveal material or particle specific attributes that are particularly relevant in driving nanomaterial toxicity, therefore allowing identification of key characteristics that can influence safety. In an attempt to achieve this, available information regarding the exposure conditions and characteristics of nanomaterials used within the described studies will be outlined. However, it is worth noting that this information is not always stipulated by investigators. In addition, there are discrepancies regarding the dose metrics used when expressing the concentration of particles exposed to cells or animals; specifically whether dose is based upon the mass, surface area, or particle number administered. This is of relevance as it has been repeatedly demonstrated that the toxicity of particles is related to their size, so that as particle size decreases, toxicity generally increases, which is thought to be driven by their surface area. However, nanomaterials are a diverse group of materials, and it has become evident that other particle dimensions are also important in driving their toxicity, such as length. Furthermore, the tendency of nanomaterials to agglomerate or aggregate is of concern, and has encouraged investigation into improving nanomaterial suspensions, including the use of dispersants, solvents, surface modification or mechanical processes.

Exposure to nanomaterials is expected to primarily occur through dermal, inhalation, ingestion or injection routes. Accordingly, a particular focus on the consequences of exposure of the lungs, skin, gastrointestinal tract (GIT), or blood has been employed, with inclusion of both *in vitro* and *in vivo* models for each route. However, the realisation that nanomaterials can distribute from their exposure site, within the blood or even nerves, means that nanomaterial toxicity may be exerted at a number of targets including, for example, the liver, brain, spleen, and kidneys.

Studies that can be employed to assess the toxicity of a wide variety of nanomaterials include the utilisation of both *in vivo* (within mice and rats) and *in vitro* models (using cell lines and primary cells). Rodent models have been used extensively to study a number of disease states, including particle induced lung disease (including quartz, and asbestos induced disease), and so there is already an understanding of the limitations of such models, in terms of extrapolating hazard assessment to humans. Current expertise does not perfectly allow for inclusion of species differences, but the existing database of particle toxicology combined with the use of such models provide a useful series of protocols to allow benchmarking of new nanomaterials to the relative potential toxicity of other substances of known hazard.

Cell lines are frequently used to investigate the effects of potentially toxic substances. The types of cells available are diverse and represent a wide range of organ and cell types, including tumour derived and transformed cells. Their response is often representative of the *in vivo* response, but careful comparisons and controls are required to ensure relevance. One of the biggest problems with *in vitro* models is the use of excessive concentrations to assess toxicity or mechanisms of toxicity. Although a wide range of concentrations are useful in order to generate comparative values such as LD₅₀, NOEL etc., observation of an effect *in vitro* should not be taken as indicative of an effect *in vivo* without consideration of the relevance of the dose or the endpoint measured. The use of *in vitro* cytotoxicity (cell death) for risk assessment purposes, even if benchmarked against a material of 'known' risk, is questionable, since very few particle induced diseases are associated with acute immediate cell death. Instead, sub-lethal effects measured *in vitro* are more likely to be useful for risk assessment purposes than focussing on the cytotoxic potential of nanomaterials. Most pathological particles act via the induction of cellular and molecular changes such as oxidative stress and/or the induction of inflammation, both of which can lead to disease. These endpoints have therefore been assessed with greatest interest and highest priority, when assessing the toxicity of

nanomaterials. Therefore measures of cytotoxicity are, more useful to ascertain sub-lethal concentrations for further investigation rather than for risk assessment purposes.

Some of the anomalies of cell lines have been attributed to their phenotypic changes associated with immortality. The use of primary cells can be used to overcome this problem. Primary human cells are also used to address species differences when using animal models. Many *in vitro* experiments use just a single cell type, which has the advantage of providing a simple well controlled system, but often lacks the potential consequences of co-operation between different cell types, including the presence of defence systems (such as immune cells) that may reduce or exacerbate toxicity.

Both single and co-culture systems allow the assessment of toxicity within a controlled system, where a number of different concentrations and time points can be evaluated. In comparison, *in vivo* study design requires consideration of strategies to reduce the use of animals, resulting in a more limited number of doses and timepoints within an individual study. In order to allow the safe development of nanotechnology while limiting the use of *in vivo* models in the future, it is clear that the development and validation of the predictive capability of *in vitro* models of nanomaterial toxicity is vital.

6.2 CARBON FULLERENES

6.2.1 Introduction

The studies that have investigated the potential toxicity associated with fullerene exposure will be outlined in this review, in an attempt to identify which attributes of fullerenes drive toxic responses, as well as the mechanisms underlying any observed toxicity. However, identifying hazards related to fullerene exposure is complicated by the fact that there are a number of fullerene derivatives available, which stems from the different number of carbon atoms used to generate fullerenes, the diverse array of moieties that can be attached to the fullerene surface, and the different preparations processes utilised to render fullerenes water soluble, in order to enable their exposure within aqueous suspensions.

Although fullerenes are often depicted as particles consisting of a cage of 60 or more carbon atoms, in reality, these are often structures that crystallise into larger particles. Therefore exposure is often to clusters of crystals, termed nano or colloidal fullerenes. In order to enhance dispersion, and to minimise the cluster/crystal size it is common to use dispersants. The potential toxicity of these dispersants will be commented upon throughout the following review as this has been a focus of a number of studies. Due to the importance of fullerene solubility to its exploitation, fullerene derivatives have been generated that exhibit greater water solubility than their pristine (i.e. unmodified) counterparts. For example, fullerols (also termed fullerlenols) are often produced, whereby the surface of the fullerene molecules are polyhydroxylated to render fullerenes more water soluble. Therefore numerous fullerene types exist and their impact on fullerene properties and toxicity will be discussed. The phototoxic behaviour of fullerenes will also be included, which derives from the ability of fullerenes to absorb light and generate reactive oxygen species (ROS) (Neilson *et al.* 2008).

It is necessary to highlight that a number of the described studies are relatively old, and so their focus was not on the 'nano' dimensions of fullerenes, and instead were preliminary investigations into fullerene toxicity, and biocompatibility. A particular focus of the more recent studies has been to determine the antioxidant properties of fullerenes, and how to improve their dispersion within aqueous suspensions.

6.2.2 *In vivo* assessment of fullerene toxicity

As a consequence of the number of expected applications that contain fullerenes, it is anticipated that exposure could occur via oral, dermal, pulmonary or injection routes, so that toxicity at the site of exposure is of particular interest (namely the skin, lungs and gastrointestinal tract), but it is also relevant that fullerenes may distribute throughout the body and accumulate within sites distal to their portal of entry such as the liver and spleen. The *in*

in vivo studies identified have either administered fullerenes via the lungs, via injection (intravenous or intraperitoneal administration), or dermally. These studies are few in number and so the available data relating to *in vivo* toxicity is rather limited. It is necessary to highlight that when considering the risk associated with human exposure to fullerenes, the ENRHES review is primarily concerned with an occupational setting, so that a particular focus on pulmonary and dermal exposure to fullerenes is relevant, with injection studies used to address the toxicokinetics and biocompatibility of fullerenes.

None of the studies conducted with fullerenes have used standard protocols (e.g. OECD or ISO), and are unlikely to be conducted in GLP laboratories. There is still much debate about how to do such studies, for example how to disperse the particles prior to injection. Therefore, these limitations must be included when considering the relevance of the studies reviewed and the confidence of the conclusions generated.

6.2.2.1 Pulmonary exposure to fullerenes

Previous studies have highlighted that particle size is an important determinant of particle toxicity, specifically that particles with nano dimensions (< 100 nm) are more toxic than their larger equivalents (see for example, Ferin *et al.* 1992), and the relevance of this to fullerenes is worthy of consideration, due to their definition as nanoparticles. Accordingly, Baker *et al.* (2008) exposed rats to nanoparticle (55 nm diameter, 2.22 mg m⁻³) and microparticle (0.93 µm diameter, 2.35 mg m⁻³) forms of C₆₀ via nasal inhalation. The exposures were conducted for 3 hours per day, for 10 consecutive days, and toxicological assessments were conducted up to 7 days post exposure. The lung burden of particles was also assessed, which was generally greater for the nanoparticle exposure group. Specifically, the pulmonary deposition fraction of C₆₀ nanoparticles was 14.1%, and for C₆₀ microparticles, was 9.3%. However, the half life for C₆₀ within both treatment groups was similar, being 26 days for nanoparticles and 29 days for microparticles, thus suggesting that similar elimination processes were involved during their removal from the lungs. The exposures did not result in detectable gross or microscopic lesions at necropsy, and minimal hematology and serum chemistry changes were observed. Within the lungs, no cellular infiltration (indicative of an inflammatory response) was observed, although C₆₀ was internalised by alveolar macrophages. Therefore, the study did not reveal any inflammatory or toxic potential for C₆₀ in the lungs of rats, nor did it reveal any differences in toxicity when generated in the nanoparticle or microparticle forms.

Many studies have demonstrated that a range of nanoparticles induce pro-inflammatory effects in the lung (see for example Donaldson and Stone, 2003 for a review), but a study by Roursgaard *et al.* (2008) assessed the anti-inflammatory potential of fullerols at doses of 0.02 to 200 µg per mouse. This was achieved by evaluating their ability to attenuate the pulmonary inflammatory response elicited by α-quartz in mice. In fact, intratracheal exposure to fullerols at a dose of 200 µg (equivalent to 10 mg kg⁻¹) elicited a neutrophil driven pulmonary inflammation, which was associated with increased macrophage inflammatory protein (MIP)-2 production. This inflammatory response was however less pronounced than for quartz. Mice pre-treated with fullerol (<20 µg (equivalent to 1 mg kg⁻¹)), demonstrated an attenuation of the subsequent inflammatory response elicited by quartz. This was proposed by the authors to be due to the ability of fullerols to reduce ROS mediated inflammation, but this finding was only relevant with the lower doses of fullerols studied. Therefore the results implied that at low concentrations, fullerols may have protective, anti-inflammatory properties, but at higher concentrations they exhibit a pro-inflammatory response.

Fujita *et al.* (2009) treated rats with C₆₀ via whole body inhalation, for 6 hours per day, 5 days per week, for a total of 4 weeks. Observations continued for a period of up to 1 month post exposure, during which time the authors observed, using DNA microarrays, an up-regulation in a small number of genes involved with the stimulation of inflammation, oxidative stress, apoptosis and metalloendopeptidase activity. C₆₀ was also observed within alveolar macrophages and epithelial cells. However, the authors concluded that the inflammatory response and tissue injury induced was not severe in magnitude, despite the fact that only gene changes were measured. Similarly, Sayes *et al.* (2007) reported no pulmonary toxicity was associated with intratracheal exposure to C₆₀ or C₆₀(OH)₂₄ of rats (up to 3 mg kg⁻¹, for a period

of up to 3 months following exposure), which is in contrast to the response induced by α quartz, which was pro-inflammatory and pro-fibrotic in nature.

Therefore, the findings from this small number of available studies demonstrate that following exposure via the pulmonary route, fullerenes are capable of eliciting localised responses that are pro- or anti-inflammatory in nature, with the type of response initiated likely to be reliant on the fullerene in question, exposure method and the dose used. Consequently, insufficient evidence is currently available to make definitive conclusions about what drives the pro- and anti-inflammatory responses, associated with fullerene pulmonary exposure. No studies were identified that addressed uptake of fullerenes from the lungs to the cardiovascular system.

6.2.2.2 Intraperitoneal exposure

Chen *et al.* (1998a) generated fullerene-protein conjugates, using bovine thyroglobulin, bovine or rabbit serum albumin, or derivatives of lysine, and investigated their antigenicity. Mice exposed to the particles intraperitoneally generated antibodies against the C₆₀ derivatives, suggesting they exhibited antigenic behaviour. The findings were expanded upon by Erlanger *et al.* (2001) who demonstrated that anti-C₆₀ antibodies were able to interact with SWCNT, which was imaged using atomic force microscopy. The findings insinuated that C₆₀ derivatives may act as sensitising agents and thus have the potential to modulate subsequent immune responses.

Chen *et al.* (1998b) administered rats with water soluble, polyalkylsulfonated C₆₀, via intraperitoneal injection, in an acute (up to 1000 mg kg⁻¹, for 24 hours) or subacute (up to 60 mg kg⁻¹, with daily exposures for 12 consecutive days) setting. Specifically, within 24 hours, 5 out of 6 rats died when administered a dose of 750 mg kg⁻¹ of fullerene, and 100% of exposed rats died in the 1000 mg kg⁻¹ treatment group. The fullerene was found to have an LD₅₀ of 600 mg kg⁻¹. The kidney was recognised as a primary site of fullerene elimination and toxicity within the acute study, which was reproduced within the subacute study. In addition, macrophages within the liver, spleen were observed to be laden with particles in the subacute group. Within preliminary studies, liver cytochrome P450 activity was also observed to be suppressed. It is necessary to highlight that exceptionally high doses were utilised within this study (in order to attain LD₅₀) that could explain the pathology and mortality that transpired.

In the very limited number of studies that have been conducted, the intraperitoneal injection of fullerenes has been used to assess fullerene biocompatibility and tissue distribution. It would appear that fullerenes are able to elicit an antigenic response, due to its potential to modulate inflammatory responses, but the applicability of this to other fullerene derivatives requires assessment. In addition, the kidney, liver and spleen have been demonstrated to be a target of fullerene toxicity, and so their transport within the blood is anticipated following intraperitoneal injection, but this requires further investigation to determine how universal this finding is to all fullerenes.

6.2.2.3 Dermal exposure

Only one investigation studying the potential skin effects of fullerenes was found. Specifically Huczko *et al.* (1999) used patch testing to assess the skin irritant potential of fullerene soot within 30 volunteers (who reported irritation and allergic susceptibilities) for a 96 hour exposure time. No skin irritation was found.

Studies that purport to study the consequences of dermal exposure to fullerenes are lacking, with the only available investigation suggesting that no detrimental outcome on the skin is apparent, but this requires more extensive investigation.

6.2.2.4 Oral administration

Yamago *et al.* (1995) investigated the distribution of ¹⁴C labelled, water soluble C₆₀ within rats, following oral administration, for a period up to 160 hours post exposure. Subsequent to oral exposure, C₆₀ was not effectively absorbed, but instead the majority was excreted in the faeces

within 48 hours. However, it is of interest that trace amounts of fullerene were observed within urine, therefore implying that some fullerenes were able to pass through the gut wall.

Mori *et al.* (2006) used fullerite, a mixture of C₆₀ and C₇₀, to evaluate the acute toxicity (up to 14 days) of fullerenes, subsequent to the oral exposure of rats, at a dose of 2000 mg kg⁻¹. No lethality, or other signs of toxicity in terms of behaviour or body weight were evident during the observation period, despite the high dose that was administered, with fullerene elimination within faeces evident.

Chen *et al.* (1998b) demonstrated that polyalkylsulfonated (water soluble) C₆₀ was not lethal, subsequent to oral exposure of rats in acute (50 mg kg⁻¹, single administration) or subacute (50 mg kg⁻¹ daily for 12 days) exposure set ups, and as a consequence was considered to be non-toxic. These findings are in contrast with the lethality associated with intraperitoneal exposure, as mentioned previously. However, perhaps sub-lethal toxicity should be a focus of future studies.

The limited number of investigations that evaluated the consequences of oral administration, suggested that fullerenes are primarily eliminated within faeces. However, it has also been suggested that a small but unspecified proportion of the fullerene dose is able to pass through the gut wall, and thereby enter the circulation. Such studies are inadequate in number to make definitive conclusions regarding the transfer of fullerenes into the circulation, and therefore their systemic availability following oral exposure.

6.2.2.5 ADME profile of fullerenes

Determining the kinetics of fullerenes within the body, subsequent to exposure (via the lungs, gut and skin) is necessary to identify potential targets of fullerene toxicity, and thereby direct relevant *in vitro* assessments of their toxicity at particular target sites. This is necessary, as the delivery of fullerenes to target organs, such as the liver or kidneys requires their transfer into blood from their exposure site, and so their likelihood of accessing different sites within the body is of relevance. Accordingly, a number of barriers (at the exposure site and those apparent within secondary targets) are in place to prevent against uptake, and it is necessary to determine if this is surmounted by fullerenes to determine their systemic uptake and therefore availability.

Studies that provide evidence for the absorption of fullerenes into the blood from their exposure site are few in number, and as such this question should be a focus of future investigations. Baker *et al.* (2008) did not detect fullerenes within the blood, following inhalation by rats, suggesting that they do not translocate from their exposure site. However, this was suggested to occur due to their potential biotransformation within the lung, and insensitivity of the detection method. Particles were presumably eliminated due to the action of alveolar macrophages and mucociliary escalator, but this requires further consideration. Perhaps radioisotope or fluorescent labelling could allow for the better detection of fullerenes when evaluating fullerene kinetics. In contrast, Yamago *et al.* (1995) suggested that fullerenes were able to pass into the blood, from the gut. Chen *et al.* (1998b) also illustrated that the kidney, liver and spleen were associated with the toxicity or accumulation of fullerenes following intraperitoneal injection suggestive of their transport via blood. Targeting of these organs is likely to be driven by the resident macrophage populations that sequester foreign particles,

The metabolism of fullerenes has been suggested to occur, following their accumulation within the liver (Gharbi *et al.* 2005), with the metabolites, as yet, unspecified, this therefore required further investigation.

The elimination of fullerenes within urine (Yamago *et al.* 1995) and faeces (Mori *et al.* 2006, Yamago *et al.* 1995) has been demonstrated, suggesting that they may be eliminated, in part, from the body following exposure via a number of routes.

Information regarding the ADME profile of fullerenes is generally lacking, and therefore warrants further investigation in future studies. In the small number of studies described here, it would appear that the majority of fullerenes remain at the deposition site (specifically within the lungs

and gut), but that it is also possible for fullerenes to cross cell barriers and to be transported within the blood. Accumulation appears to be predominant within the liver, kidneys and spleen, with evidence of toxicity also manifesting at sites of accumulation. Metabolism of fullerenes has also been suggested, and the consequences of this require consideration. Elimination of fullerenes within the faeces and urine has also been demonstrated, which may reduce their propensity for distribution and toxicity. However, it is relevant to note that the representative nature of the limited number of findings, for all fullerene derivatives is unknown at this time.

6.2.2.6 Distribution of C₆₀ following injection

Fullerene distribution following injection into blood has been studied both due to their potential use as carriers for drugs, and to assess their distribution and localisation sites should they enter the blood via other routes (e.g. following inhalation).

Yamago *et al.* (1995) investigated the distribution of ¹⁴C labelled, water soluble C₆₀ within rats, after intravenous injection. Subsequent to exposure, the fullerenes were rapidly removed from the blood (only 1.6% of the administered dose remained in the blood after an hour) and accumulated within the liver, which was the primary site of localisation, although some localisation was also evident within, for example the kidney, lungs spleen, heart and brain. In a similar study, Bullard-Dillard *et al.* (1996) also exposed rats via intravenous exposure to radiolabelled C₆₀ (0.2 µM). Clearance of C₆₀ from the blood was again rapid, with only 1% of the administered dose of pristine C₆₀ remaining within the circulation after 1 minute. However, the clearance of quaternary ammonium salt-derivatised C₆₀, was slower, with 9% of the dose remaining at 1 minute post exposure, which was attributed to its more hydrophilic, water soluble character. Again the majority of the unmodified particles were contained within the liver (over 90%) at 120 minutes post exposure, with minimal accumulation within the spleen, lung and muscle. The water-soluble C₆₀ had a wider tissue distribution, with only 50% of the administered dose evident within the liver, with the remaining dose contained in the spleen, lungs, muscle and cellular component of blood. After 120 hours, it was apparent that the majority (95%) of unmodified C₆₀ still remained within the liver, with no evidence of elimination within urine or faeces, highlighting that the liver is a potential target for fullerene accumulation and toxicity.

In line with these findings, Gharbi *et al.* (2005) demonstrated that C₆₀ was able to accumulate within the liver following the intraperitoneal exposure of rats, which was also indicated by a colour change (to dark brown) of the liver. However C₆₀ localisation within the liver decreased with time (nearly all was eliminated by day 13), and so it was suggested that the liver was capable of either eliminating C₆₀ (within the faeces) or biochemically transforming C₆₀, as C₆₀ metabolites were identified within the liver. Histological analysis revealed that no inflammation or fibrosis was associated with the hepatic accumulation of particles, which was primarily accounted for by their uptake by Kupffer cells.

The findings from the different studies therefore share the commonality, that subsequent to injection, fullerenes preferentially accumulate within the liver. Therefore it is of high relevance to evaluate the impact of fullerene accumulation on liver function, and to assess the contribution of the liver to the metabolism of fullerenes and, in addition to considering the ability of the liver to facilitate the removal of fullerenes from the body within bile, and therefore the faeces.

6.2.3 In vitro investigations of C₆₀ toxicity

As for the *in vivo* assessment of fullerene toxicity, there are a limited number of investigations that describe the toxic potential of fullerenes *in vitro*, which have concentrated on the dermal, and cardiovascular toxicity of fullerenes.

6.2.3.1 Dermal models

Scrivens *et al.* (1994) demonstrated that ¹⁴C-labelled C₆₀ (1.3 µM) were internalised by immortalised human keratinocytes, so that after a 6 hour exposure time, 50% of the applied concentration was contained within the cells. Despite the internalisation, C₆₀ exposure (20 nM

to 2 μM) did not impact on cell proliferation. A similar effect was observed by (Bullard-Dillard *et al.* 1996) who observed that C_{60} and quaternary ammonium salt-derivatised C_{60} (up to 2 μM) were internalised by keratinocytes, with the process being slower for derivatised particles. However, it was apparent that C_{60} elicited a decrease in cell proliferation that was evident at high concentrations (2 μM) and over an extended period of time of 8 days. (Rouse *et al.* 2006) found that phenylalanine derivatised C_{60} (up to 0.4 mg ml^{-1}) were internalised by HEK keratinocytes, and elicited an inflammatory response, indicated by an increase in IL-6, IL-8 and IL-1 β production. The fullerene ultimately initiated dose dependent cytotoxicity via a necrotic mechanism. These results were expanded upon by Rouse *et al.* (2007) who illustrated that there was a relationship between C_{60} penetration and skin flexing within an *ex vivo* pig skin preparation. Specifically, a fullerene-peptide conjugate was internalised into epidermal and dermal layers (not reaching microvasculature or blood), and this effect was more pronounced within flexed skin (experienced, for example when walking barefoot), than unflexed skin. It is also of interest that the penetration of the particles did not occur via their direct transport through cells, but indirectly between skin cells via intercellular spaces. Sayes *et al.* (2004) found that the cytotoxic potential (mediated by lipid peroxidation) of different forms of derivatised fullerenes to human dermal fibroblasts, HepG2 hepatocytes and NHA astrocytes, was dependent on the type and level of functionalisation (see below).

The findings from the discussed studies suggest that the fullerene type, skin condition, and experimental protocol (cell type, concentration and duration) are able to influence the inflammogenic and cytotoxic potential of fullerenes to the skin, in vitro. No clear conclusion regarding uptake potential or toxicity can be generated for skin at this time, and it is possible that different fullerenes will behave differently in this target organ.

6.2.3.2 Models of cardiovascular effects

As fullerenes may have the potential to translocate from their site of exposure into the circulation, or be directly administered into the blood through injection, they are likely to encounter the endothelial cells that line blood vessels, to potentially cause vascular injury. As a result, Yamawaki and Iwai, (2006) investigated the ability of $\text{C}_{60}(\text{OH})_{24}$ (1-100 $\mu\text{g ml}^{-1}$) to induce endothelial damage within the HUVEC cell line. Following an acute exposure (24 hours) fullerenes were internalised by cells, and elicited a dose dependent decrease in cell viability, which was suggested to be autophagic in nature (and was demonstrated to be non-apoptotic). Subsequent to a chronic exposure (10 days), fullerenes detrimentally affected cell attachment and slowed cell growth. It was therefore speculated (by the authors) that exposure to fullerenes is a potential risk for cardiovascular disease initiation or progression. However, further investigations *in vivo* would be required to confirm such a suggestion.

Radomski *et al.* (2005) demonstrated that a number of engineered particles and urban particulate matter (0.2 to 300 $\mu\text{g ml}^{-1}$, for 8 minutes) were able to elicit the aggregation of platelets (to varying extents). However, C_{60} was not effective in this assay, suggesting that they are relatively less thrombogenic than other nanoparticles.

The limited number of available investigations provided conflicting results, regarding the pro-thrombogenic potential of fullerenes. It is therefore likely that the fullerene derivative, and experimental set up (including the model used, particle concentration and exposure time) were able impact on the findings.

6.2.3.3 Additional targets

Interest in investigating the ocular toxicity of fullerenes derives from the potential to exploit fullerenes as drug carriers that bypass blood-ocular barriers to enable their delivery to the blood (Roberts *et al.* 2008). Fullerols ($\text{C}_{60}(\text{OH})_{22-26}$) have been observed to accumulate within human HLE-B3 lens epithelial cells within *in vitro* and *ex vivo* models, and this accumulation was associated with cytotoxicity (Roberts *et al.* 2008). The cytotoxicity of fullerols was observed to be enhanced with UVA and visible light exposure during treatment, illustrating that there is a photosensitive aspect to fullerol toxicity. The endogenous antioxidant lutein was able to offer some protection against the photo-oxidative cytotoxicity induced by fullerol, thus suggesting an ROS component to the response. However this was not conclusive since neither ascorbic acid

or N-acetylcysteine antioxidants could achieve the same effect. It was also observed that fullerol was able to bind to the lens protein α -crystalline (which is likely to increase its retention within cells), so that interactions with biological molecules is a realistic possibility. Consequently, the potential for fullerene internalisation, enhanced ROS production, and interactions with cellular components were highlighted within this study.

However, it is relevant that Huczko *et al.* (1999) used the Draize rabbit eye irritation test to reveal the potential toxicity of fullerenes to the eye. Instillation of a fullerene soot suspension (for up to 72 hours) was observed to have no toxicity within the eye.

The two studies that determined the toxicity of fullerenes to the eye, were contradictory in nature, which is likely to derive from the use of different fullerenes, and models. Consequently, definite conclusions regarding the ocular toxicity of fullerenes cannot be made without the completion of further investigations.

6.2.4 The biological mechanisms driving fullerene toxicity

A number of investigators have demonstrated that fullerenes are capable of eliciting toxicity that is mediated via the stimulation of an inflammatory response, and the involvement of oxidative stress. Therefore it may be possible to generate broad conclusions regarding the mechanisms underlying fullerene toxicity. Processes underlying fullerene toxicity will therefore be discussed. In addition, the uptake of fullerenes by cells will be addressed, as this has the ability to promote their clearance, but also toxicity. The genotoxic and reproductive toxicology of fullerenes will also be considered.

6.2.4.1 Fullerene mediated inflammatory responses

Nanoparticles of a variety of types have been demonstrated to induce inflammation and so it is often believed to be a common response associated with exposure (for reviews see for example, Kagan *et al.* 2005; Donaldson *et al.* 2005; Donaldson and Stone 2003). As such, the pro-inflammatory potential of fullerenes requires consideration, due to their definition as nanoparticles. There are *in vitro* investigations that indicate that an inflammatory response may be instrumental to the toxicity of fullerenes, as demonstrated by the enhanced production of pro-inflammatory mediators such as IL-8 and TNF α (see for example Rouse *et al.* 2006). However, there is a lack of information available regarding *in vivo* inflammatory mediated responses, which should be a focus of future experiments. Furthermore a concentration dependent effect is likely, as Roursgaard *et al.* (2008) demonstrated that fullerol has an anti-inflammatory effect within the mouse lung at lower doses but a pro-inflammatory effect at higher concentrations, within mice.

Some studies suggest that fullerene derivatives may in fact be capable of suppressing inflammatory responses. Huang *et al.* (2008) generated C₆₀ based fulleropyrrolidine-xanthine molecules. It was anticipated that the fullerene component would act as a free radical scavenger, and the xanthine attachment would be capable of suppressing inflammatory reactions. Pre-treatment of LPS stimulated J774 macrophage-like cells with the fullerene was effective at scavenging LPS induced nitric oxide and TNF α production. These findings therefore suggest that fullerene derivatives could be exploited as anti-inflammatory agents. However, more work is required to investigate this hypothesis further.

Additionally, Tsao *et al.* (1999) demonstrated that carboxyfullerene (2 mg ml⁻¹) pre- or post-treatment (up to 40 mg kg⁻¹) was able to attenuate *E. Coli* mediated meningitis within mice, following intraperitoneal injection. It was suggested that *E. Coli* induced inflammation increased permeability of the blood brain barrier, thus permitting the access of fullerenes into the brain, in order to enable their protective behaviour to emerge. Carboxyfullerenes may therefore have a protective effect against bacterial meningitis, which was more effective than dexamethasone (anti-inflammatory steroid) treatment. However, it is worth highlighting that the concentrations of fullerenes used within this experiment were high.

Harhaji *et al.* (2008) investigated the impact of fullerene treatment on TNF α mediated cell death. It was observed that C₆₀/C₇₀ and polyhydroxylated fullerene preparations (up to 250 $\mu\text{g ml}^{-1}$) for 24 hours) were cytotoxic to the mouse L929 fibroblast cell line, but that C₆₀/C₇₀ was more potent. Furthermore, a combined treatment of C₆₀/C₇₀ with TNF α was more toxic than observed for each treatment alone, thus suggesting a synergistic interaction. Paradoxically, it was evident that a co-treatment of polyhydroxylated fullerene with TNF α was able to reduce the cytotoxic effect of TNF α , thus insinuating that functionalised fullerenes acquired a protective activity. This finding was further supported by the observation that C₆₀/C₇₀ exposure enhanced, and polyhydroxylated fullerenes prevented, TNF α mediated ROS production and mitochondrial depolarisation. It was speculated that the capacity of fullerenes to modulate TNF α mediated toxicity was dictated by their ability to modulate TNF α mediated ROS production. Specifically C₆₀/C₇₀ was suggested to enhance ROS production increasing the cytotoxic response associated with TNF α exposure, whereas polyhydroxylated preparations attenuated ROS production, and thereby had a cytoprotective effect, by antagonising TNF α mediated cytotoxicity. The study therefore highlighted that two fullerene preparations can behave very differently, which is a logical conclusion but means that it is difficult to make generalisations about fullerene behaviour, and therefore predict their behaviour.

It is often assumed that nanoparticles stimulate a response that is inflammatory in nature, and this has been demonstrated, to a very limited extent, for fullerenes. However, in contrast, the anti-inflammatory nature of fullerenes has been a focus of investigations due to opportunity to exploit this phenomenon within therapeutic interventions. The findings indicate that the concentration of fullerene, the fullerene derivative in question (which, on occasions were purposefully altered to integrate an anti-inflammatory aspect) and experimental model used are able to impact on the inflammatory potential of fullerenes.

6.2.4.2 Fullerene mediated oxidative responses

The ability of nanoparticles to enhance ROS production within cells, and thereby stimulate the development of oxidative stress (see for example Stone *et al.* 1998), has prompted investigations to determine whether fullerenes have the same pro-oxidant potential, due to their classification as nanoparticles.

Sayes *et al.* (2005) demonstrated that nano-C₆₀ (0.24 – 2400 ppb) exerted cytotoxicity that was mediated through enhanced ROS production, lipid peroxidation, and membrane damage in a variety of cell lines (dermal fibroblasts, hepatocytes and astrocytes). The damage to cell membrane integrity was confirmed by evidence that lactate dehydrogenase (LDH) was released from cells, and that fullerene exposed cells were more permeable to dextran. The involvement of ROS was confirmed by the observation that the administration of the antioxidant ascorbic acid could prevent against the appearance of fullerene mediated cytotoxicity. Similar observations were made by Kamat *et al.* (2000) who observed that C₆₀, and C₆₀(OH)₁₈ could elicit membrane damage under photosensitive conditions, which was accounted for by the appearance of lipid peroxidation within isolated rat liver microsomes. Furthermore the oxidation of proteins, indicated by the formation of protein carbonyls, depletion of membrane enzymes, and attenuation of the toxic response using antioxidants, all provided further confirmation of an oxidant driven response. Although both fullerene species were capable of eliciting a pro-oxidant response, the toxicity was greater for C₆₀(OH)₁₈ than for C₆₀.

Paradoxically Xia *et al.* (2006) illustrated fullerol (C₆₀(OH)₂₂₋₂₆) exposure was incapable of stimulating ROS production, depletion of GSH or stimulation of HO-1 expression within RAW 264.7 macrophages, despite the fact that it was a powerful ROS producer in cell free conditions, and that the fullerols were internalised by cells. No TNF α production was associated with fullerol exposure, but an increase in mitochondrial calcium levels was observed which implied mitochondrial damage occurred, although no changes in mitochondrial membrane potential were realised. A panel of ambient and manufactured nanoparticles were tested within the same study and importantly, it was recognised that they differed in their ability to be internalised, stimulate ROS production, deplete cellular antioxidants, induce mitochondrial toxicity and cytotoxicity.

The findings outlined are often contradictory, and suggest that in some conditions fullerenes may induce pro-oxidant effects, but in others they do not, and this is likely to be dictated by the fullerene in question, the cell type being investigated, and the experimental set up.

6.2.4.3 Antioxidant properties of fullerenes

There has been a focus on investigating the potential free radical scavenging activity of C₆₀ which has prompted some fullerene derivatives to be described as 'radical' sponges (Xiao *et al.* 2006). This is driven by the knowledge that fullerene administration may be exploited to protect against radical mediated damage, that is associated with toxicant exposure or a number of disease states.

Wang *et al.* (1999) demonstrated that lipid soluble and water soluble C₆₀ derivatives prevented superoxide and hydroxyl radical initiated lipid peroxidation within rats, to a greater extent than the natural antioxidant, vitamin E. While Dugan *et al.* (1996) observed that fullerols were potent antioxidants, and were able to decrease excitotoxic mediated neuronal cell death exerted by N-methyl-D-aspartic acid (NMDA) or α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA).

Gharbi *et al.* (2005) observed that the pre-treatment of rats with C₆₀ (0.5 – 2 g kg⁻¹) via intraperitoneal injection, protected the liver from carbon tetrafluoride mediated liver damage, with the free radical scavenging activity of C₆₀ assumed to drive the protective effect observed. It was also suggested that the antioxidant potential of C₆₀ is dependent on its degree of dispersion. The authors suggested that aggregates of C₆₀ will not exhibit antioxidant properties due to the lack of availability of the unsaturated bonds contained within the molecule's structure, so that the antioxidant behaviour of fullerenes is improved within water soluble forms. The authors did not discuss the potential for C₆₀ to bind to carbon tetrafluoride, therefore reducing its bioavailability. A decrease in aggregate size would also increase surface area which would make this rather non-specific mechanism even more potent. In addition, the authors highlighted that it was necessary to inject exceptionally high doses of C₆₀, in order to obtain a reproducible level of accumulation within the liver and thereby allow the exertion of its protective effects. However, as no toxicity was associated with this exposure the use of such high doses was justified ethically, but not perhaps in terms of relevancy.

In a different study, Yin *et al.* (2009) investigated the free radical scavenging activity, and therefore cytoprotective properties of a number of fullerene derivatives. It was observed that a gadolinium based metallofullerol (fullerene cage encapsulates metal) Gd@C₈₂(OH)₂₂, C₆₀(OH)₂₂ and C₆₀(C(COOH)₂)₂ all aggregated, so that the average size of the particles contained within the aqueous suspensions was 78 nm (Gd@C₈₂(OH)₂₂), 123 nm (C₆₀(OH)₂₂) and 170 nm (C₆₀(C(COOH)₂)₂), and that the extent of aggregation dictated their antioxidant behaviour. Specifically, the electron spin resonance (ESR) spin trap technique was used as an *in vitro* cell free method to show that the fullerene derivatives could efficiently scavenge a number of free radicals. It was then demonstrated that hydrogen peroxide induced cytotoxicity, within A549 cells and rat brain capillary endothelial cells (rBECs) was reduced by a pre-treatment with the derivatised fullerenes (up to 100 μ M), with C₆₀(C(COOH)₂)₂ being the least protective and Gd@C₈₂(OH)₂₂ exhibiting the greatest protective effect. Yin *et al.* (2009) concluded that fullerene derivatives exerted antioxidant-like behaviour, and that suspensions of (C₆₀(C(COOH)₂)₂) contained larger sized particle aggregates that limited their antioxidant potential. This observation therefore supports the suggestion that better dispersed C₆₀ suspensions exhibit a more effective antioxidant capacity. The therapeutic exploitation of the antioxidant properties exhibited by Gd@C₈₂(OH)₂₂ have also been explored *in vivo*, where its administration was been demonstrated to inhibit the growth of malignant tumours within mice, following intraperitoneal administration, and that this was due to their ROS scavenging activity (Yin *et al.* 2008).

Xiao *et al.* (2006) prepared a polymer-wrapped (polyvinylpyrrolidone) fullerene, and named it a Radical Sponge. It was found that treatment of the cells with the fullerene derivative (up to 75 μ M), prior to UVA irradiation, was able to protect HaCaT keratinocyte cells from UVA mediated cytotoxicity, thus demonstrating their cytoprotective behaviour. Further investigations revealed that this antioxidant effect was a consequence of the ROS scavenging activity of the

fullerene derivative, and not due to its UVA sheltering or UVA absorbing activity. Similarly Lin *et al.* (2002) observed that the systemic administration of carboxyfullerenes (6 mg kg^{-1}) prior to ischemic/reperfusion injury of the rat brain was unable to impact on the size of the infarction area that developed, perhaps due to its inability to cross the blood brain barrier. In contrast, local pre-treatment with carboxyfullerenes (up to 0.3 mg) was associated with a reduction in infarct area and lipid peroxidation associated with ischemia-perfusion. However, the local administration of 0.3 mg fullerene was associated with toxicity; namely adverse behavioural changes, and in a few cases, death. Therefore, the neuroprotective antioxidant properties of carboxyfullerenes were demonstrated, but investigation into the toxic response associated with fullerene exposure requires further assessment, as this may limit their therapeutic exploitation within the treatment of ischemic/reperfusion injury. Tykhomyrov *et al.* (2008) also investigated the neuroprotective effects exhibited by fullerenes, whereby they co-administered water soluble, hydrated fullerenes to rats (30 nM, in drinking water) along with alcohol. The fullerenes protected against the oxidative damage within brain tissue that is associated with chronic alcohol exposure. In addition, Mori *et al.* (2007) demonstrated that fullerenes could be used as an effective pre-treatment at reducing the lethality associated with methamphetamine and morphine co-exposure of mice, which are also known to occur via oxidative stress. This protective effect was equivalent, or superior to that of more traditional treatments, including cooling and the administration of phospholipase (PLA)₂ inhibitors.

The use of fullerenes as therapeutic agents has also been explored in relation to their antioxidant driven cytoprotective behaviour. Injac *et al.* (2009) investigated the protective effect of fullerol ($\text{C}_{60}(\text{OH})_{24}$) pre-treatment (25, 50 and 100 mg kg^{-1} , following intraperitoneal injection) on doxorubicin toxicity (which is oxidant mediated) within the heart and liver of rats with colorectal cancer. The protective effect of fullerol was witnessed within macro, and microscopic observations, ECG evaluation, serum biomarkers for myocardial or hepatic damage, and oxidative stress development. In general, an improvement in doxorubicin associated toxicity was observed within the heart and liver, with fullerol pre-exposure. However, lower doses of fullerols exhibited a greater protective effect, and this may be accounted for by the fact that higher doses of fullerols were less well absorbed from the gut, or that the high doses administered contributed to the toxicity that was apparent. Therefore, it was again demonstrated that fullerols are able to exhibit protective effects against oxidative mediated injury, thereby promoting their exploitation as antioxidants, and that this was a dose dependent phenomenon. Trajkovic *et al.* (2007) investigated the protective effects of fullerol (10 and 10 mg kg^{-1} , for up to 30 days), administered via intraperitoneal injection, against ionising radiation within rats, and compared this to the traditional radioprotector, amifostine. Fullerol pre-treatment was able to improve survival rates within rats. The radioprotective effect exerted by fullerols was most pronounced at a dose of 100 mg kg^{-1} , and was comparable to that of amifostine. As the harmful effects of radiotherapy are known to be mediated by ROS, fullerols were anticipated to be effective radioprotectors by a mechanism that was antioxidant in nature. The potential therapeutic exploitation of fullerols was addressed further by Bogdanovic *et al.* (2008) who assessed the ability of $\text{C}_{60}(\text{OH})_{24}$ to protect against ionising radiation mediated ROS production *in vitro*. The protective effects of fullerol ($10 \text{ }\mu\text{M}$) were investigated within irradiated malignant cultured K562 erythroleukemia cells. The survival rate of irradiated cells was improved by fullerol pre-treatment, which was suggested to occur due to increased antioxidant defences within irradiated cells that counteracted the oxidative damage associated with radiation that acted to preserve cell viability.

Although the free scavenging activity of fullerenes is accepted as being potentially beneficial, in certain circumstances it can be problematic. For example, Ueng *et al.* (1997) observed that a single intraperitoneal injection of fullerol; at concentrations of 0.1, 0.5 and 1 g kg^{-1} , induced mortality of 10%, 22% and 54% respectively, within 3 days of the treatment. Fullerol administration, of greater than 0.5 g kg^{-1} , also elicited a decrease in cytochrome P450 content and activity within liver microsomes (isolated from fullerol exposed animals). The mechanism of this decrease is not known but could be due to the electron scavenging behaviour of polyhydroxylated C_{60} , or binding of fullerol to the enzyme thus promoting enzyme destruction, or prevention of enzyme synthesis as a result of cell injury. In addition, mitochondrial function was observed to be diminished by fullerol exposure, as indicated by the suppression of oxidative phosphorylation, which is likely to derive from a reduction in the transfer of electrons. However

the high doses used are likely to account for the mortality and cell injury observed and are unlikely to be encountered by humans.

Overall, the studies relating to antioxidant properties of fullerenes suggest that contrary from being toxic, C₆₀ and its derivatives could actually exhibit beneficial health effects. However, it appears that the antioxidant properties exhibited by fullerenes are restricted to particular fullerene forms, and therefore a number of conditions being met in order to allow its manifestation. Water solubility is likely to impact on their antioxidant/cytoprotective potential, so that the better dispersed the fullerene is, the more likely it is that it will exert free radical scavenging activity, which is likely to derive from their derivatisation, as they are specifically generated to improve fullerene water solubility. The concentration administered is therefore also key to dictating the free radical activity, as fullerenes exposed at high concentrations are more likely to interact to form larger structures, which is known to detrimentally impact on its antioxidant behaviour. Many of the studies reported have used exceptionally high exposure concentrations and are therefore difficult to interpret in terms of relevance unless large doses are used in such applications.

6.2.4.4 Uptake of fullerenes into cells

Uptake studies have investigated the behaviour of both professional phagocytes, such as macrophages, as well as non-phagocytic cells. Determining the uptake of fullerenes by cells is of relevance as phagocytic cells, at exposure sites are responsible for the clearance of particles. Secondly, the uptake of fullerenes by cells has the potential to impact on normal cell physiology and function, which requires assessment. When addressing the uptake of fullerenes, a variety of cell types have been considered. In addition, computer simulations have been conducted that act to predict the interactions of fullerenes with cell membranes and their subsequent penetration.

It has been observed that subsequent to pulmonary exposure fullerenes are evident within alveolar macrophages (Fujita *et al.* 2009, Xia *et al.* 2006). Furthermore, subsequent to intraperitoneal administration fullerenes enter the circulation, and have been observed to accumulate within Kupffer cells in the liver (Gharbi *et al.* 2005). Macrophages therefore appear to be capable of taking up particles, to thereby fulfil their role within host defence. However, the consequences of fullerene uptake require attention as oxidative or inflammatory events may be stimulated. In addition, a number of other cell types have been demonstrated to internalise fullerenes, such as keratinocytes (Rouse *et al.* 2006), epithelial cells (Fujita *et al.* 2009) and eye lens cells (Roberts *et al.* 2008) often with oxidative and lethal consequences.

Computer simulation has been exploited to reveal the mechanism of fullerene permeation through cell membranes (Wong-Ekkabut *et al.* 2008). It was identified that small fullerene clusters (<10 molecules) were able to localise within the membrane lipid bilayer, where they disaggregated, and that this process was passive and spontaneous, and that even at high concentrations no mechanical damage to the membrane was observed. However, although the computer simulations provide insight into the possible behaviour of fullerenes, caution is required when extrapolating the results as the interactions of fullerenes with other membrane components, such as other lipids, carbohydrates and proteins were not considered within the model, and it is therefore necessary to verify the results. However, the tendency for fullerenes to interact with the lipid tails within the model, parallels the finding that lipid peroxidation is a prominent feature of their exposure to cells.

In addition, Bedrov *et al.* (2008) used molecular dynamic models to investigate the interaction and transport of C₆₀ within a plasma membrane model. C₆₀ was observed to interact with the lipid head groups, and lipid core of the membrane. Consequently, fullerenes were predicted to have a high permeability, within the simulations conducted. For this reason, the authors suggested that fullerenes could be exploited as efficient carriers to enable drug entry into cells. Similarly, Qiao *et al.* (2007) used molecular dynamics to study the translocation of fullerenes across a model cell membrane. Pristine C₆₀ was observed to readily translocate across the lipid membrane. This was achieved due to the ability of C₆₀ to create a cavity (termed transient micropores) within the membrane. C₆₀ molecules were then speculated to 'jump' into the membrane, which enabled the penetration of the molecule through the membrane. In contrast,

$C_{60}(OH)_{20}$ derivatives barely penetrated the membrane, which was explained by their absorption onto the membrane surface, due to its hydrophilic nature. This interaction discouraged $C_{60}(OH)_{20}$ interaction with the lipid core of the membrane, so that it did not enter the membrane, but instead the strong interactions with the membrane surface head groups, caused a 'pinch' to form in the plasma membrane. Therefore, pristine fullerenes, and fullerene derivatives exhibit differences, with regards to their translocation through the model membrane, and perhaps this can explain differences in their toxicity, and why fullerene derivatives exhibit reduced toxicity than their pristine counterparts (see for example Sayes *et al.* 2004). However, in the main, the simulations did not consider the aggregation state of fullerenes, and depict the consequences of membrane exposure to individual molecules, which would be anticipated to more easily enter cells due to their small size (<1 nm).

Porter *et al.* (2006) found that C_{60} did not elicit cytotoxicity within human derived macrophages ($0.16-10 \mu\text{g ml}^{-1}$) *in vitro*, despite the fact that they were internalised, and contained within the cytoplasm, nucleus and lysosomes. It was observed that C_{60} crystals and aggregates were contained within lysosomes (perhaps as a consequence of their internalisation by phagocytosis or endocytosis), in which C_{60} were degraded into smaller structures. C_{60} aggregates were also apparent along the plasma membrane, which was suggested by the authors to promote the development lipid peroxidation observed by other investigators. It was highlighted that there was a difficulty in imaging particles, due to the fact that it is difficult to distinguish them from artefact presence, and so the results require further verification.

The uptake of fullerenes has been demonstrated on numerous occasions, within a variety of cell types. The implications of uptake are relatively unknown, and therefore worthy of consideration in the future, but are likely to be oxidative or cytotoxic in nature. Computer simulations have also been used to predict the penetration of fullerenes within the plasma membrane, and attributes of particles that encourage such an interaction, but their relevance requires confirmation.

6.2.4.5 Genotoxicity of fullerenes

Genotoxicity tests are conducted to reveal damage to DNA elicited by fullerene exposure, by for example detecting mutations, and changes in chromosome structure or number. A number of assays can be adopted to detect genotoxicity, including the Comet assay, Ames test, and determining tumour development within animals.

Dhawan *et al.* (2006) investigated whether C_{60} was able to inflict DNA damage within human lymphocytes, and was detected using the Comet assay, when exposed at concentrations ranging from 0.42 to $2100 \mu\text{g l}^{-1}$, for up to 6 hours. To ensure that residual solvents used to prepare C_{60} suspensions were not responsible for any observed toxicity (see later), the experiments were conducted using preparation methods that were free of organic solvents. These included prolonged mixing of C_{60} in water ($aquC_{60}$), or the solvent to water exchange method using ethanol ($EtOHC_{60}$). It was demonstrated that solvents were more effective at dispersing C_{60} , as demonstrated by the fact that the size of C_{60} clusters was smaller (122 nm diameter) than those produced within $aquC_{60}$ suspensions (178 nm diameter). Both samples were able to cause DNA damage within lymphocytes, with $aquC_{60}$ being more effective. The results therefore highlight that the dispersion method is able to impact on the toxicity of C_{60} , whereby fullerenes prepared by mixing in water were more capable of eliciting a genotoxic response than those produced using the solvent to water exchange method. In addition, Sera *et al.* (1996) investigated the mutagenic effect of fullerene exposure (up to $30 \mu\text{g}$ per plate, for 48 hours) on *Salmonella typhimurium*, in light and dark conditions using the Ames test. If exposure occurred within the dark, no mutagenic responses were evident. In contrast, a mutagenic effect was observed, when exposure occurred in the presence of visible light, due to the production of ROS, which interact with DNA to elicit damage, and was typified by the formation of 8-hydroxydeoxyguanosine. Lipid peroxidation was also increased by fullerene exposure in light, further highlighting the oxidative consequences associated with light irradiation. The study therefore illustrated the phototoxic, and mutagenic properties of fullerenes.

Contrary to the ability of C_{60} to induce genotoxic events within cells, fullerene derivatives have been demonstrated to have potential therapeutic properties for the treatment of cancer. Chen

et al. (2005) observed that $\text{Gd}@C_{82}(\text{OH})_{22}$ inhibited skin tumour growth within hepatoma implanted mice, with this inhibitory effect apparent on treatment of mice for 6 days or more (via intraperitoneal injection, up to $228 \mu\text{g ml}^{-1}$). The anti-tumour efficiency of $\text{Gd}@C_{82}(\text{OH})_{22}$ was greater than that of the conventional antineoplastic agents cyclophosphamide and cisplatin. The administration of C_{60} was associated with very low toxicity *in vivo*, and with no cytotoxicity evident within hepatocyte cell lines *in vitro*. Furthermore Tabata *et al.* (1997) aimed to target radiolabelled ^{125}I C_{60} -polyethylene glycol (PEG) conjugates ($424 \mu\text{g kg}^{-1}$) to tumours, following intravenous injection within tumour bearing mice. The distribution of the fullerene was determined following exposure, and 78% of the administered dose was eliminated from the body within 24 hours. No marked accumulation within a particular organ was observed (probably as a consequence of its derivatisation), although localisation within the liver and GIT was observed, with no toxicity associated with exposure. The fullerene was able to accumulate within tumours, due to its large size (which also relied on the hyperpermeability of tumour vasculature). Light irradiation (to achieve photoactivation of the fullerene) at the tumour site allowed the specific destruction of the tumour by the fullerenes, with no damage to the overlying normal skin. Tumour destruction was not apparent with C_{60} -PEG administration alone, therefore light irradiation was essential for the tumour destructive effect to manifest. Accordingly, the phototoxic property of fullerenes was exploited within the destruction of tumours, and fullerenes may therefore be considered for photodynamic therapy of tumours. However, its exploitation is reliant on the utilisation of particular C_{60} derivatives; accordingly PEG conjugation to C_{60} was necessary to increase the size of the molecule, and to increase its water solubility. In addition, Zhu *et al.* (2008) evaluated the tumour inhibitory effect of fullerols (0.2 and 1 mg kg^{-1} , administered daily via intraperitoneal injection, for up to 17 days) within the mouse H22 hepatocarcinoma model. $C_{60}(\text{OH})_x$ exhibited anti-tumour activity, with treatment more effective if administered from the time of tumour inoculation onwards, as opposed to when treatment was initiated after tumour growth reached 1cm.

Furthermore, genotoxicity has not been associated with fullerene exposure in a number of models. Mori *et al.* (2006) investigated the mutagenicity of a C_{60}/C_{70} mixture. It was illustrated that no mutagenic responses were evident within a variety of *Salmonella typhimurium* and *Escherichia Coli* strains, using the Ames test (up to $5000 \mu\text{g}$ per plate). In addition, within the chromosomal aberration test (in CHL/IU hamster lung cells) no aberrations within the structure or number of chromosomes were apparent. The separate tests therefore reached the same conclusion that the fullerene investigated was not mutagenic. Furthermore, Jacobsen *et al.* (2008) investigated the mutagenicity associated with a number of carbon based nanoparticles, including C_{60} within the mouse FE1-Muta epithelial cell line. It was demonstrated that C_{60} exposure (0 - $200 \mu\text{g ml}^{-1}$, 24 or 576 hours) was associated with a slight increase in ROS production in cells and in cell free conditions, but no impact on cell viability was observed. C_{60} was not capable of eliciting strand breaks, and no alterations in mutation frequency were observed when using the Comet assay.

The genotoxicity of fullerenes is therefore difficult to interpret from the studies conducted so far, with contradicting results reported. Again these are likely to be influenced by the dose, dispersion, model and endpoint measured. An important component of the genotoxic response exhibited by fullerenes is anticipated to be their photoactivity which is able to promote such a response. However, the opportunity to exploit fullerenes as anti-tumour agents is of interest, and warrants further investigation.

6.2.4.6 Reproductive Toxicology of fullerenes

Evaluation of fullerene effects on the reproductive system are limited to a small number of *in vivo* and *in vitro* studies. Most of these have focused on how treatment can affect the developing embryo. A study by Tsuchiya *et al.* (1996) intraperitoneally administered polyvinylpyrrolidone solubilised C_{60} (up to 137 mg kg^{-1}) to pregnant mice, on day 10 (vaginal plug: day 0). After 18 hr of exposure all the embryos were examined and found to be dead. A variety of doses were examined with C_{60} apparently distributed into the embryos at 50 mg kg^{-1} resulting in head region and tail abnormalities. The yolk sac appeared with shrunken membrane and narrow blood vessels which may suggest insufficient blood supply to the embryos. At 25 mg kg^{-1} , one embryo had abnormal enlargement of the head, whereas all other embryos appeared normal. The no-observed-adverse-effect level (NOAEL) was 16.7 mg kg^{-1} .

The study recommends further work due to the low number of animals per exposure group. This is an unusual route of administration, using a relatively high exposure dose and it covers only a small part of the pregnancy period.

A study by Zhu *et al.* (2008) assessed embryonic development using the zebra fish model. The group exposed newly fertilized eggs to 50 mg l⁻¹ fullerol (C₆₀(OH)₁₆₋₁₈), nano aggregates of C₆₀ (1.5 mg l⁻¹; particle size: ~100 nm) or a combined treatment of nanoC₆₀ (1.5 mg l⁻¹) and glutathione ((GSH), 30 mg l⁻¹) for 96 hours. Observed endpoints were survival, hatching rate, heart beat rate and pericardial oedema. The fullerol solution had no adverse effect on the endpoints, whereas the C₆₀ suspension had a conspicuous adverse effect on all parameters that was lessened by the addition of GSH. Zhu *et al.* (2008) concluded that the adverse effects of C₆₀ were due, at least partly, to free radical-induced mechanism or another form of oxidative stress. Similarly, Usenko *et al.* (2008) also used zebra fish embryos to examine the potential of C₆₀ to elicit oxidative stress responses. The group's findings showed reduced light and therefore reduced photo catalytic activity during C₆₀ exposure significantly decreased mortality and the incidence of fin malformations and pericardial edema at 200 and 300 ppb C₆₀, 24 hours post fertilisation. Embryos co-exposed to the glutathione precursor, N-acetylcysteine (NAC), also showed reduced mortality and pericardial edema; however, fin malformations were not reduced.

Han and Karim, (2009) examined cytotoxicity of fullerene C₆₀ particles in Chinese hamster ovary mammalian cell line (CHO). Particles, 10 mg, were dissolved in 250 ml tetrahydrofuran (THF) which was previously sparged with N₂ to remove oxygen. Upon resealing the THF-fullerene particle mixture, the suspension was stirred at room temperature for 24 hours, to allow it to be saturated with soluble fullerene particles. Upon saturation the solution was then vacuum filtered using a 0.45 µm nylon membrane filter. The dissolved fullerene particles in the THF solvent could be extracted into a water solution at a significantly increased solubility. Results show that once the concentration of the fullerene aggregates reaches a certain level, the cells start to die. The lethal dosage LD₅₀, within 24 hours, was determined at 33 mg l⁻¹. Furthermore, the percentage of cell mortality increased with increasing fullerene concentration and incubation time yielding a negative effect on cell viability. CHO cell mortality was reported as a function of the incubation time (0–6 days), at five different fullerene particle dosages (7.6–69.8 mg l⁻¹). The group conducted two controls to highlight THF were not interfering with cytotoxicity as THF itself is a toxic agent. The first via HPLC analysis showed no THF spectrum detected in the prepared fullerene aqueous solution and secondly the group generated 'prepared water' whereby THF without fullerenes was processed as described above and was shown to induce no toxic effect on cells. These results, illustrate the potential toxicity of fullerene particles in mammalian ovary cells.

The reviewed literature examines the effects of fullerenes during pregnancy, highlight effects on developing embryos, however studies are extremely limited in number and in sample size. Only one study identified examined effects on an ovarian cell line model with no studies focused on other organs or cell types in the female reproductive system. No specific in vitro or in vivo studies were found examining fullerene effects in male reproductive system.

6.2.5 Linking the physico-chemical attributes of fullerenes to pathogenicity or toxicity

As for other particles, the physico-chemical properties of fullerenes are likely to impact on their toxicity. Such characteristics include composition, crystal or aggregate size, water solubility and surface modifications/functionalisation.

6.2.5.1 Surface modifications

Surface modification is also termed derivatisation or functionalisation and is used to achieve a specific change in particle properties. Common examples of particle functionalisation include hydroxylation (-OH) and carboxylation (-COOH). In general, the surface of fullerenes is completed for a specific purpose, such as improving water solubility. In addition, it is recognised that such changes may also influence toxicity, as for example fullerene

functionalisation has been observed to promote the appearance beneficial properties such as antioxidant, or anti-inflammatory activity.

Accordingly, 'pristine' (unmodified) C₆₀ has been demonstrated to be more toxic than the functionalised water soluble counterparts (including C₆₀(OH)₂₄) when examined *in vitro* with keratinocyte and hepatocyte cell lines (Sayes *et al.* 2004). In fact, as the fullerene cage became more derivatised, and therefore more water soluble, the corresponding toxicity was observed to decrease. However, in a separate study, Sayes *et al.* (2007) exposed rats via intratracheal instillation (0.2 – 3 mg kg⁻¹) to C₆₀ or C₆₀(OH)₂₄ and found that both fullerene types did not induce toxicity from 1 day to 3 months post exposure, as indicated by LDH release, neutrophil infiltration, oxidative stress or histopathological analysis. Although none of the fullerene derivatives inflicted toxicity, the administration of quartz as a control produced a pro-inflammatory and pro-fibrotic response. Therefore, although functionalisation may affect *in vitro* toxicity, this may not translate *in vivo* effects, highlighting the potential difficulty in extrapolating and interpreting experimental findings (Sayes *et al.* 2007). However, there is also a difference between the *in vitro* models and the *in vivo* models with respect to target organ under investigation; the *in vivo* study investigated pulmonary responses, while the *in vitro* cell lines included keratinocytes and hepatocytes. Therefore, fullerenes may not be universally toxic to different tissue or cell targets, if they are able to be systemically available and thereby reach cells.

In contrast to the results of Sayes et al. (2007), Kamat et al. (2000) demonstrated that C₆₀(OH)₁₈ was more toxic than underderivatised C₆₀ in vitro (see earlier), although both were capable of eliciting oxidative damage. Therefore, the influence of surface attachments on fullerene toxicity may be dependent upon the target cell/organ and/or the fullerene type under investigation.

6.2.5.2 The impact of fullerene dispersion on toxicity

C₆₀ has a hydrophobic character, and therefore unless rendered water soluble through derivatisation of its surface (see above), a stable suspension of C₆₀ in aqueous solutions is difficult to achieve (Dhawan *et al.* 2006). However, 'pristine' C₆₀ molecules are able to form a stable water soluble suspension that is comprised of C₆₀ clusters or aggregates, that generally range from 5-500 nm in diameter (Fortner *et al.* 2005, Dhawan *et al.* 2006). To obtain C₆₀ suspensions a number of techniques can be employed (Dhawan *et al.* 2006). A 'solvent to water exchange' protocol can be followed whereby fullerenes are mixed with a solvent (such as THF), which is then removed via evaporation or distillation. Alternatively fullerenes can be dispersed in a solvent (such as toluene or ethanol) mixed with water, and then sonicated to transfer the fullerenes to the water phase. In addition fullerenes can be mixed with water for an extended period, which is often termed aqueousC₆₀. It has been suggested that the toxicity associated with C₆₀ exposure may derive as a consequence of the presence of residual solvent (or their derivatives), which have the potential to become intercalated into the lattice C₆₀ structure or are released into the aqueous phase. This has implications for fullerene preparation as such impurities may impact upon the observed toxicity.

Isakovic *et al.* (2006) dispersed C₆₀ using THF (termed THFC₆₀), and subjected the suspension to γ irradiation in order to decompose residual THF. The non-irradiated THFC₆₀ sample (0.125-1 $\mu\text{g ml}^{-1}$) elicited cell death, via a necrotic mechanism, within all cell types tested (mouse fibroblast L929, mouse B6 melanoma cells, rat glioma C6, and human glioma U251 cell lines, primary rat astrocytes, mouse and rat macrophages). The authors concluded that this cytotoxic response was associated with increased ROS production, as the toxicity was associated with lipid peroxidation and pre-treatment with the antioxidant N-acetyl cysteine prevented the THFC₆₀ induced toxicity. In contrast, the irradiated THFC₆₀ sample failed to elicit a cytotoxic effect, and was instead deemed to be cytoprotective in nature due to its antioxidant properties as demonstrated by its ability to prevent against hydrogen peroxide mediated toxicity in cells.

Markovic *et al.* (2007) compared the toxicity of C₆₀ when prepared within different solvents (THF or ethanol) or within an aqueous solution. All fullerene preparations contained particles that had a diameter of less than 36 nm. Cytotoxicity, ROS production and mitochondrial depolarisation was evident (within human NTCC 2544 keratinocyte, human NHDF dermal fibroblast, mouse B6 melanoma, and mouse L929 fibrosarcoma cell lines), with THFC₆₀ exhibiting the greatest

toxicity, and aqueous C₆₀ the least. No species, cell type or assay dependence was observed to influence the toxicity. It was also proposed, by the authors that THF was able to intercalate into the structure of fullerenes, to increase its toxic potential.

Zhang *et al.* (2009) generated THFC₆₀ and the samples either remained unwashed or were washed with water, in order to investigate whether residual solvent presence contributed to any observed toxicity. In addition a C₆₀ suspension was generated by first preparing C₆₀ within toluene. The major THF derivative contained within the THFC₆₀ suspension was THF peroxide, which accumulated within the water phase of the sample and was introduced during the preparation of THFC₆₀ suspensions. Washing of the sample was successful in effectively removing THF peroxide. Washed THFC₆₀ and C₆₀ in toluene did not exhibit oxidative properties but unwashed THFC₆₀ increased ROS production and the THF derivative was held responsible for this effect. Unwashed THFC₆₀ was the only sample that had a bacteriocidal action to *E. Coli*, and again its THF peroxide content was suggested to be accountable for this effect.

Fortner *et al.* (2005) investigated whether the size and stability of C₆₀ suspensions is related to the conditions surrounding their formation, namely, the rate of water addition and pH of the solution. It was found that a higher pH promotes the formation of smaller C₆₀ aggregates. In addition, by slowing the rate of water addition, when conducting solvent to water exchange, the average particle size increases. These are important observations as fullerene aggregation may influence toxicity (see below).

Lyon *et al.* (2006) prepared four different preparations of C₆₀; namely THFC₆₀, C₆₀ in toluene, aqueous C₆₀ and poly(vinylpyrrolidone) functionalised C₆₀ (PVPC₆₀), and determined if the size and morphology of the aggregates were able to impact on their bacteriocidal behaviour. The method of preparation was observed to impact on the size of the aggregates, so that particles ranged from 50-150 nm for THFC₆₀, 30-100 nm for aqueous C₆₀, with the C₆₀ in toluene and with the PVPC₆₀ samples more uniformly dispersed (10-25 nm diameter). THFC₆₀ displayed the most potent antibacterial behaviour, although all samples were capable. It was also demonstrated that the solvent controls (i.e. contained no C₆₀) exhibited no evidence of toxicity. In addition, the THFC₆₀, and aqueous C₆₀ samples were centrifuged to separate larger and smaller aggregates from the suspension and it was apparent that suspensions containing smaller aggregates had a greater propensity to display antibacterial effects. It is difficult to draw a firm conclusion from this study, as the solvent for dispersion is altered in order to influence the particle size. It is therefore not clear whether the toxicity differences between samples might be due to solvent or aggregate size differences.

In general, the findings suggested that C₆₀ preparations are more toxic when prepared using a solvent to water exchange method, in comparison to those prepared through the extended stirring in water. Together these studies clearly demonstrate that trace contamination with THF is sufficient to significantly increase the toxicity of C₆₀. One explanation appears to be the formation of reactive species due to interaction between the THF and C₆₀; these studies therefore highlight the need to stipulate the method of fullerene dispersion.

6.2.6 Comments on the experimental designs

Relevant fullerene exposure concentrations to be used within *in vitro* and *in vivo* experiments cannot be obtained without information regarding the human exposure levels, which is information that is currently lacking. However, some concentrations used have been exceptionally high, and unlikely to be encountered by humans; for example (Gharbi *et al.* 2005) exposed rats to fullerenes at a dose of up to 2 g kg⁻¹, via intraperitoneal exposure. In addition, it is often the case that a single dose has been administered to animals or cells, and toxicity assessed at a number of post exposure time points, but it is likely that the utilisation of fullerenes within occupational or consumer settings will involve the exposure of humans for extended periods, and this should perhaps be considered within future studies. The uncertainty regarding human exposure levels to fullerenes also makes interpretation of the observed hazards difficult.

In addition, the most relevant way of expressing fullerene concentrations is debatable; and within the studies outlined has been expressed as ppm, µM, mg kg⁻¹, mg ml⁻¹ etc (which in

some circumstances is likely to derive from the exposure scenario). The toxicity of nanoparticles has been demonstrated in a number of studies to be related to their small size and therefore high surface area (Brown *et al.* 2001, Duffin *et al.* 2002, Duffin *et al.* 2007, Stoeger *et al.* 2006). The high propensity for fullerenes to aggregate means that not all the potential calculated surface will be available at the surface. Introducing a standard way of expressing fullerene concentration would also allow the opportunity to more easily compare the findings of different investigators.

There are a diverse number of fullerenes available; which derives from the number of carbon atoms contained within the cage structure, surface modification, and the preparation processes utilised to promote their water solubility. Therefore, fullerenes used by different investigators are likely to behave differently, even if described as *THFC*₆₀, *aquC*₆₀ etc, as the processes underlying their generation by different groups will be distinct. Full physico-chemical characterisation is therefore essential to allow comparisons between fullerene toxicity to be made. Toxicity is likely to be dependent upon the fullerene under investigation as the toxicity of C₆₀ has been demonstrated to not be assay, cell and species specific (Markovic *et al.* 2007). So far there is evidence of a lack of correlation between *in vitro* and *in vivo* studies (Sayes *et al.* 2007), although this can in part be explained by the differences in cell targets studied between the *in vitro* study (Sayes *et al.* 2004) and the *in vivo* model (rat lung; Sayes *et al.* 2007).

6.2.7 Summary

There are a number of factors that appear to be implicated in fullerene behaviour and toxicity, including chemical structure, surface modifications, and preparation procedure. Ultimately these factors drive fullerene water solubility which appears to be related to antioxidant/cytoprotective or pro-oxidant/cytotoxic properties. Generally the greater water solubility exhibited by a fullerene sample, the lesser the toxicity associated with exposure. However, the situation is complicated by the findings that residual solvents (or their derivatives) used within the preparation of fullerene samples are able to contribute to the observed toxicity, which negates improving water solubility by particular methods. In addition, the preparation of surface modified fullerenes is conducted to improve an aspect of fullerene function, and also modifies the toxicity of fullerenes. However, the fact that fullerene derivatives do not always behave similarly to their unmodified counterparts is expected, and may allow for the safe integration of fullerenes into products, by revealing which attributes of particles are most influential in driving toxicological findings.

The studies conducted so far suggest that fullerene toxicity involves an oxidant driven response, suggesting that toxicity evaluations should evaluate the potential of fullerenes to cause oxidative stress and related consequences such as inflammation or genotoxicity. The studies conducted with fullerenes thus far are rather limited in terms of models used, targets investigated and mechanisms of toxicity. Much more work is required to generate sufficient knowledge to inform a risk assessment. Accordingly, it is unrealistic to make generalisations about the behaviour of fullerenes from the limited number of studies that have been conducted, as investigations into the toxicity of fullerenes via specific routes of delivery, or at particular cell and organ targets, are often too few in number to make definite conclusions about fullerene behaviour. In addition, the quality (including the concentrations used, experimental model), of conducted experiments are of relevance to consider, which is of vital importance when considering the risk associated with fullerene exposure.

6.3 CARBON NANOTUBES

6.3.1 Introduction

Relatively little is known about the potential human exposure to, and toxicity of carbon nanotubes (CNT) during their manufacture, use or disposal. Revealing the hazards associated with CNT has been a focus of existing *in vivo* and *in vitro* studies due to the expected promise surrounding CNT exploitation. However, without relevant exposure information, interpreting the relevancy of available toxicity data is difficult, and so the implications of human exposure are uncertain at this time. Consequently, although exposure via inhalation or dermal contact is

expected during manufacture, which drives the majority of toxicity studies, little information regarding the dose or characteristics of exposure are available (Maynard *et al.* 2004). A thorough review of exposure information is required in order to use the following review of the hazard literature to generate the first steps towards a risk assessment.

To date, research has addressed a number of aims, which include determination of which of the following might contribute to CNT toxicity; 1) physical characteristics such as diameter (determined by wall number) and length, 2) aggregation/agglomeration status, 3) composition, including contaminant presence and surface modifications, and 4) the potential for confounding findings due to an inappropriate experimental design. It has been suggested that a thorough physico-chemical characterisation of nanomaterials, including CNT, is provided within publications in order to allow comparisons between different studies, and therefore analysis of the attributes most influential in driving CNT toxicity. A future discussion of appropriate experimental design is also essential in this area. Experimental design can impact upon the physico-chemical characteristics of the CNT, in terms of aggregation and surface chemistry. In addition variations between studies in terms of the concentrations, exposure duration, methods of dispersion, and endpoint analysed can greatly influence the conclusions drawn. The limited studies conducted to date are rather disparate in terms of the nanotubes studied, the physico-chemical characteristics measured, the dispersants used, the models employed and the toxicology endpoints measured. Therefore, at this time it is difficult to generate any clear consensus regarding the potential toxicity of these particles. However, this review aims to bring together a majority of the research published so far in this area in order to identify key factors that might be useful when considering CNT hazard, and that could contribute towards a risk assessment.

6.3.2 *In vivo* assessment of CNT toxicity

As described above, research regarding CNT hazard has been driven primarily by the potential for exposure via respiratory and dermal routes during manufacture and utilisation. In addition, the longevity at the exposure site, and potential for distribution within the body, subsequent to exposure has also been addressed within a limited number of studies. Some studies also assess the biocompatibility of CNT used in biomedical applications such as drug delivery.

6.3.2.1 Pulmonary exposure to carbon nanotubes

By far, the largest proportion of studies that evaluate CNT toxicity have been conducted within pulmonary models, including instillation, aspiration and inhalation techniques, to enable the exposure of rats and mice.

One of the first studies published in this area was conducted by Lam *et al.* (2004), who investigated the toxicity of SWCNT obtained from different sources. These included (i) a sample, abbreviated by the authors as CNT, obtained from CarboLex, which contained 0.53% iron and 25.99% nickel; (ii) HiPco, SWCNT, manufactured by Rice University in their raw form (26.9% iron; termed RNT), (iii) or a purified form (reduced iron content to 2.14%, known as PNT); both the RNT and PNT samples contained a low nickel content. The different SWCNT samples were used in order to determine if the manufacturing method or metal content of SWCNT impacted on their toxicity. Mice were exposed to the SWCNT samples suspended in heat inactivated mouse serum via a single intratracheal instillation at a 'low dose' of 3.3 mg kg⁻¹, or 'high dose' of 16.7 mg kg⁻¹, and toxicological assessments made 7 or 90 days post exposure. Nanotubes do not disperse well in aqueous media (see later), and so the serum was used to aid dispersion. All types of nanotube studied were capable of producing interstitial 'epithelioid' granulomas (which were associated with particle aggregates) and interstitial inflammation in the lungs, which were most frequently observed in the high dose group and more pronounced at the 90 day time point. Mortality was evident only in mice exposed to the highest dose of CarboLex CNT (5/9 mice, 4-7 days after instillation). This mortality was associated with congestion of the airways due to the large size of the SWCNT aggregates and therefore is most likely to reflect their inadequate dispersion, caused by the administration of a high dose of particles, rather than a specific toxic effect. However, the authors also suggested that nickel release from CarboLex CNT, as a consequence of their sonication prior to exposure, may contribute to the observed mortality. Limited toxicity was observed for the RNT or PNT samples. However, granuloma

development, was the most prominent lung lesion manifested, for all three nanotube samples tested. This information suggests that SWCNT acute toxicity is dependent upon dose and probably lung distribution, while the importance of factors such as the manufacture process and composition are less clear. The same study also included silica quartz and carbon nanoparticles as particle controls. The results demonstrated that SWCNT were relatively more potent than these controls, in terms of the formation of pathological markers such as inflammation or granulomas. However, although these SWCNT were illustrated to be more pathogenic than quartz, only a limited number of endpoints were evaluated; these did not include endpoints such as fibrosis and tumour induction which are associated with chronic exposure to quartz. No information is available regarding the dimensions (diameter and length) of the SWCNT used in this review, or many of those that follow.

Warheit *et al.* (2004) expanded on the observations of Lam *et al.* (2004), and suggested that granuloma formation within the lungs of rats arose due to the presence of SWCNT aggregates. The study of Warheit *et al.* (2004) exposed rats to a single intratracheal instillation of SWCNT, at a concentration of 1 or 5 mg kg⁻¹, for a period of up to 3 months. The SWCNT used were characterised as being 1 µm long and having an individual SWCNT diameter of 1.4 nm, which when coalesced into larger rope-like structures had a diameter of 30 nm. A transient neutrophil driven inflammatory response was observed at 24 hours post exposure, and was also associated with markers of cell damage (lactate dehydrogenase (LDH) release) and was followed by a fibrotic response. Multifocal, macrophage containing granulomas were evident at 1 month, and surrounded CNT ropes. Of the samples tested, only crystalline silica quartz was capable of inducing a sustained response over the whole observation time, suggesting that quartz was more potent than the SWCNT in this study. A mortality rate of 15% was observed within the high dose SWCNT group, which reinforces the need to avoid using CNT doses that congest the airways, leading to death via asphyxiation, rather than a specific toxic effect. Death via asphyxiation due to SWCNT or MWCNT exposure of humans is highly unlikely due to the difficulty in generating sufficient concentrations of airborne respirable aggregates. The results of Warheit *et al.* (2004) also contradict those of Lam *et al.* (2004), in relation to toxic potency of the different particles (whereby Lam *et al.* (2004) demonstrated that SWCNT were more potent than quartz), but this could relate to the endpoint(s) studied, the doses used, or the characteristics of the quartz sample used as a control.

Shvedova *et al.* (2005) used pharyngeal aspiration as a means of delivery of SWCNT to mice. Pharyngeal aspiration is considered by some, but not all researchers to be more physiologically relevant than intratracheal instillation (see for example Mercer *et al.* 2008). The SWCNT were reported to have a diameter of 1-4 nm and an iron content of 0.23%. Animals were exposed to a single dose, of up to 40 µg per mouse, and were studied for a period of up to 60 days post exposure. The concentrations of particles used were substantially lower than those utilised by previous investigators, and yet similar pathological hallmarks were observed. Specifically, SWCNT exposure generated an acute transient inflammatory response that was characterised by the increased infiltration of neutrophils at day 1, lymphocytes at day 3 and a subsequent elevation in macrophages at day 7, after which time the inflammatory response resolved. Increased pro-inflammatory cytokine production was characterised by an elevation of tumour necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β (maximal at 1 day post exposure)) and transforming growth factor beta (TGFβ (maximal at day 7)). Both aggregated and dispersed CNT were evident within the administered dose of SWCNT, and appeared to be accountable for the manifestation of different pathologies. Specifically granulomatous inflammation (first apparent at day 7) was associated with SWCNT aggregates, so that aggregates were surrounded by hypertrophied epithelial cells. Conversely, dispersed SWCNT were associated with interstitial fibrosis and alveolar wall thickening at 28 days post exposure onwards. This study therefore highlights that both well and poorly dispersed SWCNT result in pulmonary pathological responses following delivery via aspiration, but the nature of the response is influenced by the effectiveness of the SWCNT dispersion. Biochemical markers of lung cell damage, were also evaluated within the bronchoalveolar lavage fluid (BALF), and included increased protein presence, and raised LDH activity for up to 28 days post exposure. Furthermore, an increase in the lipid peroxidation product, 4-hydroxynonenal, with a concurrent depletion in glutathione (GSH) within the BALF was observed (and was most severe at 1 day post exposure) both of which are indicative of oxidative stress. These biochemical data suggest that the SWCNT induced pulmonary cell death/damage and oxidative stress, in addition to the

pathological/inflammatory changes described. This study ranked particle toxicity in the order SWCNT > ultrafine carbon black (ultrafine carbon black) > silica. While such a ranking can be useful for risk assessment, such a study design does not allow identification of the physico-chemical properties that are responsible for adverse effects.

Muller *et al.* (2005) aimed to investigate the contribution of CNT agglomerate formation to their pulmonary toxicity. This was achieved through the exposure of rats via a single intratracheal instillation of MWCNT, at relatively high doses of 0.5, 2 or 5 mg per animal, with toxicological assessments made up to 60 days post exposure. The MWCNT remained unground or were ground using a ball mill, to reduce their tendency to interact to form larger structures, but this also significantly shortened the MWCNT. The ability of grinding to influence the characteristics of the MWCNT was witnessed by the fact that agglomerates of unground CNT (5.9 μm length) were predominantly entrapped within the airways, but that their ground (0.7 μm length) counterparts were observed to be better distributed throughout the lungs, which suggested that the ground particles were exposed in a less agglomerated form. At day 3 of the response, inflammation, as indicated by neutrophil infiltration and TNF α production, was greatest for ground MWCNT. There was also increased LDH activity and protein content within BALF, indicating damage to the airway epithelium, which was greatest for the ground sample. Therefore, overall, the ground MWCNT were suggested to have the greatest inflammogenic potency. From this study it is not possible to really ascertain whether the difference in inflammation induced by grinding is due to a change in fibre length, or a modification in surface properties leading to a change in agglomeration. The pathology associated with the MWCNT exposure at the 60 day time point was manifested as a dose dependent fibrotic response, with unground MWCNT more capable of stimulating such a response. For unground MWCNT it was illustrated that collagen rich granulomas (formed from multinucleated giant cells and macrophages) fully or partially blocked the airways, and surrounded MWCNT agglomerates. Ground samples appeared to be better dispersed, but were still able to encourage granuloma development within the interstitium. These findings of (Muller *et al.* 2005) therefore support that of (Shvedova *et al.* 2005) who demonstrated that the pathology associated with better dispersed CNT was restricted to the interstitium, and that of the agglomerates manifested within the airways where they were trapped. These two studies therefore suggest that the toxicity exhibited by different MWCNT is agglomerate form, and lung location specific.

As mentioned above, the method of delivery of CNT to the lung may impact upon their toxicity. Li *et al.* (2007a) illustrated that the pulmonary lesions that developed as a consequence of MWCNT exposure varied with intratracheal versus inhalation exposure, of mice. Accordingly, with a single intratracheal instillation exposure (0.05 mg per mouse for 8, 16 or 24 days), clumps of MWCNT were found within, but did not block the bronchi. The instilled MWCNT were found within the bronchial wall at day 8 where they stimulated an inflammatory response at day 24. In addition, instilled MWCNT clumps (at a similar size to that witnessed within the bronchi) were able to deposit within the alveoli, which culminated in alveolar destruction. This suggests that instillation of poorly dispersed CNT is not a suitable technique for assessing toxicity. In fact, the majority of these 'early' CNT studies using intratracheal instillation as a route of administration have used unrealistically large doses of particles that are unlikely to be encountered by humans. Therefore, lower dose studies, including inhalation exposures, are required to verify the observations made, and to study them in more detail.

Inhalation studies conducted to date are few, the first of which has been published by Li *et al.* (2007a). Subsequent to inhalation (6 hour exposure per day, at a mean concentration of 32.61 mg m⁻³ for 8,16 or 24 days) Li *et al.* (2007a) identified that MWCNT were better dispersed when compared to that encountered for intratracheal exposure. They did however observe some CNT agglomerates adhered to, and within the bronchial wall, but no inflammatory cells were observed to surround them. Smaller MWCNT agglomerates were present within alveoli, and promoted the thickening of the alveolar wall (indicative of a fibrotic response), but their structure remained intact. Thus the pathological lesions that developed within inhalation groups were different to that evident within intratracheal instillation groups, and were suggested to occur due to differences in the size and therefore distribution of CNT within the lung.

Mitchell *et al.* (2007) assessed both the pulmonary and systemic immune response of mice to the inhalation of MWCNT. The whole body exposure occurred for 6 hours per day and for a

duration of 7 or 14 days, at concentrations of up to 5 mg m^{-3} , with agglomeration increasing with concentration. It was observed that MWCNT were engulfed by alveolar macrophages. However, no increases in inflammatory cell infiltration were found, with the lack of inflammation, granuloma formation, fibrosis and tissue injury confirmed using histopathological analysis, despite the fact that MWCNT distribution throughout the lung was observed. However, despite the lack of local, pulmonary effects, systemic immunity was affected, so that spleen derived cells showed suppressed T cell dependent antibody response, decreased proliferation of T cells following stimulation, altered natural killer cell killing, with increased oxidative stress and IL-10 release (responsible for a suppression in immune responses and therefore increases the susceptibility to disease and infection). The appearance of effects within the spleen was suggested by the authors to occur due to the potential of MWCNT to bypass pulmonary defences, and reach the circulation. Although this is an inhalation study, the results are also actually difficult to interpret, especially those relating to the systemic immune response. A whole body exposure allows animals to orally ingest particles due to efficient and extensive cleaning of their fur and skin, and may also allow for dermal exposure, both of which may allow particles to ultimately access the circulation. In addition, mucociliary clearance of CNT from the lung is also anticipated to deliver CNT to the GIT, and worthy of consideration when considering the potential for the eventual transfer of CNT into the blood. Therefore it is suggested that the systemic responses revealed within this study need to be compared with those following an ingestion, gavage or dermal study, which has not been conducted to date.

Shvedova *et al.* (2008a) also exposed mice via whole body inhalation, using an aerosol of SWCNT of 5 mg m^{-3} , at 5 hours per day, for 4 days and conducted toxicological investigations at 1, 7 and 28 days after exposure. The SWCNT sample contained 17.7% iron and possessed dimensions of 0.8-1.2 nm diameter and 100-1000 nm length. The pulmonary response to inhalation was compared to that following exposure via pharyngeal aspiration. The results indicated that inhalation of SWCNT elicited a transient accumulation of neutrophils that peaked at day 1, and resolved after this time, and an increase in macrophages was apparent at day 7. Inhalation exposure was also associated with granulomatous inflammation in the alveolar region of the lungs. Protein and LDH release was also stimulated by exposure which was maximal at day 1, but were elevated up to 28 days post exposure. Pro-inflammatory cytokine production (TNF α and IL-6) was enhanced following exposure, and was maximal at day 1, with TGF- β elevated at day 7. Granulomatous (epitheloid macrophage mediated) inflammation was most evident at day 28. Collagen deposition, and alveolar connective tissue thickness was observed to increase with time, which is indicative of a fibrotic response. Oxidative damage (indicated by GSH depletion, increased lipid peroxidation, elevated oxidatively modified proteins) was also a feature of the response that was apparent from day 7 onwards. Mutagenicity was evident, with a higher frequency of mutations apparent with inhalation exposure, highlighting their potential to exhibit genotoxicity. Consequently, inflammation and oxidative stress were suggested by the authors to act concomitantly to eventually culminate in the development of granulomas, fibrosis and mutagenesis. Overall, it was demonstrated that although the pathology exhibited within both exposure scenarios was similar, inhalation was most effective at producing inflammatory, oxidative, fibrotic and mutagenic responses, as the magnitude of toxicity was consistently lower when mice were exposed via aspiration. However, it was highlighted that generating CNT aerosols was difficult, due to the tendency of CNT to agglomerate and form entangled structures which need to be adequately aerosolised, through the removal of these 'clumps' to avoid asphyxiation.

Assessment of the deposited doses is not provided in any of the inhalation studies, due to the difficulty in chemically or physically analysing the tissue content of carbon based structures. The airborne concentrations used are relatively high if the exposure study by Maynard *et al.* (2004) proves to be representative of real occupational exposure concentrations (whereby airborne concentrations of SWCNT were $0.53 \mu\text{g m}^{-3}$). However, the exposure duration is relatively low and such a cumulative exposure could potentially be expected over the working lifetime of an individual. Such hypotheses require investigation in order to link this hazard data into a risk assessment.

In addition, with respect to inhalation, the deposition of CNT will be dependent upon their aerodynamic properties, for example long, thin fibres will penetrate deeper into the airways than aggregates. Deposition of aggregates in the upper airways is likely to allow effective removal

via the actions of the mucociliary escalator. In comparison, deposition in the deeper regions such as the alveoli will require removal via phagocytic cells, the efficiency of which will be dependent upon the dimensions of the agglomerate or individual particles. It is therefore more likely that particles depositing deeper within the lung airways and alveoli are more likely to translocate from the lung, reaching the circulation (Warheit *et al.* 2004). The translocation of CNT from any route of exposure necessitates that to reach distant sites within the body they must cross an epithelial barrier. It is possible that exposure to either CNT themselves, other toxic substances, or disease could inflict damage to the epithelium and thereby lead to an increase in cellular permeability. MWCNT (110-170 nm diameter, 5-9 μm long) have in fact been demonstrated to impair the paracellular permeability of lung airway Calu-3 epithelial cells, but it was observed that this effect was not demonstrated with SWCNT (0.7-1.2 nm diameter, 2-20 μm long) or ultrafine carbon black exposure Rotoli *et al.* (2008). However, such penetration has not yet been demonstrated *in vivo*.

A majority of the studies exposing animals via the pulmonary route have focused on identification of pulmonary responses. However, if CNT translocate to other locations either via the cardiovascular system, or into the pleura, additional endpoints and targets of study will be required in future studies. Alternatively, it is also possible that the pulmonary response to CNT involves the release of systemically acting factors, which could exhibit toxicity at sites distal to the lungs.

Many of the published studies demonstrate that CNT exposure can result in an acute, neutrophil driven inflammatory and fibrotic response, with granuloma development associated with CNT aggregates (Lam et al. 2004, Warheit et al. 2004, Shvedova et al. 2005, Shvedova et al. 2007). Thus far, this response is predominantly derived from studies focused on SWCNT. There are however some studies (Mitchell et al. 2007, Li et al. 2007a) that show no evidence of granuloma on exposure to CNT. Explanations for this discrepancy, other than method of delivery, include differences in dose, differences in the physico-chemical characteristics (i.e. number of walls, diameter, length, aggregation) of the CNT studied, and differences in the models employed, thus the finding that not all CNT tested behave similarly is logical, and therefore reasonable. Therefore at this time it may not be appropriate to make generalisations about CNT toxicity. Instead, future studies will need to use multiple techniques, doses and CNT manipulated systematically to vary in specific characteristics in order to determine the factors that might drive pathogenicity.

6.3.2.2 Intraperitoneal injection

Due to the cost and technical difficulty of tracing CNT translocation following inhalation or instillation, very few studies of this nature have been studied to date. Intraperitoneal exposure of the mesothelial lining of abdominal cavity to CNT has been used as a surrogate for the mesothelial lining of the pleural cavity surrounding the lungs. Direct injection into the pleural cavity is technically difficult and so the intraperitoneal model provides a convenient model for investigating mesothelial responses. The mesothelial response is of interest in order to assess whether CNT have the capacity to elicit toxicity that is comparable with that of other pathogenic fibres such as asbestos. Such studies have aimed to investigate whether CNT conform to the pathogenic fibre paradigm, which dictates that long (>approximately 15 μm), relatively straight and biopersistent fibres are more pathogenic than short fibres. Poland *et al.* (2008), within a pilot study, investigated the toxicity of a variety of MWCNT with varied morphology; specifically long (>20 μm rigid and straight) or short and entangled (<20 μm length) forms, and compared the response to that of asbestos 'controls' (long and short fibre amosite), subsequent to their intra-peritoneal injection within mice. All particles were dispersed with albumin (in saline) and injected into mice at a dose of 50 μg , with analysis conducted at 24 hours or 7 days post-exposure. It was observed that the inflammatory response, characterised by neutrophil influx and protein exudation, induced by the long straight MWCNT, advanced to granuloma formation on the peritoneal surface of the diaphragm. In addition, foreign body giant cell formation was observed, which paralleled the response that was achieved with long fibre amosite asbestos exposure. The short or entangled MWCNT and short asbestos were incapable of eliciting such a response, therefore suggesting that the ability of MWCNT to possess asbestos-like properties is dependent upon their length and morphology.

Takagi *et al.* (2008) utilised the p53 mouse model to investigate the carcinogenicity of MWCNT, crocidolite asbestos or fullerenes (3 mg per animal) administered via intraperitoneal injection. It was concluded that MWCNT and asbestos had the greatest carcinogenic potential, which was based on the visualisation of mesotheliomas, with no tumours evident within control and fullerene treated mice. However, although mesothelioma development was observed to be a cause of death, peritoneal adhesion (and fibrous thickening) which causes constriction of the ilius, was also considered to contribute to the observed mortality. A foreign body response to MWCNT was also observed, so that granulomas were evident, with fibrosis also being a feature of the response. However the relevance of this study is debatable due to the excessively high concentration of MWCNT that were administered to the mice, which is made especially controversial due to the fact that the model used is considered to be sensitive to carcinogenicity. Furthermore, the mice were monitored until 100% mortality was reached in one of the treatment groups (MWCNT), at which point the study was terminated (week 25). Therefore, it may have been more relevant to have used an acute or chronic time frame for the exposures, to allow sub-lethal effects to be assessed, which were only considered within a satellite study, at day 10.

Finally, if CNT are able to induce mesotheliomas, as suggested by (Poland *et al.* 2008) then translocation from the airways to the pleura (or peritoneum) must be demonstrated following exposure, especially via inhalation. No such peer reviewed publications has been generated to address this issue, but an abstract presented at the USA Society of Toxicology meeting has been presented by NIOSH and claims to demonstrate translocation of nanotubes to the pleura (http://www.cdc.gov/NIOSH/blog/nsb031909_mwcnt.html accessed 05/05/09). This information requires clarification in the near future.

For the fibre paradigm to hold-up, it is essential to determine the solubility or dissolution of CNT, as this is able to impact upon their biopersistence. However, to date little evidence of biopersistence has been provided. The resistance of CNT to stringent procedures such as boiling in nitric acid however, suggests that such particles are likely to be biopersistent. In addition, further support for the fibre paradigm applicability to nanotubes is required in the form of evidence of translocation to the pleural cavity following inhalation, and development of a mesothelial pathology associated with this translocation. No such peer reviewed studies have been published to date that provide such evidence.

6.3.2.3 Dermal exposure

In vivo assessment of the dermal effects of CNT are limited to a small number of rodent studies. The first, conducted by Koyama *et al.* (2006), implanted carbon based nanomaterials (2 mg), into the subcutaneous tissue of mice for up to 3 months. The aim of this study was not to assess dermal irritation or toxicity, but to gain an understanding of their biocompatibility for use in medical devices. Both SWCNT and MWCNT implantation were able to affect peripheral CD4+ and CD8+ cell numbers, which was reliant on the post-implantation time. After one week, SWCNT exposure induced the activation of major histocompatibility complex (MHC) class 1 within CD4+/CD8+ T-cells. Subsequently (from 2 weeks), SWCNT activated MHC class II. However, MWCNT appeared to have an inhibitory effect on MHC expression. Therefore, depending on the CNT in question, these findings could have implications for the recognition and removal of foreign material, and therefore requires further assessment. Granulomas (from 3 weeks onwards) were observed to encapsulate nanotube agglomerates in both the SWCNT and MWCNT treated animals. It is relevant that the authors noted that the toxicity exhibited by CNT was substantially lower than that of asbestos (of an unspecified form).

In a separate study Yokoyama *et al.* (2005), implanted hat-stacked carbon nanofibres (30-100 nm diameter, 100 nm to 1µm in length) into rat skin, and observed the generation of a 'mild' inflammatory response characterised by the infiltration of neutrophils. They too observed the appearance of granuloma-like structures, but no necrosis. Sato *et al.* (2005) also demonstrated that clusters of MWCNT were surrounded by granulomatous tissue that contained macrophages, giant foreign body cells and fibroblasts, subsequent to the subcutaneous exposure (0.1 mg) of rats. The inflammatory response was greater for MWCNT that were 825 nm in length, compared to that observed for 220 nm MWCNT. The longer length is not sufficient to fit the fibre paradigm, but instead this difference is thought to derive from the greater propensity of the longer CNT to aggregate. Sato *et al.* (2005) also observed that

aggregated 825 nm MWCNT were contained within the macrophage cytoplasm and were not membrane bound. However, the 220 nm MWCNT were internalised by phagocytic cells into lysosomes, thus suggesting that the cells attempted to degrade the internalised MWCNT. The resistance of nanotubes to chemical induced degradation suggests that this is unlikely, but the impact of such intracellular localisation upon the physico-chemical characteristics of the nanotube requires investigation.

Murray *et al.* (2009) dermally exposed mice (daily, at doses of 40, 80 or 160 µg per mouse) to unpurified SWCNT, for 5 days and demonstrated an increase in skin bi-fold thickness, which is a measure of oedema and inflammation development. Inflammation was confirmed by an increase in cells in the epidermis, mast cells in the dermis, and neutrophil influx. Oxidative stress was also associated with exposure, and was indicated by a decrease in GSH, and oxidation of protein thiols and carbonyls.

The limited number of available studies suggest that there is a hazard associated with the exposure of skin to CNT, with the response being primarily inflammatory in nature. In future studies it is necessary to consider the systemic availability of CNT following dermal exposure. The recurrent appearance of granulomas following subcutaneous exposure is interesting as it suggests that this pathology is not limited to the respiratory system or mesothelium, but that such a response may occur in many different tissue types, independent of the route of delivery. Furthermore, the results of many studies suggest that the granuloma formation might be related to the agglomeration status of the nanotubes, with greater agglomeration being more likely to induce more extensive pathology. In addition, as demonstrated in the lung, inflammatory and oxidative driven responses appear to be replicated within the skin, thus reinforcing the importance of their development when assessing CNT toxicity.

6.3.2.4 Cardiovascular CNT exposure and toxicity

It is important to evaluate the implications of CNT administration, subsequent to their intravenous exposure, as their circulation within the blood may affect blood components (specifically cells) or allow for their distribution to a number of targets that are potentially affected. Entry into the blood could of course occur in a medical application via direct intravenous injection, but might also occur if their translocation into the circulation from other organs (including the lungs, skin or gastrointestinal tract) is realised.

Yang *et al.* (2008) investigated the inflammatory effects of SWCNT (10-30 nm diameter, and 2-3 µm length) following intravenous exposure of mice to doses of 40, 200, and 1000 µg per mouse, with analysis conducted at 90 days post exposure. Inflammation was assessed in the liver, lung, and spleen due to the high accumulation levels of the particles in these organs. According to histopathological assessment, TNFα production and complement activation assessment, minimal effects were observed, except for some inflammation at the highest dose. However, the lack of an inflammatory response was, in some ways expected, due to the 90 day time point that was utilised. Previous studies (conducted within the lung) indicated that inflammatory responses are generally resolved within 90 days, being generally acute, and transient in nature. In the study by Yang *et al.* (2008), at sites of SWCNT accumulation, oxidative stress (GSH depletion) and lipid peroxidation were evident, emphasising the potential of SWCNT to elicit damage at a number of sites. Hepatic injury was also evident when considering serum biochemical parameters (such as transaminase). Consequently, the delivery of SWCNT within the blood to a number of target organs has been illustrated to have a detrimental impact on their function.

In the study by Li *et al.* (2007b) the extrapulmonary responses to a single intrapharyngeal instillation of SWCNT (up to 40 µg per mouse, mostly aggregated), were investigated for up to 8 weeks post exposure in hemeoxygenase-1 (HO-1)-luc reporter mice. The induction of the surrogate oxidative stress marker, HO-1 was increased in the lung, aorta and heart tissue (maximal at day 7). Subsequently, studies conducted within C57BL/6 mice, revealed increased aortic mitochondrial DNA damage and protein oxidative products (protein carbonyls) and decreased mitochondrial GSH levels, all of which are suggestive that oxidative damage occurred. The results therefore highlight that there are secondary targets for toxicity of SWCNT, subsequent to pulmonary exposure. In addition, using a repeated exposure regime (20 µg per

mouse, administered once every 2 weeks for 8 weeks), SWCNT administration was observed to accelerate plaque formation in ApoE^{-/-} mice (mouse model of cardiovascular disease) fed an atherogenic diet. The inflammatory response elicited by SWCNT was also evaluated within these mice, however no increases in inflammatory mediators such as IL-6, IL-10, macrophage chemotactic protein (MCP)-1, interferon gamma (IFN- γ) and TNF α were observed within the plasma, suggesting that a systemic inflammatory response was not initiated by the SWCNT. Therefore, it is unknown whether the extra-pulmonary toxicity was developed either as a consequence of the migration of SWCNT from the lung, or due to the release of, as yet, unknown factors.

Radomski *et al.* (2005) investigated the potential for vascular thrombosis formation within rats exposed to carbon nanomaterials (50 $\mu\text{g ml}^{-1}$) via injection. It was found that, infusions of carbon nanoparticles, SWCNT or MWCNT, significantly accelerated the time and rate of carotid artery thrombosis development (in the ferric chloride model of carotid thrombosis), in the order carbon nanoparticles > SWCNT > MWCNT. The results observed *in vivo* were confirmed *in vitro* so that the ability of CNT to amplify platelet aggregation was paralleled within both experimental setups (see later).

There are very few studies relating to the cardiovascular toxicity of CNT, however, when considered together the studies by Yang et al. (2008), and Radomski et al. (2005), suggest that exposure to CNT via the cardiovascular system could be associated with a significant hazard. However, this is reliant on CNT becoming systemically available following exposure, which is currently only applicable to intravenous exposure, as evidence of CNT translocation from the lungs, skin and GIT is lacking at this time. The evidence that CNT are capable of inducing vascular damage and blood clotting requires further investigation, in relation to dose, characteristics of the nanotubes used and the potential increased risk of cardiovascular disease subsequent to occupational and medical CNT exposures. In addition, the ability of CNT to stimulate the release of factors into the circulation, that mediate toxicity at sites distal to the exposure site, is also worthy of consideration within future investigations.

6.3.2.5 ADME profile of CNT

Determining the distribution of CNT within the body, subsequent to exposure (via the lungs, gut and skin) is necessary to identify potential targets of CNT toxicity, and thereby drive appropriate *in vitro* assessments of their toxicity, at relevant tissue and cellular targets. Accordingly, the delivery of CNT to particular targets is reliant on their transfer into blood from the exposure site. A number of barriers at the exposure site are in place to protect against the transfer of CNT into the circulation, and thereafter prevent their access into other secondary target sites. As a result, it is necessary to determine if this protection is overcome by CNT, to evaluate their systemic uptake and therefore availability.

The absorption of CNT from the exposure site into the blood is necessary to consider, due to its propensity to allow the delivery of CNT to multiple target sites, where they may accumulate and elicit toxicity. Thus far, no studies have addressed whether CNT can translocate into the blood from the lungs, skin or GIT, and so their propensity to become systemically available should be addressed within future studies. One *in vitro* study Rotoli *et al.* (2008) has suggested that MWCNT may increase lung paracellular permeability, which may therefore allow for the greater translocation of CNT from the lung into blood, but this finding requires further confirmation *in vivo*.

The distribution of CNT to various organs has been reported following intravenous exposure (Yang *et al.* 2008), with predominant localisation within the liver, lungs and spleen. The propensity of CNT to elicit toxicity at sites of accumulation is therefore of great interest and relevance. It is conceivable that a similar distribution pattern would therefore be expected following exposure by other routes, but this would be dependent on confirmation of the fact that CNT can actually access the blood from the lungs, skin and GIT, which at this time is unknown.

No information regarding the metabolism of CNT is available, but it is unlikely that CNT are degraded due to their biopersistent nature.

The elimination of CNT has the propensity to limit their longevity within the body, and thereby potentially reduce their capacity for toxicity. Limited evidence is available that describes the elimination of CNT. In a study conducted by Singh *et al.* (2006), mice were injected with radiolabelled and ammonium functionalised SWCNT (up to 400 µg per mouse) and their biodistribution and subsequent clearance was followed. The purpose of the CNT functionalisation was to make them more water soluble, and as a result promote their exploitation within biomedical applications by supporting their biocompatibility. A rapid excretion via the kidneys was evident, so that SWCNT were demonstrated to have a blood half-life of 3 hours. Therefore incorporating such functionalisation would be extremely attractive when avoiding CNT accumulation in the body, as it appears to facilitate their clearance to thereby reduce their potential for toxicity. However, it is also necessary to draw attention to the fact that the rapid clearance of CNT is anticipated to be a disadvantage for drug delivery purposes due to the decrease in circulation time associated with their rapid excretion. In addition, it is important to note that the CNT used were relatively short (1 nm diameter, 300-1000 nm long) and functionalised in such a way to ensure that their removal from the body was promoted, and so the applicability of the findings to other CNT requires assessment. In addition, as described previously, Wang *et al.* (2004) have also demonstrated similar results using hydroxylated SWCNT which were excreted into urine.

Information regarding the ADME profile of CNT is severely lacking. It would appear that CNT can be eliminated within urine, but this is likely to be driven by particular physico-chemical characteristics. Therefore, at present it is not possible to make definite conclusions regarding the adsorption, distribution and longevity of CNT within the body, but is necessary to direct the most appropriate in vitro approaches when determining their toxicity. This is necessary due to the need to determine the relevancy of toxicological findings within different target sites, in vitro, as if CNT are unable to access the systemic circulation, addressing their toxicity at the secondary target sites becomes irrelevant. However, the behaviour of CNT is likely to be affected by their physico-chemical characteristics, and experimental set up used (including exposure route, concentration, duration).

6.3.2.6 Distribution and biopersistence of CNT

CNT localisation following direct injection into blood has been studied due to their potential exploitation as drug delivery devices, and to assess their tissue distribution following exposure. Determining the behaviour of CNT following their direct administration into blood is of relevance due to the assumption that if CNT actually access the circulation, then they are expected to behave similarly.

A limited number of studies have investigated the biodistribution of CNT following intravenous injection. For example, Cherukuri *et al.* (2006) injected (20 µg kg⁻¹) unmodified SWCNT (1 nm diameter, 300 nm long) into rabbits and followed their biodistribution using near-infrared fluorescence. The SWCNT were cleared from the blood, with a half life of one hour. The liver was the primary site of accumulation, the consequences of which remain unknown. The liver is often shown to accumulate many different types of particles injected into the blood, and in general is accounted for by the phagocytic nature of the Kupffer cell (liver macrophage) population (see for example Deng *et al.* 2007). However, some of these studies have also shown the presence of nanoparticles within hepatocytes (Gharbi *et al.* 2005). The cellular localisation of nanotubes within the liver is not known, but is likely to involve the participation of Kupffer cells.

Yang *et al.* (2007) demonstrated that bundles of radiolabelled 'pristine' SWCNT (10-30 nm diameter, 2-3µm long) were distributed within the body after intravenous administration (200 µg) but were not contained within the faeces and urine. This led to the conclusion that the CNT in question were biopersistent in nature, which was confirmed by histological analysis which illustrated that CNT were distributed throughout the body. They were particularly evident within the liver (dominant site of accumulation), lung, and spleen, and were retained there for the observation time of 28 days. It was also evident that serum transaminases were increased, thus indicating damage to the liver that may be associated with their accumulation.

Wang *et al.* (2004) investigated the biodistribution and kinetics of radio-labelled water soluble, hydroxylated, SWCNT (1.4 nm diameter, 300 nm long) in order to assess their drug delivery capabilities. The influence of different administration models on this parameter was also assessed, namely intraperitoneal, sub-cutaneous, intravenous, and oral administration. It was observed that subsequent to intraperitoneal administration, SWCNT distributed throughout the body (except the brain) quickly with predominant localisation within bone, as well as the stomach and kidneys. The ability of SWCNT to pass through a number of body compartments was therefore illustrated, with elimination apparent within urine. The pattern of distribution was mimicked following the other administration routes used.

In a separate study, the utilisation of MWCNT as drug delivery vehicles was also explored by Deng *et al.* (2007), who evaluated the distribution of radiolabelled taurine conjugated MWCNT, subsequent to exposure via a variety of routes. It was demonstrated that taurine attachment to the MWCNT surface promoted their water solubility, while TEM revealed that the MWCNT structure remained intact. After intravenous injection, MWCNT accumulated predominantly within the liver, with limited lung and spleen localisation. Accumulation of MWCNT within the liver was accounted for by the fact the MWCNT were phagocytosed by Kupffer cells, with no apparent toxicity associated with the high level of MWCNT accumulation, as indicated by serum indicators and histopathological analysis. Subsequent to intratracheal administration, the MWCNT were eliminated with time so that by day 28 only 20% of the administered dose remained within the lung, illustrating the removal of deposited MWCNT. After oral administration, the majority of MWCNT were evident within the faeces and also remained within the stomach, small and large intestines, with no detectable transport into the blood. It was also observed that the MWCNT remained unchanged within the tissues in which they resided, and within faeces, suggesting that they are resistant to degradation or transformation, and therefore could be biopersistent if not excreted.

This study and others suggest that biopersistence of CNT could be considerable and therefore could contribute to their pathogenicity. Intact, unground MWCNT have been observed to be slowly removed from the lungs with 81.2% remaining at 60 days (Muller *et al.* 2005). However, in the same study ground MWCNT were more rapidly cleared with 36% remaining at 60 days, indicating that modification of physico-chemical characteristics can impact upon biopersistence. It was therefore suggested that the length of CNT was able to modulate their clearance kinetics, with shorter fibres being more easily cleared by macrophages.

When considering the distribution of CNT, it is of note that the attachment of different moieties, or utilisation of CNT of different dimensions, may alter their behaviour. Detection of CNT within organs will be influenced by their aggregation status; accordingly individual CNT may not be visible by conventional imaging used to quantify the burden of particles within organs, which could hamper attempts to visualise the accumulation of CNT. For this reason, CNT are often radiolabelled or fluorescent probes are attached to their surface which could in turn alter their physico-chemical characteristics and hence translocation or toxicity. Stability of the label attachment over time is also essential, and requires confirmation. It is also important to realise that distal effects of CNT may not be due to direct action of the particles on the cells of that organ, but instead they could be mediated by the release of systemically acting factors.

The small number of available studies suggest that CNT are able to distribute within the body, following intravenous exposure, with predominant accumulation evident within the liver. The localisation of CNT within particular organs (lungs, liver, spleen) is likely to derive from their accumulation within resident macrophage populations, the consequences of which require further investigation, including the initiation of an inflammatory response. However, to achieve more definitive conclusions it would be necessary to conduct more extensive investigations. Subsequent to pulmonary exposure, it is evident that the majority of CNT remain within the lung, suggesting that they are biopersistent, which is likely to derive from their composition, and dimensions. The biopersistence of CNT can also be suggested due the recurrent finding that granulomas are associated with their exposure.

6.3.3 *In vitro* investigations of CNT toxicity

In vitro studies have used cell types from the lung, skin, cardiovascular system and immune system to investigate CNT toxicity. No *in vitro* studies of the impact of CNT on the central nervous system, gastrointestinal tract, kidney or liver were identified.

6.3.3.1 Lung models

In vitro, the assessment of the toxicity of CNT to the lung, has concentrated on the impact of exposure on epithelial cells that line the airways or that form the alveoli, or on resident alveolar macrophages.

Manna *et al.* (2005) demonstrated that SWCNT when exposed at 0.1 to 10 $\mu\text{g ml}^{-1}$ for 72 hours, elicited a dose dependent decrease in cell viability, within A549 and H1299 lung epithelial cell lines. Their toxicity was postulated to be mediated through an oxidative mechanism, if the cell response to SWCNT is comparable to that elicited within keratinocytes (see later). In addition, Magrez *et al.* (2006) investigated the toxicity of MWCNT at 0.002 to 0.2 $\mu\text{g ml}^{-1}$, for a period of up to 4 days, to lung epithelial cell lines (H596, H446, and Calu-1). MWCNT were cytotoxic to all cells tested, but the H596 cells were most sensitive to the toxicity exerted by MWCNT. However, the toxicity exhibited by carbon nanoparticles was greater than that of CNT, thus insinuating that the morphology and dimension of particles were able to impact on their toxicity. Muller *et al.* (2005) observed that MWCNT were cytotoxic (via an apoptotic mechanism) to the RLE lung epithelial cell line, at concentrations of 50, 100 and 150 $\mu\text{g ml}^{-1}$. Within the same study, MWCNT were also demonstrated to induce mutations within lung cells, thus illustrating their genotoxic potential. Ye *et al.* (2009) illustrated that MWCNT were cytotoxic to A549 and BEAS-2A (bronchial) epithelial cells, in a dose dependent manner (25-200 $\mu\text{g ml}^{-1}$). The underlying molecular mechanisms responsible for the cytotoxic response were investigated, and were characterised by enhanced cellular ROS production, and the induction of an inflammatory response.

Davoren *et al.* (2007) assessed the toxicity of SWCNT to A549 lung epithelial cells, at concentrations ranging from 1.56 to 800 $\mu\text{g ml}^{-1}$, for 24 hours. It was observed that SWCNT were cytotoxic, in both serum and serum free exposure conditions, only at concentrations of 400 and 800 $\mu\text{g ml}^{-1}$, but that the cytotoxicity observed was less pronounced in serum containing conditions. The inclusion of serum was also demonstrated to promote the dispersion of SWCNT, so that a lesser extent of agglomeration was evident (based on visual considerations). It is therefore relevant that the findings from this study were in agreement with that of Wick *et al.* (2007), whereby SWCNT aggregation appears to promote CNT toxicity. In addition, although SWCNT exhibited a very low level of toxicity, and therefore a hazard was identified in association with SWCNT exposure, it was only apparent at high concentrations, and therefore its relevancy therefore requires consideration.

Wick *et al.* (2007) demonstrated that SWCNT at concentrations ranging from 7.5 to 30 $\mu\text{g ml}^{-1}$, for a duration of 3 days, were cytotoxic to the MSTO211H pleural mesothelial cell line, which was dependent on the aggregation state of the SWCNT; so that the greater the extent of aggregation, the greater the toxicity observed. In line with these findings, Pacurari *et al.* (2008) examined the underlying molecular mechanisms responsible for driving the cytotoxicity, and genotoxicity associated with exposure of mesothelial cells to SWCNT (up to 500 $\mu\text{g ml}^{-1}$, for up to 24 hours). ROS production was enhanced by SWCNT exposure, which could be partially inhibited by metal chelators, indicating that their metal component (20.6% nickel, 0.07% iron, 6.2% yttrium) was able to contribute to the oxidant response observed. In turn, ROS sensitive signalling pathways and transcription factors (including activator protein (AP)-1, nuclear factor kappa beta (NF κ B) and mitogen activated protein kinase (MAPK)) were activated, and were suggested to have potentially carcinogenic consequences. Although the response was more pronounced with asbestos exposure, the underlying processes involved within their toxicity were similar.

Other cellular components of the lung have been demonstrated to be susceptible to the toxicity of CNT, including alveolar macrophages. Jia *et al.* (2005) determined the impact of a panel of carbon based nanomaterials namely, SWCNT (1.4 nm diameter, 1 μm length), MWCNT (10-20

nm diameter, 0.5-40 μm length) and C_{60} on primary guinea pig alveolar macrophage function. The SWCNT were the most capable of inducing cytotoxicity, although MWCNT were also capable of eliciting such a response, but C_{60} was not. However, the higher impurity of SWCNT samples was suggested by the authors to contribute to its enhanced toxicity, and so this suggestion requires further investigation. Morphological assessment (using TEM) suggested that cytotoxicity occurred via apoptosis. The ability of macrophages to retain their phagocytic function was also assessed by determining the ability of macrophages to internalise latex beads, subsequent to particle exposure. SWCNT were able to impair phagocytosis to the greatest extent, while MWCNT and C_{60} required higher concentrations to induce such a response. In contrast Pulskamp *et al.* (2007) demonstrated that SWCNT and MWCNT elicited low acute (cyto)toxicity to NR8383 alveolar macrophages and A549 cells when exposed to cells at concentrations of 5-100 $\mu\text{g ml}^{-1}$, for 24 hours. However, CNT were internalised by macrophages and were observed to negatively affect mitochondrial function, and enhance intracellular ROS production, but no inflammatory response was associated with exposure within both cell types. The toxicity of CNT was also related to their purity, so that a decrease in metal content of samples attenuated their (oxidative) toxic potency. In addition, it is of interest that the assessment of the cytotoxic potential of CNT was demonstrated to be dependent on the assay used to assess cell viability, which is a finding that has also been observed by Casey *et al.* (2007a).

A variety of lung derived cell types have therefore been demonstrated to be sensitive to the toxicity of CNT, which is manifested as being inflammatory, oxidative and cytotoxic in nature. However, the toxic response exhibited by CNT is not consistently reproduced by different investigators, and may therefore be related to the physicochemical characteristics of the CNT utilised, or the experimental set up (specifically the cell investigated, CNT concentration, or exposure time, and assays used to assess toxicity) used to determine their toxic potential.

6.3.3.2 Dermal models

The *in vitro* assessment of the dermal toxicity of CNT has predominantly used cell lines, and primarily focussed on the impact of CNT on keratinocytes. For example, Shvedova *et al.* (2003) have shown that treatment of the human HaCaT keratinocyte cell line with SWCNT (30% iron, at a concentration of 0.06-0.24 mg ml^{-1} , for up to 18 hours) lead to oxidative stress as implied by the accumulation of lipid peroxidation products and the depletion in cellular GSH and vitamin E. In addition they identified ultrastructural alterations to both mitochondria and nuclei as well as evidence of cytotoxicity.

Monteiro-Riviere *et al.* (2005a) exposed HEK keratinocytes to MWCNT for up to 48 hours, at concentrations ranging from 100 to 400 $\mu\text{g ml}^{-1}$. MWCNT were observed, by TEM, within cytoplasmic vacuoles apparent as early as 1 hour post exposure indicating that the cells were able to internalise the CNT. All concentrations stimulated an increase in IL-8 production by 8 hours, suggesting that an inflammatory response was integral to the response, with MWCNT exposure eventually culminating in significant (dose dependent) cytotoxicity at 24 hours.

Witzmann and Monteiro-Riviere, (2006) demonstrated that protein expression of HEK keratinocytes was influenced by MWCNT exposure, at a concentration of 0.4 mg ml^{-1} , for up to 48 hours. Elevated IL-8 and IL-1 β production was also observed, thus indicating that MWCNT may initiate an inflammatory response, following dermal exposure. Alterations in protein expression, associated with MWCNT exposure was assessed using two-dimensional gel electrophoresis and mass spectrometry. Proteins that were most profoundly affected were those related to metabolism, cell signalling, the cytoskeleton and vesicle trafficking.

Zhang *et al.* (2007) exposed human epidermal HEK keratinocytes *in vitro* to 6-aminohexanoic acid-derivatized SWCNT at concentrations of 0.00000005-0.05 mg ml^{-1} , for up to 48 hours. The derivatisation was used to promote their water solubility. It was observed that SWCNT were able to decrease cell viability in a dose and time dependent manner. A pro-inflammatory response was also observed in the form of IL-6 and IL-8 protein production, on exposure to SWCNT, but there was no evidence for an increase in IL-10, TNF α , or IL-1 β production. However the adsorption of IL-6, IL-8, and IL-10 to the SWCNT was apparent, therefore the cytokine levels measured may actually have been greater than were estimated. Large

aggregates of the SWCNT were observed within the cells, and were contained within cytoplasmic vacuoles, but importantly smaller structures (10 nm in size) were also observed within the cytoplasm, so that uptake was not solely restricted to large aggregates. It was also observed that SWCNT had a propensity to aggregate within the cell medium, and for this reason surfactants were added to aid in their dispersion. Pluronic F127 was able to decrease the size of aggregates that formed within the cell culture medium, whereas DMSO was not effective at dispersing SWCNT aggregates. In addition, the toxicity of SWCNT (in terms of their cytotoxicity and inflammogenicity) was reduced, on the inclusion of Pluronic F127, in comparison to that apparent on exposure of cells to SWCNT dispersed in Pluronic F127 free medium. Therefore, this finding implied that aggregates of SWCNT are responsible for driving toxicity, and this can be minimised by the inclusion of a dispersant.

The skin tissue model, EpiDermFT, has been recently used by Murray *et al.* (2009) to investigate the toxicity of SWCNT. Unpurified SWCNT induced an increase in collagen and a greater thickness of the dermis, as well as increased cytokine (IL-12, IL-6, IFN γ) production. These effects were supported by *in vivo* observations of inflammation in a mouse model.

Together, these studies suggest that inflammatory and oxidative responses are involved in the response of skin cells, such as keratinocytes, to SWCNT and MWCNT. However, such studies were predominantly conducted using a single cell type. Therefore although CNT can be recognised as representing a dermal hazard, this is likely to be reliant on their capability of penetrating through the stratum corneum barrier in vivo, which requires consideration in future studies (Monteiro-Riviere and Inman, 2006). Consequently, although excellent in vitro skin models are commercially available, such models tested with CNT could not be identified. Such models would provide a useful comparison for future studies

6.3.3.3 Models of cardiovascular effects

The two *in vitro* cardiovascular models published to date are quite different, and include the use of isolated platelets (Radomski *et al.* 2005) and cultured heart muscle cells (Helfenstein *et al.* 2008). The study by Radomski *et al.* (2005) investigated the effects of urban particulate matter and a number of engineered particles, including carbon nanoparticles, C₆₀, SWCNT and MWCNT on platelet aggregation, at concentrations up to 300 $\mu\text{g ml}^{-1}$ for 8 minutes. Platelet aggregation plays a key role in blood clotting and therefore such a response is essential to assess for particles gaining access, or being injected directly into the cardiovascular system. With the exception of C₆₀, all of the particles tested were capable of promoting platelet aggregation *in vitro*, in the order carbon nanoparticles > SWCNT > MWCNT > particulate matter. The mechanisms underlying thrombus development were investigated, and it was found that the activation of GPIIb/IIIa (glycoprotein integrin receptor) was increased by particle treatments, and P-selectin was translocated to the platelet surface, both of which are known to be crucial events in the aggregation of platelets. *In vivo* work confirmed the effectiveness of different nanoparticles at inducing thrombosis development, thus illustrating that this *in vitro* model can be predictive of blood clotting *in vivo*.

Helfenstein *et al.* (2008) investigated whether diesel exhaust particles (DEP) and engineered particles (SWCNT and TiO₂) were capable of affecting heart cardiomyocyte function, at concentrations ranging from 0.25 to 50 $\mu\text{g ml}^{-1}$, for 24 hours. It was demonstrated that all particle types were capable of altering electrical impulse conduction within the cells. *In vivo* this may result in reduced effectiveness of the heart due to decreased contractility and/or heart beat irregularities. The most pronounced impact on normal cell function was actually demonstrated for TiO₂ and DEP, and not SWCNT. This involved ROS production associated with TiO₂ and DEP exposure, and a decrease in the organisation of the myofibrillar structure.

The limited number of conducted studies available, suggest that CNT are able to elicit pro-thrombotic responses, and detrimentally affect normal cardiac electrophysiology. Therefore CNT may have the potential to contribute to initiation or progression of cardiovascular disease. However, more extensive investigations are required to make definite conclusions regarding the cardiovascular consequences of CNT exposure.

6.3.3.4 Immune cell models

Immune phagocytic cells such as macrophages are often responsible for the clearance of foreign particles from different tissues in the body. They therefore play a key role in determining the biopersistence and tissue distribution of CNT. Furthermore, such cells are responsible for inducing and implementing inflammation. Inflammation is required for the clearance of foreign particles, but inflammation which is inappropriate in amplitude or duration can lead to disease. In addition, inflammation in an individual with a pre-existing disease can lead to exacerbation of the disease symptoms, as is hypothesised for air pollution (Donaldson and Stone, 2003, MacNee and Donaldson, 2003).

As described previously, a study conducted by Shvedova *et al.* (2005) used the RAW 264.7 macrophage cell line to evaluate the contribution of this cell type to the SWCNT induced pulmonary fibrogenic responses previously observed in mice. Production of the pro-fibrotic cytokine TGF- β was enhanced, and comparable to that induced by the classical stimulant zymosan, thereby implicating macrophages in the mediation of fibrotic responses on exposure to SWCNT. However production of TNF α and IL-1 β was markedly lower than that induced by zymosan, and therefore the release of these pro-inflammatory cytokines was considered to be a potentially less important component of macrophage exposure to SWCNT.

Muller *et al.* (2005) exposed primary, rat peritoneal macrophages to the ground or unground MWCNT (see previously) for 6-24 hours, at concentrations of up to 100 $\mu\text{g ml}^{-1}$. While ground MWCNT were relatively well dispersed within the cell culture medium, the unground MWCNT formed large aggregates that floated within the medium and as a result were not in direct contact with the cells. Ground MWCNT, chrysolite asbestos and ultrafine carbon black were all capable of inducing production of the pro-inflammatory cytokine TNF α and stimulating cytotoxicity. MWCNT were found to be more pathogenic than ultrafine carbon black, and were either equivalent to, or less toxic than asbestos. In contrast, the unground MWCNT sample had no effect, possibly due to lack of contact with the cells. There is therefore evidence, both *in vivo* and *in vitro* that the toxicity of MWCNT is dictated by their characteristics and/or dispersion. In addition Murr *et al.* (2005) investigated the impact of SWCNT or MWCNT aggregate exposure, at concentrations of 0.005 to 10 $\mu\text{g ml}^{-1}$, for 48 hours, on the murine RAW267.9 macrophage cell line. Cell death was apparent from a threshold concentration of 2.5 $\mu\text{g ml}^{-1}$, with cytotoxicity of an equivalent magnitude to that exerted by chrysolite asbestos and nanoparticle carbon black.

Brown *et al.* (2007) demonstrated that the physical structure of MWCNT and nanofibres was able to influence the way in which they interacted with human peripheral mononuclear cells, *in vitro*. Specifically, long (50 μm), straight MWCNT were too long to be internalised by macrophages, which stimulated the onset of frustrated phagocytosis, with an associated increase in ROS and TNF α production. These effects were determined at sub-lethal concentrations of 62 and 31 $\mu\text{g ml}^{-1}$. In the same study, entangled MWCNT formed aggregates of approximately 10 μm length, that were easily ingested by the macrophages resulting in little ROS or TNF α production. Next, the ability of THP-1 differentiated monocytes to internalise *E. Coli* was assessed, in order to determine the impact of the samples on bacterial clearance. It was observed that bacterial clearance was impaired by all of the MWCNT samples tested. These results therefore highlight that the morphology and dimensions of CNT are able to impact on the effectiveness of CNT uptake and subsequent inflammatory response, which complements well the *in vivo* findings of Poland *et al.* (2008).

Dumortier *et al.* (2006) investigated the uptake of ammonium and/or PEG functionalised SWCNT by a mixture of primary mouse lymphocytes and macrophages, derived from the spleen. All of the particles (10 $\mu\text{g ml}^{-1}$ for 24 hours) were internalised and were evident within the cytoplasm, but not the nucleus, and uptake was not associated with cytotoxicity. As lymphocytes are known to proliferate on activation, T and B cells were also exposed to SWCNT (up to 50 $\mu\text{g ml}^{-1}$) and their proliferation assessed, but no proliferative responses were observed, and no IL-2 or IFN- γ release was detected. In addition, the ability of lymphocytes to respond to other physiological stimuli subsequent to SWCNT exposure was also determined. Ammonium functionalised SWCNT did not impact on the effects of the potent stimulus of bacterial lipopolysaccharide (LPS). The ammonium functionalised SWCNT were unable to

stimulate primary peritoneal macrophages, however PEG associated SWCNT were able to promote TNF α and IL-6 production, suggesting a pro-inflammatory response. In addition, the ability of macrophages to respond to physiological stimuli, subsequent to pre-treatment with functionalised SWCNT, was assessed, and it was found that macrophages could respond to LPS exposure to the same extent when exposed to ammonium functionalised SWCNT, but the stimulatory response was diminished when LPS stimulated macrophages were pre-treated with PEG associated SWCNT. The authors suggested that the ability of PEG functionalised SWCNT to modify immune responses could be explained by their poorer solubility, in comparison to that of ammonium functionalised CNT. As described previously, improved water solubility through functionalisation, has also been observed to improve excretion of SWCNT by rodent models (Singh *et al.* 2006) suggesting that functionalisation and water solubility are key factors in determining effects *in vivo* and *in vitro*.

The immune system is anticipated to play a key role in the removal of CNT from the body, which has primarily been evidenced by the contribution of resident macrophage populations to CNT uptake at the exposure site or within secondary targets, the consequences of which require assessment. However, the efficiency of the process is likely to be driven by the morphology and dimensions of CNT, and cannot be regarded as universally applicable to all CNT forms. In addition, the involvement of immune cells within granuloma development further emphasises their ability to contribute to CNT pathology. Furthermore, interference with immune cell function has been demonstrated, and thus the ability of CNT to increase susceptibility to infection requires further assessment in vivo.

6.3.4 Summary of the biological mechanisms of CNT induced toxicity

A number of investigators have now demonstrated that CNT (despite differences in terms of their wall number, source, metal contamination and particle dimensions), are capable of eliciting toxicity, which includes the initiation of an acute, neutrophil driven inflammatory response, oxidative stress, granuloma formation and fibrosis. Therefore it may be possible to generate broad conclusions regarding the mechanisms underlying CNT toxicity. These results, have also been mimicked in part by *in vitro* experiments, providing further support for such effects and mechanisms. Many of the studies outlined below repeat information provided above in relation to routes of exposure. The following section will therefore not provide details of the studies conducted, but instead will use this information to summarise the current understanding pertaining to mechanisms of toxicity including uptake, cellular effects, disease production and cytotoxicity.

6.3.4.1 Oxidative stress and inflammation

Oxidative stress (an imbalance between oxidant production and antioxidant defences, which favours oxidant presence) is clearly implicated in the induction of inflammation in a number of studies using particle and non-particle insults. Oxidative stress, at sub-lethal levels, activates specific transcription factors (such as NF- κ B), which switch on the genes that code for pro-inflammatory cytokines. Such effects have been observed for environmental particulate air pollution (PM10) (Jimenez *et al.* 2000), ultrafine carbon black (Brown *et al.* 2004), nanoparticle polystyrene beads (Brown *et al.* 2001) and also pathogenic fibres such as asbestos (Brown *et al.* 1999, Janssen *et al.* 1995). Experimental evidence suggests that the consequences of oxidative stress are 'tiered' in relation to the severity of the oxidative insult, with relatively low levels inducing a protective response such as antioxidant upregulation, moderate levels initiating inflammation, and if antioxidant defences are overwhelmed then a damaging response ensues involving cell death (Nel *et al.* 2006).

CNT have been found to induce an increase in ROS production and/or antioxidant depletion in the lung and heart (Shvedova *et al.* 2005, Li *et al.* 2007b) *in vivo*, while *in vitro* oxidative stress has been observed in models of the skin (Shvedova *et al.* 2003) and lung (Manna *et al.* 2005). The contribution of oxidative stress is further exemplified by the fact that the pre-treatment of cells/animals with antioxidants has the potential to diminish the toxic response of SWCNT (Shvedova *et al.* 2007).

Inflammatory responses have been identified in a number of *in vivo* investigations into CNT, including the pulmonary exposure of mice (Shvedova *et al.* 2005) and rats (Muller *et al.* 2005), as well as intraperitoneal exposure of mice (Poland *et al.* 2008). *In vitro*, a variety of CNT exposed cell types, including keratinocytes (Monteiro-Riviere *et al.* 2005a), and macrophages (Brown *et al.* 2007), have also been shown to enhance production of pro-inflammatory mediators such as IL-8 and TNF α . Such a response *in vivo* would be anticipated to drive the activation and recruitment of inflammatory cells. The *in vitro* experiments suggest that inflammation might not be limited to the lungs following administration *in vivo* via different routes. Again this requires further investigation. However, it is relevant that inflammatory mediators, specifically IL-8 has been demonstrated to adsorb onto the surface of SWCNT, thereby preventing its accurate detection (Davoren *et al.* 2007).

Studies linking oxidative stress and pro-inflammatory responses have also been conducted. Manna *et al.* (2005) demonstrated that SWCNT induced ROS production in HaCaT keratinocytes, which prompted the activation of the nuclear factor NF- κ B which is responsible for activating the increased expression of a variety of pro-inflammatory genes. Consequently, NF- κ B activation was held responsible for eliciting the inflammatory responses mediated by CNT that were observed by previous investigators, with such events ultimately compromising cell viability. These findings were corroborated by Shvedova *et al.* (2007), who illustrated that SWCNT elicited a robust inflammatory response within mouse lungs (subsequent to intratracheal exposure) that was virtually eliminated by pre-treatment with the antioxidant ascorbic acid, thereby implicating oxidative stress as a stimulus for the response. This therefore suggested that oxidative stress development is an integral component of the inflammatory response of the lung to CNT exposure, and that the two processes are inherently linked. Similarly, Ye *et al.* (2009) also investigated the mechanisms underlying the inflammatory response elicited by MWCNT within lung A549 and BEAS-2B epithelial cells. It was demonstrated that IL-8 and ROS production was increased with MWCNT exposure, but that pre-treatment with an antioxidant (N-acetylcysteine) was able to attenuate the level of IL-8 production associated with MWCNT exposure. The findings thereby implicated ROS production in mediating CNT driven inflammatory responses within the lung. Furthermore, the signalling processes that were responsible for driving the inflammatory response were investigated, and it was observed that NF- κ B activation was associated with MWCNT exposure, and that the degradation of I κ B (which ordinarily inhibits NF- κ B function) was apparent, and that NF- κ B inhibitors were able to block IL-8 production that was provoked by CNT exposure.

Nutritional manipulations involving vitamin E deficiency within mice, have also been shown to impact on the toxicity of SWCNT (Shvedova *et al.* 2007). The change in vitamin E levels resulted in alterations in antioxidant status, that increased susceptibility to oxidative stress, and thus a higher sensitivity to SWCNT induced inflammation and fibrosis. Shvedova *et al.* (2008b) demonstrated that NADPH oxidase deficiency, leading to a decreased superoxide production by neutrophils, enhanced the acute inflammatory response initiated by SWCNT exposure (40 μ g per mouse) by pharyngeal aspiration exposure to mice. It was observed that the magnitude of the inflammatory response was greater in NADPH oxidase deficient mice as neutrophils accumulated within the lung and were not cleared, and that the production of inflammatory cytokines (TNF α , IL-6, MCP-1) was enhanced. There was also a suppression of the anti-inflammatory and pro-fibrotic response (due to decreased TGF- β and collagen deposition).

A study conducted by Ding *et al.* (2005) had a slightly different emphasis, and demonstrated that exposure of skin and lung fibroblasts to MWCNT (0.06-0.6 mg l⁻¹) initiated an increase in IFN γ production, and a decrease in cell number. The authors hypothesised the response to be a consequence of the small size of particles leading to a comparable response invoked by a viral infection. A significant down-regulation of genes involved in cell growth and metabolism was observed, while an increase in genes involved in protein degradation, interferon mediated inflammatory responses and apoptosis were also detected. Multi-walled nano-onions (giant, nested fullerenes), used for comparison, did not mediate an interferon mediated response, and were found to be substantially less toxic, suggesting that different cells respond to nanomaterials according to their structure. No other studies have been identified that test this hypothesis further, and so the potential for CNT to induce a viral-like response is not clear.

Together these studies provide clear evidence to support that oxidative stress stimulates the activation of signalling pathways via the transcription factor $NF\kappa B$, which then triggers the initiation of gene expression leading to an inflammatory response. Initiation of oxidative stress and inflammation by CNT therefore suggests that the mechanism of action of these particles *in vivo* and *in vitro* could be comparable to other pathogenic particles. However, different approaches are becoming evident whereby a viral-like response to CNT may be associated with exposure.

6.3.4.2 Granuloma formation and fibrosis

The development of granulomas has been demonstrated on numerous occasions (see earlier), and is therefore assumed to be a common response to CNT exposure, and it is of note that their development is associated with exposure to foreign, biopersistent materials such as CNT. The study by Poland *et al.* (2008) has used the endpoint of granuloma formation in the peritoneal cavity to distinguish between the pathogenic potential of different nanotubes, varying in length and morphology. The results suggest that long, straight MWCNT are more potent than shorter or entangled specimens. Further studies are required to test this hypothesis in the pleural cavity and to demonstrate translocation of nanotubes from the airspaces following inhalation. It is worth noting that for pathogenic fibres such as asbestos, not all mesotheliomas are limited to the pleural cavity, but that in fact many have also been observed in the peritoneum of humans (Selikoff *et al.* 1990, Mossman *et al.* 1996). Therefore perhaps the relevance of the intraperitoneal injection is greater than has been suggested.

The fact that granuloma formation is a prominent feature associated with CNT exposure emphasises their biopersistent nature, whilst also insinuating that a common toxicity associated with CNT exposure may be evident at a variety of exposure sites. However, the development of granulomas is known to be related to the aggregation state of the CNT, and is therefore perhaps not universally applicable to CNT, as a whole.

6.3.4.3 Uptake of CNT into cells

Uptake studies have investigated the behaviour of both professional phagocytes, such as macrophages, as well as non-phagocytic cells. Determining the uptake of CNT by cells is important for a number of reasons. Probably the most important uptake scenario relates to phagocytic cells as they are responsible for the clearance of particles from sites of deposition. The study by Brown *et al.* (2007) clearly demonstrated that MWCNT length and shape impact upon uptake, with long fibres resulting in frustrated phagocytosis associated with the production of reactive oxygen species and pro-inflammatory cytokines. Secondly the uptake of CNT by cells may impact on normal cell physiology and function, and so the consequences of CNT internalisation requires assessment.

In a separate study, Hirano *et al.* (2008) linked the uptake of MWCNT to the ability of these particles to cause macrophage cell death. The uptake of MWCNT by both J774.1 and CHO-K1 macrophage cell lines was associated with mechanical rupture of the cell membrane (Hirano *et al.* 2008). The interaction of MWCNT with the plasma membrane was partly mediated by their association with the macrophage receptor with collagenous structure (MARCO) scavenger receptors, on the cell surface. The authors suggested that MWCNT bind to macrophages via the MACRO receptor, which encouraged the plasma membrane to extend along the particle, causing damage to the membrane, leading to cell death. The cytotoxicity observed, however, was not dose dependent. At low concentrations (10-100 $\mu\text{g ml}^{-1}$) of MWCNT, Hirano *et al.* (2008) observed a decrease in cell viability, in a time dependent manner (up to 32 hours). However, at higher MWCNT concentrations (100-1000 $\mu\text{g ml}^{-1}$), cytotoxicity was evident, but occurred to a lesser extent than that exerted by lower concentrations. This in contrast to the effect of crocidolite on macrophage viability, where cell viability decreased in a dose and time dependent manner. It was suggested that this unusual dose response relationship is likely to derive from the agglomeration of MWCNT, which would occur more extensively at higher concentrations, reducing their interaction with cells, and therefore reducing their cytotoxic potential. It was also apparent that pre-treatment of cells with the antioxidant N-acetyl cysteine,

was unable to prevent the detrimental impact of MWCNT on macrophage viability, suggesting that ROS were not involved in the observed cytotoxicity.

Shvedova *et al.* (2005) observed minimal uptake of SWCNT by the RAW macrophage cell line, which was paralleled by limited pro-inflammatory cytokine release. However the SWCNT did enhance TGF- β production, and cytotoxicity. One explanation for the relatively low uptake of SWCNT by the RAW macrophage cell line could have been the fact that the SWCNT were relatively well dispersed. Therefore, perhaps larger structures are better recognised and internalised by macrophages, and therefore more capable of stimulating a toxic response than their smaller counterparts. However, in contrast Davoren *et al.* (2007) found no evidence of SWCNT uptake by A549 lung epithelial cells, which was thought to derive from the exposure of cells to SWCNT aggregates that were too big to be internalised. On the contrary, Chou *et al.* (2008) suggested that the uptake of SWCNT by differentiated THP-1 monocytic cells, triggered the release of ROS which was responsible for the activation of the transcription factor NF- κ B, which in turn stimulation the production of pro-inflammatory cytokines and chemokines. Macrophages *in vivo* (in the mouse lung) and *in vitro* were observed to be loaded with SWCNT, which was associated with a cytotoxic response. Furthermore Lam *et al.* (2004) demonstrated that SWCNT aggregates were evident within alveolar macrophages subsequent to the intratracheal instillation of mice. The SWCNT laden macrophages then clustered to form epitheloid granulomas, which could impair lung function and give rise to a fibrotic reaction, highlighting how the uptake of SWCNT can dictate the toxicological endpoints that occur. Poland *et al.* (2008) also observed frustrated phagocytosis by peritoneal macrophages isolated from mice exposed to long, straight MWCNT.

Determining the uptake of SWCNT is often achieved through the attachment of fluorescent moieties, in order to facilitate visualisation within cells, as they are difficult to distinguish from cellular structures, due to similarities within their composition or dimensions (Porter *et al.* 2007). Porter *et al.* (2007) used energy-filtered transmission electron microscopy to distinguish between cellular compartments and internalised SWCNT within macrophages, and in addition, filled some CNT with silver iodide to enable their imaging via confocal microscopy. Human macrophages were exposed to SWCNT for 2 or 4 days, at concentrations up to 10 $\mu\text{g ml}^{-1}$. A cytotoxic effect was evident at concentrations above 0.3 $\mu\text{g ml}^{-1}$, after 4 days exposure, which was associated with a greater cellular uptake of SWCNT. At 2 days, SWCNT were contained within lysosomes, and at day 4 SWCNT bundles were fused with the plasma membrane, which caused disruptions to membrane structure, to enable their uptake. Evidence to support the phagocytosis, endocytosis (due to their appearance within early endosomes), and passive diffusion of SWCNT across the lipid membrane were witnessed. The authors also demonstrated that staining of samples with metals, is not an essential requirement for the visualisation of SWCNT internalisation.

The consequences of uptake of CNT by non-phagocytic cell types are not known, perhaps internalisation is required to exert toxicity, but also uptake may be beneficial in applications such as drug or gene delivery.

In studies using the A549 lung epithelial cell line, Davoren *et al.* (2007) demonstrated that there was no evidence of SWCNT uptake. However, the SWCNT were observed to form large bundles which adhered to the cell surface. This exposure resulted in minimal cytotoxicity at concentrations between 3.125 and 800 $\mu\text{g ml}^{-1}$ for 24 hours.

Monteiro-Riviere *et al.* (2005a) illustrated that unmodified MWCNT (<3.6 μm in length) were capable of being internalised by HEK keratinocytes, either into cytoplasmic vacuoles or were found free in the cytoplasm. The uptake was associated with an increase in IL-8 release. The internalisation of MWCNT was concentration and time dependent. In addition, it is worth mentioning, that when the MWCNT suspension was filtered (to remove large aggregates), that only a few isolated cells contained MWCNT. Filtering the sample also prevented an elevation of IL-8 release, suggesting that the uptake of MWCNT was influenced by their size, and could play a role in initiating the cellular response. However the authors did not rule out the possibility that association of the MWCNT with the cell surface could also contribute to the induction of a detrimental response.

Kostarelos *et al.* (2007) investigated what parameters were important in dictating the internalisation of a panel of small bundles or individual, functionalised SWCNT and MWCNT by a variety of cell types. It was demonstrated that functionalised CNT (whether neutral, positively or negatively charged) were consistently internalised by all cell types, which was likely to be facilitated by their short length, which within the panel of CNT tested was maximal at 2 μm . The cellular localisation was restricted to the cytoplasm, with localisation predominantly occurring at the perinuclear region in the A549 epithelial cell line. The authors suggested that uptake (using Jurkat cells) was not solely dependent on endocytosis, and their penetration of the cell membrane has been proposed to occur due to their ability to act like a 'nanosyringe' whereby they are able to puncture the cell membrane to enable their internalisation. The evidence provided to support this conclusion is limited to light microscopy images which do not allow a full analysis of the mechanism of uptake. Kostarelos *et al.* (2007) highlighted that the capacity of CNT to enter cells may derive from the cell type, CNT dimensions and propensity to form larger aggregated structures.

In relation to drug delivery, Pantarotto *et al.* (2004) illustrated that SWCNT and MWCNT were able to penetrate the cell membrane of human 3T6 and mouse 3T3 fibroblasts, and thus access the cell interior. CNT uptake by cells was not associated with a toxic response, and so it was anticipated that CNT could be exploited as drug delivery devices. Cell penetration of SWCNT was also investigated by Kam *et al.* (2004) using Jurkat and HL60 cell lines. The surface of the SWCNT was carboxylated and fluorescently labelled, thus allowing their internalisation to be visualised. The mechanism of uptake was suggested to be mediated by endocytosis, due to the temperature dependence of the process, and due to the association of internalised SWCNT with stained endosomes. However, the SWCNT utilised in this study were relatively short (100 nm-1 μm) which is anticipated to promote their uptake by cells. It is relevant that the SWCNT were processed prior to exposure; specifically contaminants were removed by acid treatment and sonication, and aggregates were removed by centrifugation. This means that individual or small aggregates of SWCNT dominated within the suspensions, which could promote the entry of SWCNT into cells. It was subsequently confirmed that SWCNT were able to carry the protein streptavidin (attached to the CNT surface) into the cell. An important remit of the ability to use SWCNT as drug delivery devices is the ability of SWCNT exposure to not affect cell viability, unless this is the purpose of their administration. It was observed that carboxylated SWCNT elicited no toxicity on exposure at the times and doses investigated. However SWCNT with streptavidin attached to their surface were capable of inducing significant cell death, but this was recognised by the authors as a benefit, in order to achieve an anti-cancer role. If such technology were to be used to treat cancer, it would be necessary to evaluate the precision of the response, namely the specificity of the killing so that only the desired cells are targeted and affected by CNT exposure. A similar investigation conducted by Wu *et al.* (2005) evaluated the ability of fluorescently labelled MWCNT to deliver the antibiotic, amphotericin into Jurkat cells, again in relation to drug delivery. The MWCNT-drug conjugate was internalised by cells and localised around the nuclear membrane. Endocytosis was excluded as a mechanism of uptake, as uptake was not inhibited at 4 $^{\circ}\text{C}$, and instead it was suggested that the MWCNT behave like needles, which enables their penetration and entry into cells, which therefore supports the findings of Kostarelos *et al.* (2007). It was also confirmed that the antibiotic activity of the attached drug was maintained through its attachment to MWCNT. Toxicity was evident with drug treatment alone due to the ability of amphotericin to disrupt cell membranes, but this was not observed on treatment with the CNT-drug conjugate, where only limited toxicity was evident. This suggests that the covalent binding of amphotericin to MWCNT reduced the toxic effects of this drug, although the anti-fungal behaviour was retained.

Determining the uptake of CNT by cells is difficult as individual tubes are difficult to detect with SEM and TEM, and elemental analysis cannot be used as they are composed of carbon (Cherukuri *et al.* 2004). As a result, it is common that fluorescent moieties are covalently attached to CNT that permit their detection within cells, as previously described. However Cherukuri *et al.* (2004) demonstrated that the uptake of pristine SWCNT by macrophage-like cells could be visualised due to their unique, near infra red intrinsic fluorescence (900-1600 nm wavelength). Specifically, it was observed that their uptake was dose and time dependent and that macrophages actively internalised the SWCNT, due to the temperature dependence of the process, with no apparent toxicity associated with uptake. The results of this study were expanded upon by Cherukuri *et al.* (2006) who investigated the blood elimination of SWCNT

from rabbits using infra red fluorescence. To avoid aggregation of SWCNT they were prepared in a synthetic detergent, Pluronic F108, ultrasonicated and ultracentrifuged to achieve a suspension that was enriched with single, individual SWCNT. Such a dispersion is necessary for their visualisation using this method. The blood concentration decreased with time (half life of 1 hour) with the most concentrated localisation occurring within the liver.

It is clear that CNT have the potential to enter a wide variety of cell types, but the process of uptake is unclear, with some evidence supporting active mechanisms such as endocytosis or phagocytosis, while other authors suggest CNT behave like nano-needles. Perhaps the method of delivery to the cell interior is dictated by the CNT, dispersion and cell type in question. It is not yet clear whether uptake is required to induce cellular responses including toxicity, or whether perhaps interaction with the cell surface is sufficient. Again, the answer to this question may be CNT, dispersion and cell type specific.

6.3.4.4 Genotoxicity of CNT

Genotoxic responses are associated with DNA damage and mutations that compromise the survival and function of cells. Genotoxic responses may be primary (due to an intrinsic property of the particles themselves, such as their ability to generate ROS) or secondary (due to excessive/persistence presence of nanoparticles such as inflammatory events elicited by nanoparticles) in nature (Knaapen *et al.* 1999, Schins, 2002).

Muller *et al.* (2008), using the micronucleus assay, observed that MWCNT were able to elicit genotoxic events in the rat lung ($0.5\text{-}2\text{ mg kg}^{-1}$, 3 days post exposure) and within *in vitro* models ($10\text{-}150\text{ }\mu\text{g ml}^{-1}$, following a 6 hour exposure). It is likely that *in vivo* the increase in micronucleated cells may have been related to the marked inflammatory response that was evident within the rat lung. *In vitro* MWCNT-related genotoxicity was the result of aneugenic (chromosome loss) as well as clastogenic (DNA strand breaks) events. The results therefore demonstrate that in this study MWCNT are able to induce mutations within lung cells, which is of concern.

Pacurari *et al.* (2008) examined the genotoxic potential of SWCNT, on exposure to mesothelial cells, at concentrations up to $500\text{ }\mu\text{g ml}^{-1}$, for a period of up to 24 hours. A number of genotoxic effects were identified, including DNA damage (using the Comet assay) and H2AX phosphorylation (indicative of DNA damage) which were associated with SWCNT exposure. It was postulated that DNA damage was a consequence of the direct interaction of SWCNT with DNA, and due to SWCNT stimulated ROS production.

In order to determine the genotoxic potential of SWCNT Shvedova *et al.* (2008a) determined the appearance of *K-ras* mutations within the lungs of mice, subsequent to pulmonary exposure. The frequency of mutations was found to be dependent on the exposure method and exposure time. Specifically, the occurrence of mutations occurred with a greater frequency within mice exposed via inhalation (and increased with time), whereas aspiration of SWCNT was associated with a low frequency of mutations that were equivalent to that of the controls. These findings therefore highlight that the exposure method has the ability to impact on the evaluation of CNT toxicity.

Zhu *et al.* (2007) determined the ability of MWCNT to elicit DNA damage within mouse embryonic stem cells, at concentrations of $5\text{ or }100\text{ }\mu\text{g ml}^{-1}$, for up to 24 hours. MWCNT were demonstrated to be capable of inducing DNA damage. This was concluded due to evidence that p53 protein was phosphorylated, in a dose and time dependent manner (which is indicative of DNA damage as it triggers the arrest of the cell cycle so that DNA can be repaired and can also stimulate apoptosis if DNA cannot be repaired). DNA repair enzyme expression was also increased, therefore insinuating that DNA was damaged by MWCNT exposure. There was also evidence of damage to the DNA double strand, as DNA double strand repair protein expression was increased. The evidence therefore suggested that the MWCNT caused DNA damage, and increased the frequency of mutations, that was thought to be driven by MWCNT mediated ROS production.

However, Kisin *et al.* (2007) determined the ability of SWCNT to induce genotoxicity, within three separate *in vitro* tests; namely the Comet assay (to detect strand breaks), the micronucleus test (to identify chromosomal damage), and the Ames test (to determine DNA damage). SWCNT mediated DNA damage was found within V79 fibroblasts, using the Comet assay. There was also a trend for an increase in micronucleus formation within cells. However, no evidence of genotoxicity was observed within *S. Typhimurium* using the Ames test. Consequently, more extensive *in vitro* and *in vivo* studies would be required to confirm the genotoxic potential of SWCNT. In addition, a decrease in cell viability was also associated with the response, which interferes with the accurate detection of genotoxic responses (Kisin *et al.* 2007).

Furthermore, there is conflicting evidence available that suggests CNT, do not induce DNA damage. Di *et al.* (2009) investigated the ability of MWCNT (110-170 nm diameter, 5-9 μ m length) to cause DNA damage, using the Ames test. No cytotoxicity was associated with MWCNT exposure (up to 3.5 μ g ml⁻¹) of *S. Typhimurium* and *E. Coli* strains used. In addition, no mutations were related to MWCNT exposure. Consequently, the ability of CNT to inflict genotoxicity is debatable, when considering the discussed findings. This may derive from the knowledge that the term genotoxicity refers to damage to DNA (which can be evidenced, for example by strand breaks, the formation of DNA adducts etc), which cannot always be detected using a single test (Di *et al.* 2009). Therefore, as only the Ames test was used, the undertaking of a more comprehensive study, using a number of genotoxic tests would be required reveal the true genotoxic potential of CNT, and to confirm their lack of genotoxicity. This is also exemplified by the findings of Kisin *et al.* (2007).

The genotoxic potential of CNT is uncertain at this time, and is likely to be dictated by the experimental set up; including the model, exposure route, CNT type, concentration administered and endpoint assessed. Genotoxic events may transpire as a secondary consequence of CNT mediated inflammatory and oxidative responses, or alternatively due to their direct interaction with DNA, following internalisation by cells. Therefore the generation of more definite conclusions regarding the genotoxicity of CNT would require more extensive investigations.

6.3.4.5 Reproductive toxicology of CNT

In vivo and *in vitro* assessment of CNT effects on the reproductive system are limited to a small number of studies. Cheng *et al.* (2009) examined the effect of CNT on embryonic development, by looking at acute and long term effects of zebra fish embryos exposed to 2 ng of fluorescein isothiocyanate (FITC) labelled, bovine serum albumin (BSA) coated MWCNT at the 1 cell stage, with examinations conducted at 30 minutes, 24, 48 and 96 hours post microinjection. At early stages, the treated zebra fish embryos generated an immune response by accumulating circulating white blood cells at the trunk region. TEM analysis showed many lysosome-like vesicles in the blastoderm cells of the treated embryos, the reasons for this are unclear but could represent uptake into the cells. The embryos were able to produce normal primordial germ cells and were able to produce a second generation. The larvae of the second generation however, had lower survival rates compared to the untreated groups, suggesting a negative effect on the reproduction potential. An earlier study by Cheng *et al.* (2007) showed SWCNT induced significant hatching delay in zebra fish embryos between 52 to 72 hours post fertilisation (hpf) at concentrations of greater than 120 mg l⁻¹, but 99% of the exposed embryos hatched by 75 hpf. Double-walled CNT also induced a hatching delay at concentrations of greater than 240 mg l⁻¹. They went on to demonstrate that concentrations of up to 360 mg l⁻¹ of SWCNT, micro scaled or larger, agglomerates were unable to compromise the nanoscale pores of the protective embryo chorion after 96 hpf, indicating that the chorion is an effective protective barrier to SWCNT agglomerates. The hatching delay observed in this study likely was induced by the Co and Ni catalysts used in the production of SWCNT that remained at trace concentrations after purification.

The limited literature examining the effects of CNT during pregnancy, highlight effects on the developing foetus but are limited to the zebra fish model with no published studies looking at effects in mammals. No specific in vitro or in vivo studies were found examining CNT effects on male and female reproductive systems.

6.3.4.6 Susceptibility to toxicity

It is unlikely that all humans, or all species will exhibit the same sensitivity to CNT induced toxicity. These variations derive from both genetic and environmental factors. The role of such factors in controlling susceptibility is beyond the scope of this review, but factors such as disease status, diet and age are all likely to be important.

Inoue *et al.* (2008) investigated the inflammogenicity of SWCNT (1.2-1.4 nm diameter, 2-5 μm length) or MWCNT (2-20 nm diameter, several μm in length), following a combined CNT (4 mg kg^{-1}) and LPS (33 $\mu\text{g ml}^{-1}$) treatment, which was administered to mice via intratracheal instillation. Such a study design might be used to simulate the effects of CNT on an already inflamed lung, therefore simulating inflammatory disease or an infection. However, the doses used were relatively high leading to confusing data that was difficult to interpret, but the findings implied that combined LPS and CNT exposures increased the inflammatory response that was induced.

In a different study, Han *et al.* (2008) investigated whether pre-exposure to MWCNT (20 μg), via pharyngeal aspiration of mice, was able to influence the pulmonary response to ozone (0.5 ppm). It was anticipated that MWCNT would induce an inflammatory response that predisposed the lung to further damage by other pollutants, such as ozone. MWCNT alone induced a pronounced infiltration of neutrophils, accompanied by an increase a number of inflammatory and cytotoxicity markers in BALF. Ozone alone did not induce inflammation. Pre-treatment with MWCNT, 12 hours prior to ozone, was unable to enhance the inflammatory response to ozone exposure, and in fact an attenuation of the inflammatory and cytotoxic responses elicited by MWCNT were observed, suggesting a tolerance to the effects of pollutants on their sequential exposure, perhaps due to the initiation of a protective response.

Furthermore, as discussed previously, Li *et al.* (2007b) demonstrated that SWCNT were able to accelerate plaque formation in ApoE^{-/-} mice, and so CNT exposure may make individuals more prone to the development of cardiovascular disease.

A predisposition to the toxicity of CNT may be manifested, specifically within individuals with pre-existing pulmonary or cardiovascular disease. Exposure to CNT is therefore anticipated to enhance disease pathology and thereby exacerbate disease symptoms, in a similar manner to that observed for particulate air pollution (Pope and Dockery 1999). However, insufficient evidence is currently available to support this contention, and the susceptibility of individuals to CNT toxicity, should be addressed within future studies. It will also be necessary to encompass whether disease within other targets (other than the pulmonary and cardiovascular systems), such as the liver (such as alcoholic liver disease) would make individuals more susceptible to toxicity, due to their propensity to accumulate within different target sites.

6.3.5 Linking the physico-chemical characteristics of CNT to pathogenicity or toxicity

6.3.5.1 Length

As described previously, length has been accepted to influence fibre clearance, because it dictates the ability of phagocytic cells to completely internalise CNT. Longer fibres promote the development of frustrated phagocytosis, reduced clearance and hence the potential to persist to increase their propensity for damage. *In vitro*, the impact of MWCNT length on superoxide production, pro-inflammatory cytokine production and cytotoxicity to macrophages has been demonstrated by Brown *et al.* (2007), therefore supporting the hypothesis that MWCNT can induce frustrated phagocytosis if the fibres are long enough. *In vivo* data provided by (Poland *et al.* (2008) using the mouse peritoneal cavity, also supports the observation of frustrated phagocytosis with longer straight MWCNT, that was associated with mesothelial changes including granuloma formation. In both studies, entangled and shorter MWCNT were less potent, further supporting the role of length/dimensions/shape in influencing the pathogenicity of CNT.

Many studies published that investigate the toxicology of CNT have used particles that are only a few μm in length, therefore preventing the investigation of the role of length in dictating their toxicity. It is possible that short CNT ($<15 \mu\text{m}$) are likely to be more readily taken up by cells in general allowing on one hand a more efficient elimination via phagocytic cells, but potentially enable the delivery of an enhanced dose to cells, in general. In many publications the length of CNT was not provided, often due to the fact that CNT are usually entangled and aggregated, making accurate measurements difficult.

6.3.5.2 Wall number

Tian *et al.* (2006) investigated the toxicity of 5 engineered nanomaterials, including SWCNT (2 nm diameter, 500 nm length) and MWCNT (50 nm diameter, 5 μm length), which were exposed to human dermal fibroblasts at concentrations ranging from 0.8-100 $\mu\text{g ml}^{-1}$, for a period of up to 5 days. SWCNT had the greatest detrimental impact on cell survival and cell adhesion. In fact the more pronounced toxicity associated with SWCNT, compared to that of MWCNT has been documented on a number of occasions (see for example Jia *et al.* 2005, Radomski *et al.* 2005, Inoue *et al.* 2008).

It is difficult to draw firm conclusions from the described studies, as it is unlikely that wall number was the only physico-chemical characteristic that varied between samples.

6.3.5.3 Contribution of CNT composition to their toxicity

Nanotubes can vary with regards to their composition, which includes surface modifications and the presence of contaminants. Potential contaminants include residual metal catalysts (such as iron and nickel), amorphous carbon and hydrocarbons, that are primarily introduced during their manufacture. The metal component of ambient particulate matter has been demonstrated to contribute to (Hutchison *et al.* 2005) and to enhance toxicity (Wilson *et al.* 2002). Transition metals such as iron induce toxicity via redox cycling and the production of reactive oxygen species. Post-production processing utilised to improve the purity of manufactured CNT, includes the use of acid and heat treatments to remove metals from the CNT structure. However these processes have been demonstrated to also influence the structure of CNT (Raja *et al.* 2007) making it difficult to determine the role of contaminant metals in toxicity. The metal content of CNT may also vary in bioavailability, with some metals being trapped within the lumen of the nanotube structure, while some are free on the particle surface.

Contaminants

A number of studies have attempted to investigate the role of metals in CNT induced toxicity. Early studies conducted by Shvedova *et al.* (2003) demonstrated that SWCNT containing a high (30%) iron content was able to elicit toxicity within keratinocytes. Metal chelators were able to prevent the observed toxicity suggesting that the iron content played an important role in SWCNT toxicity. However, Shvedova *et al.* (2005) also demonstrated that SWCNT with a low iron content (0.23%), were toxic within mice, suggesting that iron may not be solely responsible for driving their toxicity. Kagan *et al.* (2006) illustrated that, in cell free conditions, iron containing (26%) SWCNT had a greater potential to produce free radicals than their iron depleted counterparts (0.23%). Similarly, when the RAW 264.7 macrophage cell line was exposed to SWCNT, the iron rich sample was able to induce markers of oxidative stress including GSH depletion and lipid peroxidation, and that iron depleted samples were less effective at this.

Lam *et al.* (2004) utilised a number of different CNT samples that varied with regards to their purity, specifically in terms of their metal content. However, this did not impact on their potential to produce granulomas. This study suggests that granuloma formation cannot be simply related to the total iron content of samples. Accordingly, it is possible that the potency derives from the tendency of CNT to aggregate, thereby stimulating the initiation of a foreign body response.

Guo *et al.* (2007) evaluated the impact of sample age and processing by sonication or oxidation, on iron release from CNT. Iron mobilisation in cell free conditions was enhanced with increased

sample age. It was also illustrated that iron can be liberated from a diverse set of commercially available CNT, and that that the released iron is redox active, a property which could be mitigated by iron chelators. However the amount of iron that could be mobilised from samples could not be predicted from the total amount of iron present in the sample. Therefore assessing the potential for iron mobilisation from samples may be more relevant than measurement of total iron content. The bioavailability of components such as metals may be of importance since Nimmagadda *et al.* (2006) demonstrated that soluble components of SWCNT contributed to their *in vitro* toxicity.

Murray *et al.* (2009) demonstrated that unpurified SWCNT were more cytotoxic to epidermal skin cells *in vitro* than their purified (by acid treatment) counterparts. The unpurified CNT were also able to induce more oxidative stress (indicated by GSH depletion), which was decreased on the addition of a metal chelator, thus implicating metals in the oxidative potential of the SWCNT. Murray *et al.* (2009) also measured activation of AP-1 and NF- κ B, transcription factors, involved with the initiation and sustainment of inflammatory responses. AP-1 was activated to a greater extent by the unpurified SWCNT than by their purified equivalents, while both SWCNT samples were able to activate NF κ B to a similar extent. These results therefore suggested that SWCNT were capable of inducing oxidative mediated effects on cells *in vitro* that were enhanced by metal presence. Murray *et al.* (2009) also observed that changes in the skin morphology of the *in vitro* skin tissue model, EpiDermFT, on exposure to unpurified SWCNT. This was accounted for by an accumulation and activation of dermal fibroblasts, and subsequent increase in collagen deposition which was characterised by a greater thickness of the dermis. In addition, cytokine (IL-12, IL-6, IFN γ) production was elevated by unpurified CNT exposure. In mice, unpurified topical CNT dermal exposure leads to an increase in skin bi-fold thickness which is a measure of oedema and inflammation development. Inflammation was confirmed by an influx of mast cells and neutrophils into the skin.

Together these studies suggest that the metal (particularly iron) content of CNT can contribute to toxicity, but that it is not solely responsible, since toxicity can still be apparent in their absence. Differences between studies may relate to the relative bioavailability of metals within the different CNT samples, with some metals trapped within the core of the nanotube structure. In addition, determining the contribution of metals to CNT toxicity is confounded by the knowledge that the processes employed to purify CNT, often affect other CNT properties (such as length or surface chemistry), which may account for the altered responses observed, when compared to that exhibited by their unpurified counterparts.

Surface Chemistry

In addition, to the investigation of metals, other studies have also looked at surface modification of CNT, and how this impacts on their toxicity. For example, Carrero-Sanchez *et al.* (2006) exposed mice (via nasal, oral, intraperitoneal and intratracheal exposure) to nitrogen doped MWCNT, and demonstrated that they were less toxic than their pure counterparts. This is thought to derive from the fact that amino groups were evident on the nitrogen doped MWCNT surface that made them more biocompatible. The results from these studies therefore illustrate that the composition of the CNT is able to influence their toxicity.

Bottini *et al.* (2006) investigated the toxicity of oxidised (with acid) and 'pristine' MWCNT (400 $\mu\text{g ml}^{-1}$) to lymphocytes. First, it was observed that the oxidation procedure shortened and straightened the MWCNT. Both MWCNT samples were capable of compromising Jurkat cell line viability, but the toxicity induced by oxidised CNT was greater in magnitude, which is exemplified by the finding that cell death was evident from 48 hours onwards for oxidised samples, and from 96 hours for pristine samples. The mode of cell death was then assessed and it was apparent that an apoptotic mechanism was dominant (using Annexin V staining), which was confirmed with morphological considerations (such as chromatin condensation and membrane blebbing) both in the Jurkat cell line and in T lymphocytes isolated from healthy volunteers. In all studies conducted oxidised CNT were more toxic than their pristine counterparts, which the authors suggested was due to their better dispersion, which derived from surface chemistry alteration. It was also suggested that the physical form/shape of the

carbon material was able to impact on its toxicity, as CNT were more toxic than amorphous carbon.

The oxidised CNT that are yielded from acid purification processes are known to be more readily dispersed due to the incorporation of hydroxyl and carboxyl groups to the surface of the CNT. The introduction of such groups also enables the coupling of other moieties such as proteins. Such additions have been exploited to facilitate the delivery of macromolecules more efficiently to cells. However, the structure and function of such moieties may be affected by their attachment to CNT. This is exemplified by the fact that the catalytic function of the enzyme, R-chymotrypsin was lost, due to perturbations of its structure on attachment to SWCNT (Karajanagi *et al.* 2004). This was not however the case with soybean peroxidase whose structure and function was retained on attachment, suggesting that alterations in the structure and activity of attached material might have to be assessed on a case by case basis, highlighting the complexity of the interactions (Karajanagi *et al.* 2004).

Wu *et al.* (2005) investigated whether processing of CNT via acid treatment impinged on their length and extent of carboxylation. The length and carboxyl loading of CNT was dictated by the duration of the oxidation procedure, with longer treatments resulting in shorter CNT and a higher incidence of carboxyl group loading into the CNT structure. Therefore, different investigators are likely to use different protocols, when processing CNT, which may account for variations in their toxicity so that they do not always behave similarly.

Sayes *et al.* (2006) compared the effectiveness of covalent modification or surfactant addition, within the suspension of SWCNT. Covalent modification included the production of SWCNT-phenyl-SO₃H and SWCNT-phenyl-(COOH)₂ with different degrees of functionalisation including carbon/phenyl-SO₃X ratios of 18, 41, and 80. In addition, SWCNT prepared using the surfactant Pluronic F108 (1%), provided the best dispersion of CNT, and in turn, enhanced the toxicity of SWCNT compared to the functionalised SWCNT. In addition, an inverse relationship existed between the toxic potential, and degree of side wall functionalisation on exposure to keratinocytes. Therefore the dispersion methods utilised by different investigators may be able to impact on the findings that illustrate their toxicity. In contrast, Magrez *et al.* (2006) demonstrated that the toxicity of MWCNT to H596 lung tumour cells was enhanced through the functionalisation of its surface (mediated via acid treatment to introduce carbonyl, carboxyl and/or hydroxyl groups).

The impact of functionalisation on the *in vivo* profile of CNT was also assessed by Lacerda *et al.* (2008). Pristine MWCNT were prepared initially in mouse serum in an attempt to improve their biocompatibility. Alternatively a MWCNT sample was functionalised with ammonium in order to render them more hydrophilic, and therefore water soluble. It was demonstrated that although the aggregation of CNT was reduced for the serum dispersed MWCNT, the ammonium functionalised MWCNT were much more individualised. The CNT were administered to mice via a tail vein injection and the lungs, kidney, spleen and liver were examined via histological analysis 24 hours post exposure. It was observed that as functionalisation of the CNT surface increased, the propensity for tissue accumulation was lessened. The functionalisation of the MWCNT promoted their rapid excretion from the body thereby reducing their accumulation within the body that was experimentally proven. Serum coated MWCNT detrimentally affected the mice as exhibited by their subdued behaviour, hunched posture, reduced activity and respiratory distress. The MWCNT dispersed with serum accumulated predominantly within the lung and liver, and were visualised as large, dark clusters. The respiratory distress associated with serum dispersed MWCNT was likely to be a consequence of the accumulation of CNT within pulmonary vasculature. Accumulation of raw CNT within Kupffer cells of the liver was also observed, so that the presence of aggregates was thought to promote their recognition and removal by macrophages.

Krajcik *et al.* (2008) functionalised SWCNT with positively charged moieties, in order to promote the binding of negatively charged molecules such as small interfering (si)RNA through an electrostatic interaction, thus enabling their delivery into cells. It was demonstrated that the functionalisation of SWCNT (with hexamethylenediamine and poly-(diallyldimethylammonium)-chloride) promoted their water solubility and were able to enter primary rat cardiomyocyte cells, where the siRNA was liberated to induce the desired effect of efficient silencing of the target

genes, with minimal cytotoxicity. The nature of the functionalisation therefore allows for interactions with moieties that require delivery into the cell interior that would otherwise not be possible.

The surface chemistry of CNT has the ability to modify their behaviour, and is relevant to consider as it is the surface of the CNT with which cells interact. The modification of the CNT surface has been demonstrated to both enhance and reduce toxicity, and therefore it is unreasonable to make definite conclusions about how the modification of CNT affects their toxicity, as it is driven by the particular modification employed, which is generally undertaken for a specific purpose.

6.3.5.4 CNT agglomeration and aggregation

The terms agglomeration and aggregation are often used interchangeably to describe the attractions that hold together a collection of particles. However some authors have suggested that it is more appropriate to consider nanoparticle aggregation and agglomeration as distinct phenomena with agglomerates formed by clusters of particles that are held together by electrostatic interactions, whereas aggregates are formed from covalently fused or sintered particles that are not easily separated (Oberdoerster *et al.* 2007).

It is often remarked by investigators that there is a difficulty in testing CNT toxicity due to their high propensity to aggregate, so that it is widely accepted that CNT tend to form bundle or rope like structures due to electrostatic attractions. Therefore CNT structures contained within samples are considerably longer and wider than the individual CNT from which they are composed, and therefore achieving a suspension or aerosol where individual CNT are contained is difficult. It is therefore important to consider how interactions between CNT, which promote the formation of larger structures, impact on the toxicity of CNT. The preparation of relatively well dispersed CNT suspensions may be achieved through the use of dispersing agents, solvents, surface attachments (that make the CNT more water soluble) or mechanical processes (such as sonication, centrifugation, grinding). Extensive efforts have therefore been made by a number of investigators to better disperse CNT, with varying levels of success. The relevance of considering monodispersed CNT also requires consideration, since, if this is so difficult to achieve experimentally, the relevance to human exposure may be questionable. It might be more useful to achieve a CNT suspension with limited or controlled aggregation, promoting uniform, more easily characterisable exposure conditions for the model under investigation. In addition, the exposure scenario is likely to impact on the aggregation and agglomeration of CNT; for example limiting the aggregation of CNT when generating aerosols is difficult, whereas a number of dispersants can be employed to improve the dispersion of CNT suspensions.

Muller *et al.* (2005) used grinding to increase dispersion of MWCNT. Specifically, unground intact MWCNT were more biopersistent in the lung, when compared to their ground counterparts, and as a consequence have a greater potential to cause inflammatory and fibrotic reactions. As described previously the grinding impacted on both length and surface properties, and therefore toxicity of the MWCNT.

Wick *et al.* (2007) investigated the relationship between SWCNT agglomeration and toxicity to the MSTO-211H mesothelioma cell line. To delineate the impact of CNT dispersion on their toxicity, four SWCNT preparations were used; the raw material (i.e. as produced CNT), CNT agglomerates (that originated from the raw material but was heat and acid purified), a well dispersed CNT bundle sample (generated using polyoxyethylene sorbitan (PS80), sonication and centrifugation), and lastly the pellet obtained from the centrifugation of the CNT bundle sample was prepared, that did not contain CNT. Due to the fact that the CNT raw material was the starting material for all SWCNT samples, they were biochemically very similar. As a consequence it was anticipated that comparisons of their toxicity could be based predominantly on the efficiency of their dispersion, however, the different preparation procedures utilised will inevitably impact on their physico-chemical characteristics. It was observed that the CNT raw material and CNT agglomerates were able to decrease cell activity and proliferation, whereas CNT bundles did not cause toxicity. The CNT agglomerates elicited toxicity that was comparable to that of asbestos, which might be considered to further support the hypothesis

that CNT, in a specific form may behave like pathogenic fibres. However, the endpoints measured here are not really representative of the mechanism of fibre induced toxicity and so the relevance of this comparison is questionable. Overall, the toxic potential of CNT was therefore related to their degree of dispersion. Wick *et al.* (2007) also demonstrated that amorphous carbon (pellet) samples induced toxicity that was comparable to that of the CNT agglomerates, therefore the content of amorphous carbon within CNT samples should be assessed, as this contaminant has a high propensity to contribute to any observed toxicity.

Mercer *et al.* (2008) investigated the impact of CNT dispersion on their deposition and toxicity within the lungs subsequent to pharyngeal aspiration. The SWCNT were equivalent to those used by Shvedova *et al.* (2005), however to achieve a 'better' dispersed treatment, the CNT were treated with acetone, in an attempt to reduce Van der Waals attractions between CNT. Acetone decreased the average diameter of the SWCNT aggregates from 15.2 μm to 0.69 μm . It was therefore suggested that solvents are worth consideration when attempting to better disperse CNT, however the contribution of any residual acetone to the toxicity requires assessment. It was observed that untreated CNT (in an aggregated form) could be observed within lung micrographs of SWCNT exposed mice, and were encased by macrophages to form granulomatous inflammation, however these lesions were absent from mice treated with the acetone dispersed SWCNT. However, exposure to acetone treated CNT tended to induce a fibrotic response that was distinct from aggregate induced lesions. This supports the findings of Shvedova *et al.* (2005), who demonstrated that the pathology associated with aggregates versus better dispersed tubes was different. The distribution of CNT within the lung could not be achieved by looking at lung sections, as the acetone dispersed SWCNT were too well dispersed and could not be visualised. Therefore, in an attempt to reveal the distribution of CNT within the lung, the CNT were labelled with gold or quantum dots to allow their visualisation. Unmodified SWCNT were found to be concentrated in proximal alveolar region, and acetone dispersed SWCNT had a 'finer a more diffuse' deposition, with minimal appearance within the conducting airways but instead were situated within the interstitium, air spaces, and were able to enter the alveolar walls.

Monteiro-Riviere *et al.* (2005b) evaluated the effects of five commonly used surfactants (namely Pluronic L61, Pluronic L92, Pluronic F127, Tween 20, and Tween 60, at concentrations of 0.1-10%) on the dispersal of MWCNT, and investigated whether their inclusion within MWCNT suspensions was able to influence their toxicity to HEK keratinocytes. Preliminary studies illustrated that all surfactants (with the exception of Pluronic F127) reduced cell viability at all concentrations, and were therefore deemed inappropriate to use despite their ability to reduce CNT aggregation. Pluronic F127, due to its low toxicity, was used in further studies in order to assess the impact of MWCNT exposure to HEK cell function. The Pluronic F127 dispersed MWCNT generated a response comparable to that elicited by MWCNT in cell medium alone. MWCNT in the absence or presence of surfactant were equally able to compromise cell viability, however the IL-8 production stimulated by the MWCNT exposure was lower when dispersed with Pluronic F127, than without. The relevance of this difference is difficult to ascertain, but could suggest that aggregated MWCNT induce a greater pro-inflammatory signal in keratinocytes than the dispersed nanotubes.

Casey *et al.* (2007b) investigated whether cell culture medium components were able to interact with SWCNT in terms of impacting upon their de-bundling and how this might influence their toxicity. Serum inclusion improved the dispersion of CNT as indicated by reduced settling. However, their appearance, as illustrated using Raman spectroscopy demonstrated the continued existence of bundled aggregates, and not individual tubes, regardless of the composition of dispersing solution. Therefore, although serum is capable of interacting with the CNT it does not completely promote their de-bundling.

Filtering can be used as a technique to reduce the presence of aggregates within the dispersing solution as used by Raja *et al.* (2007). The CNT aggregates in non-filtered suspensions settled randomly onto aortic smooth muscle cells, so that cells were not in uniform contact with the CNT; this exposure was associated with a decrease in cell proliferation. However, this effect was generally of a smaller magnitude when cells were exposed to filtered CNT samples, and therefore the results implied that the presence of SWCNT aggregates is likely to account for the enhanced toxicity of the unfiltered sample. These differences between filtered and unfiltered

were only relevant at the lower concentrations tested, while at the highest concentration tested (0.1 mg ml⁻¹), both SWCNT samples induced a similar toxicity.

The aforementioned studies highlight that improving the dispersion of CNT is able to affect their behaviour/toxicity subsequent to exposure, which also is likely to be dependent on the exposure setting, as this influences the preparation of particles for exposure. Some authors assume that the aggregation of CNT is able to reduce the concentration of CNT available to cells, and thus avoiding toxicity, however this has not always been the case. Decreasing the propensity for CNT aggregation within the suspensions administered to cells, has been illustrated to both increase and decrease their toxicity, and therefore predicting the impact of CNT aggregation on their toxicity is difficult. These differences may derive from the differences between CNT samples, dispersants and cell types studied.

6.3.5.5 Manufacture process and CNT toxicity

As a consequence of the fact that CNT can be made by different methods, by different manufacturers who use different catalytic metals, carbon sources and processing conditions (such temperature or pressure), the produced CNT characteristics vary between manufacturers and even within different batches from CNT produced by the same manufacturer (Lam *et al.* 2006). Therefore it is plausible that the CNT produced will not be identical in nature each time they are produced, with post-manufacture processing adopted by different investigators capable of further altering the physico-chemical characteristics of CNT (Lam *et al.* 2006). This highlights that CNT are a heterogenous population of materials, and that there is the potential (that has been realised within experiments) that they are not universally toxic. The potential influence of different manufacturers on toxicity was exemplified by Grubek-Jaworska *et al.* (2006), who demonstrated that four different sources of MWCNT did not elicit the same extent of inflammatory cell infiltration and IL-8 expression after intratracheal exposure of guinea pigs. Despite this, all CNT tested induced pneumonitis and granuloma formation. Studies comparing CNT from different sources and of different lengths and entanglement have already been described (Poland *et al.* 2008 and Brown *et al.* 2007).

Of course different manufacture methods lead to different physico-chemical characteristics such as length, shape and functionalisation, which according to the small number of studies conducted to date, can influence toxicity. However, at this time there are insufficient studies to provide a clear systematic analysis of such influences. Further work, in which the physico-chemical characteristics are systematically manipulated are required in order to identify which features are more likely to make a CNT pathogenic or toxic. With respect to length and shape, the fibre paradigm requires further work to investigate translocation to the pleural cavity or peritoneal cavity following inhalation, as well as evidence of an impact on the pleural mesothelium. As stated previously, provisional data from NIOSH suggests that such translocation could happen (http://www.cdc.gov/NIOSH/blog/nsb031909_mwcnt.html accessed 05/05/09). Despite these gaps in our current knowledge the data of studies such as Poland *et al.* 2008 have been sufficient to prompt legislative modifications and the advice provided by the UK Health and Safety Executive (HSE; <http://www.hse.gov.uk/pubns/web38.pdf> accessed 05/05/09).

At this time work has predominantly focused on the lung, with some work on dermal models and immune cell models. It is therefore not possible to determine whether CNT induced effects are site, cell or organ specific within the body. Therefore work using a wider array of exposure routes and targets is require in order to explore whether CNT are universally toxic, specifically if they exert toxicity at different target sites via the same mechanisms. This will also aid in the implementation of toxicity tests that should be undertaken with highest priority, when assessing the hazard posed by the large number of CNT available.

6.3.5.6 Comments upon Experimental Designs

The experimental set up, including factors such as CNT concentration, exposure time, and test species, is likely to influence the outcome of the assessment of CNT toxicity, and vary greatly within the different investigations reported.

With respect to concentration, a number of the toxic effects observed are likely to derive from the high doses of CNT used in some studies (see for example Lam *et al.* 2004, and Takagi *et al.* 2008). At high doses, the aggregation of CNT is promoted, and so the toxicity that transpires *in vivo* is likely to result from the blockage of airways and blood vessels, rather than to a specific toxic effect. The relevance of such CNT concentrations to those encountered by humans is questionable, but difficult to confirm due to the apparent lack of relevant human exposure data. Preliminary studies conducted by Maynard *et al.* (2004) have given information regarding the likely level of exposure within an occupational setting, but further evidence is required.

It would also be of benefit to conduct studies using a range of CNT concentrations so that regulatory toxicity markers (such as the LD₅₀) or threshold doses, could be attained. However this would require the use of many animals and therefore ethically, is perhaps not appropriate. The relevance of LD₅₀ for such particles is also questionable, when in fact the risk of generating different pathological changes over a range of concentrations might be more relevant

The nature and duration of investigations also warrants consideration. It is often the case that a single dose of CNT is administered to animals or cells, when in fact it is more likely that exposure to CNT will occur over a period of time, depending on their application, so it is unlikely that a single exposure will be associated with their utilisation or accidental exposure. Most studies to date have used relatively acute or short term exposure durations, where as in fact longer durations will be required to fully test hypotheses such as the fibre paradigm. In addition, the use of chronic studies will also allow for the more relevant identification of carcinogenic consequences of CNT exposure.

There are a number of 'controls' that could be adopted in order to assess the toxicity of CNT. Benchmark controls provide a useful indication of the relative toxicity (benchmarking or ranking) of CNT versus particles or reagents of known toxicity (see for example, Warheit *et al.* 2004, Lam *et al.* 2004, Shvedova *et al.* 2005). To some extent the choice of controls can be driven by the hypothesis being tested, including determination of the physico-chemical characteristics responsible for toxicity. The use of a nanoparticle control, such as carbon black, has been reported on numerous occasions, due to the extensive background information available for this particle in humans, animals and *in vitro*. The use of nanoparticles is relevant, as at least two dimensions of the CNT structure is within the nano size range and therefore allows assessment of whether nanotubes might behave like nanoparticles with three dimensions below 100 nm. Asbestos has also been used as a control, due to reasons previously stated. In some studies the type and dimensions of the asbestos used are clearly defined, while in others it is less clear. When assessing a particular endpoint, for example inflammation, there may also be more relevant non-particle controls, such as zymosan or LPS.

As discussed previously dispersion impacts upon the potential toxicity of CNT in animals or cells. A multitude of dispersing processes have been utilised by investigators to improve the dispersion including solvents (such as acetone), surfactants (such as pluronic acid), proteins (albumin and serum) or mechanical processes (such as centrifugation, or sonication). At this time it is not possible to conclude which techniques are most appropriate, but in the future such techniques should try to mimic realistic exposure scenarios and avoid interference by dispersants.

6.3.5.7 Interference of CNT with toxicity assays

CNT have been found to interfere with some of the assays utilised to determine their cellular or toxic effects. For example, some nanoparticles may contribute to the absorbance or fluorescence of colorimetric or fluorometric assays. In addition, due to their large surface area, nanoparticles may bind to assay components including the substrates (such as the MTT reagent; Belyanskaya *et al.* 2007) or the biomarker being measured, (such as LDH and cytokine proteins, see for example Davoren *et al.* 2007). Therefore all of these factors can contribute to the production of inaccurate, misleading results that make nanoparticles appear more or less toxic than they actually are. In many of the studies reported it is not possible to ascertain whether the assays were adequately controlled to assess for interference. It is therefore advisable to use more than one assay to assess the endpoint or effect in question.

Pulskamp *et al.* (2007) utilised a number of viability tests to evaluate the cytotoxic potential of SWCNT and MWCNT to lung cells. However, the results from the different assays were not in agreement, and this was thought to be a consequence of the interference of CNT with the assays used to evaluate their cytotoxicity. However, since different cytotoxicity assays often measure different endpoints (such as decreased metabolic activity or increased membrane permeability), it is possible that the differences observed were real rather than artefacts. Belyanskaya *et al.* (2007) demonstrated that MTT formazan crystals were able to adsorb onto the CNT surface and thereby prevent MTT solubilisation. This effect was greater for sodium dodecyl sulfate (SDS) dispersed CNT ($30 \mu\text{g ml}^{-1}$ onwards) than PS80 dispersed CNT. Thus the CNT dispersion solution is able to influence the interference of CNT within the MTT assay. Casey *et al.* (2007a) assessed the ability of SWCNT to interfere with five standard viability assays, and found a lack of agreement between the different assays, all of which assess cytotoxicity *in vitro*. Cytotoxicity assays are useful for ascertaining sub-lethal concentrations that can be investigated in more detail in relation to mechanistic studies, but are not really useful for risk assessment purposes.

It is acknowledged that CNT are able to interfere with assays utilised to determine their toxicity; whether it be their ability to contribute to the absorbance of colorimetric assays, or their ability to bind to assay components (including the endpoint being measured, such as LDH and cytokines, or to the substances used to detect cytotoxicity, such as the MTT reagent). Accordingly all these factors can contribute to the production of inaccurate, misleading results that make CNT appear more or less toxic than they actually are, thus encouraging and justifying the use of appropriate controls, and more than one assay for the endpoint of interest.

6.3.6 Summary

Revealing the toxic potential of CNT has been motivated by the number of applications proposed to exploit CNT, and the benefits they could provide. However CNT exploitation may be limited by the concern regarding their toxicity, and uncertainty regarding the consequences surrounding human exposure. There has been a focus on the properties of CNT that might account for toxicity, with SWCNT often being shown to be more toxic than MWCNT, although this is difficult to confirm due to other parameters that differ between CNT samples such as length. In fact longer length ($>20\mu\text{m}$) has been demonstrated to result in greater pathogenicity in some *in vivo* models and frustrated phagocytises *in vitro*. Increased functionalisation of the surface chemistry and reduced metallic impurities have both been associated with a relative decrease in toxicity, while the consequences of aggregation/agglomeration are highly dependent upon the model used. While physico-chemical characterisation of CNT samples is clearly important for such studies, evaluating the attributes of CNT that are responsible for driving CNT toxicity is also complicated by the experimental design. While some studies indicate an ability of CNT to elicit oxidative stress and inflammation, which ultimately culminate in cytotoxicity *in vitro* or disease *in vivo*, much work is required to establish whether this is applicable for all routes of exposure and target organs.

There are a number of gaps within the literature that require addressing in future experiments. Firstly, much of the research conducted to date relates to the toxicity of CNT to the skin and lung. In contrast there is a paucity of data regarding the liver, kidneys and other organs which require consideration due to studies that have highlighted accumulation of CNT in various target sites within the body, following intravenous exposure. However, it is necessary to highlight that information regarding the systemic availability of CNT, following the exposure of the lungs, skin and gastrointestinal tract is severely lacking, and necessary to direct appropriate investigations, regarding the relevant targets of CNT toxicity. For pulmonary studies, intratracheal instillation and aspiration of CNT have been the primary mode of delivery, with limited inhalation studies due to cost and technical difficulty. Inhalation studies are required due to the questionable relevance of the instillation and aspiration models.

It is also relevant to highlight that a number of assays have been utilised to assess the toxicity of CNT. However, it is known that CNT can interfere with the assays that assess their toxicity, which necessitates that appropriate controls are conducted and that different assays measuring the same endpoint are used in order to confirm any findings of toxicity. It is also necessary to consider the quality (including the concentrations and experimental models used), of conducted

experiments, due to their vital importance to relevantly determining the risk associated with CNT exposure.

Finally, taking all of this information into account, the disparate data provided to date suggests that CNT can represent a hazard, but that hazard potential can also be manipulated, and potentially controlled by varying a number of physico-chemical characteristics. Such information, when provided in more detail, will allow for a more effective risk assessment as well as improved management of risk for workers, consumers and the environment.

6.4 METALS

6.4.1 Introduction

With regards to metal nanoparticles, by far the greatest number of studies have concentrated on evaluating the toxicity of silver particles, despite the existence, and exploitation of a variety of different metal particulates, including gold. This stems from the knowledge that silver nanoparticles are currently exploited within a number of diverse products (including clothing, and wound dressings), which primarily arises due to their antimicrobial properties. Accordingly, toxicity is therefore exerted by silver nanoparticles to microorganisms, and so it is necessary to consider the specificity of the response, and thereby determine if toxicity is mediated by silver nanoparticle within other cell types.

Within the Woodrow Wilson database of nanotechnology-based products, the most common nanomaterial encountered within product descriptions, by far, is silver (http://www.nanotechproject.org/inventories/consumer/analysis_draft/ accessed 16th October 2009, Sung *et al.* 2009). At present, there is a limited understanding into the potential detrimental outcomes of human exposure to silver nanoparticles. However, the increase in silver nanoparticle exploitation within diverse consumer products, increases the potential for human exposure in a number of settings.

As yet, there is not a great deal of available studies that purport to study the toxicity of silver nanoparticles, but the number of published studies is anticipated to tremendously increase in the near future, due to the benefits associated with the inclusion of silver nanoparticles within a number of products. What is uncertain at this time is whether silver nanoparticle toxicity emanates from their small size, or whether their release of silver ions is responsible for any observed toxicity, or a combination of both (Lubick *et al.* 2008).

Gold nanoparticles have also been used to evaluate the cellular uptake and tissue distribution of particles, on a number of occasions, due to their ease of detection, and this will be discussed in order to assess the tissue distribution of metal nanoparticles following exposure.

The toxicology of silver (ions) has recently been reviewed by (Luoma, 2008). The review focuses on environmental impacts and indicates that in the aquatic environment silver is of significant toxicity, but that in humans, silver ions are often considered not to be toxic, except at very high concentrations.

6.4.2 *In vivo* toxicity of metal nanoparticles

Few studies have been published at the time of this review which investigate the human toxicology of silver nanoparticles, although many studies are currently underway, and are likely to be published in the near future.

6.4.2.1 Pulmonary Exposure to metal nanoparticles

Takenaka *et al.* (2001) exposed rats via inhalation for 6 hours to silver nanoparticles (4-10 nm) at a concentration of 3×10^6 particles cm^{-3} (equivalent to $133 \mu\text{g m}^{-3}$), and investigations made 0,1,4 and 7 days post exposure. The particles were generated via spark discharge of silver electrodes within an argon atmosphere immediately prior to animal exposure. The particles generated were compact, spherical and electron dense. They demonstrated silver within the

lungs immediately post exposure (1.7 μg), which decreased with time down to 4% of the original lung burden within 7 days. Deposition in the lung was also associated with nasal cavity deposition and accumulation in the lung-associated lymph nodes. Silver was also detected in the blood at day zero (8.9 ng g^{-1}) suggesting translocation from the lung, and this decreased with time, suggesting clearance either via uptake into other organs or via excretion. It is not clear whether the fraction of translocated silver was in a particulate or soluble form. The authors suggest that there were a lack of particle laden macrophages within the lung tissue, however it was not clear whether they used simple light microscopy of histological stained sections or transmission electron microscopy (TEM) to make this assessment. If the study was conducted using light microscopy it would be impossible to see single particles or small aggregates at the magnification and resolution employed. Using TEM, it would still be difficult to identify single particles. Another explanation is that there was little particulate silver present within the lung tissues due to dissolution, but this seems unlikely since the nanoparticles did not dissolve in the saline solution used for the instillation studies (see below). Low concentrations of silver were also measured in other organs such as the liver, kidney, spleen, brain and heart, suggesting distribution of either the particles or the silver ions.

Takenaka *et al.* (2001) also exposed rats to suspensions of the same spark generated silver particles (collected onto a polytetrafluoroethylene filter) via an instillation (50 μg dose), with observations made at 1, 4 and 7 days post exposure. In this suspension, particles agglomerated to greater than 100 nm. As a control, they also exposed rats, via the same route, to an aqueous solution of silver nitrate (7 μg total dose, equivalent to 4.4 μg silver). The water soluble form of the silver was rapidly cleared from the lung, while the particulate form was retained within alveolar macrophages and the alveolar walls for at least 7 days. The authors concluded, that although instilled silver nanoparticle agglomerates were retained in the lung, inhaled silver nanoparticles were rapidly cleared, as was the silver nitrate instillation. No estimates of translocation into blood or other organs were conducted in the instillation study.

Hyun *et al.* (2008) investigated the consequences of repeated exposure to silver nanoparticles (13-15 nm) on the nasal respiratory mucosa of rats. Exposures were conducted via an inhalation chamber for 6 hours per day, 5 times a week for 28 days, at three different doses; low-dose (1.73 $\times 10^4$ particles cm^{-3} , 1.32 $\times 10^7$ $\text{nm}^2 \text{cm}^{-3}$, 0.5 $\mu\text{g m}^{-3}$), middle-dose (1.27 $\times 10^5$ particles cm^{-3} , 9.68 $\times 10^7$ $\text{nm}^2 \text{cm}^{-3}$, 3.5 $\mu\text{g m}^{-3}$), and high-dose (1.32 $\times 10^6$ particles cm^{-3} , 1.41 $\times 10^9$ $\text{nm}^2 \text{cm}^{-3}$, 61 $\mu\text{g m}^{-3}$). The nasal cavity and lungs from the exposed groups were comparable to the control group, although the size and number of goblet cells containing neutral mucins increased significantly in the animals exposed to the middle and high doses of silver nanoparticles. In addition, they identified a slight increase in mucins including sulfomucins, but not sialomucins. The authors concluded that while the silver nanoparticles did influence the neutral mucins in the respiratory mucosa, the results did not suggest any toxicological significance in this model.

Sung *et al.* (2009) conducted a 90 day whole body inhalation study (6 hours day for 5 days a week) to silver nanoparticles (18-19 nm), at low (49 $\mu\text{g m}^{-3}$, equivalent to 0.6 $\times 10^6$ particles cm^{-3}), medium (133 $\mu\text{g m}^{-3}$, equivalent to 1.4 $\times 10^6$ particles cm^{-3}) and high (515 $\mu\text{g m}^{-3}$, equivalent to 3.0 $\times 10^6$ particles cm^{-3}) doses. Prolonged exposure to silver nanoparticles was demonstrated to elicit an inflammatory response within the lung, and induced alterations in lung function, at all particle concentrations. The silver concentration was observed to increase in the blood, indicating that silver nanoparticles were transferred into blood from the lung, after which they subsequently became distributed within the liver, olfactory bulb, brain and kidneys. However, the silver content within these tissues may derive from nanoparticle or ion presence, which warrants further investigation. The main targets of particle accumulation and toxicity were observed to be the lungs and liver. These findings are in contrast to those of Ji *et al.* (2007), who exposed rats to silver nanoparticles (<16 nm), at low (0.48 $\mu\text{g m}^{-3}$, 1.73 $\times 10^4$ particles cm^{-3}), medium (3.48 $\mu\text{g m}^{-3}$, 1.27 $\times 10^5$ particles cm^{-3}) or high (61 $\mu\text{g m}^{-3}$, 1.32 $\times 10^6$ particles cm^{-3}) concentrations for 6 hours per day, 5 days per week, for a duration of 28 days. No hematology or biochemical indicator alterations were observed. Some toxicity was observed within the liver, but histopathological analysis did not reveal any distinct toxicity within other organs. The distribution of silver was also considered and it was found that silver was predominantly accumulated within the liver, but there was also accumulation within the brain, olfactory bulb and spleen. However, again whether this derives from particle or ion presence requires further

investigation. Overall, the inhalation of silver nanoparticles was associated with minimal toxicity within rats. Therefore, the exposure duration may be integral to the response evoked by silver nanoparticles. However, as the concentration of particles administered varied within the two investigations (they were considerably lower in the Ji *et al.* (2007) study), then this may account for the different results obtained.

There is currently limited information available regarding the pulmonary toxicity of silver nanoparticles. Inflammatory responses have been commonly associated with exposure of the lungs to nanoparticles, and so this requires investigation for silver nanoparticles. In general, the pulmonary toxicity of silver nanoparticles was observed to be driven by the exposure time, and particle size and concentration used. The studies outlined also highlight that particles may translocate from their site of exposure, and accumulate within a number of secondary targets, including the liver, spleen and brain, following pulmonary exposure, which necessitates their transfer into blood. However, at this time it is not possible to confirm that silver presence equates to particle presence or whether it derives from particle dissolution, and so the potential for ion release from particles may account for the silver content of tissues.

6.4.2.2 Dermal exposure to metal nanoparticles

Silver nanoparticle exploitation within textiles and wound dressings enables particles to come in direct contact with skin, whose structure and/or function may be compromised prior to exposure. The consequences of silver nanoparticle exposure therefore need to be assessed, specifically their ability to penetrate the skin, and any toxicity associated with exposure revealed.

Tian *et al.* (2007) used a thermal injury mouse model to determine the wound healing capabilities of a nanoparticulate silver containing (0.48 mg) wound dressing (4 x 3 cm²). The healing of burn wounds occurred more quickly when topically treated with silver nanoparticles (<100 nm), when compared to silver sulfadiazine (a standard burn treatment). The appearance of wounds was improved with silver nanoparticles, with minimal scarring apparent, and normal hair growth restored to improve the cosmetic appearance of the wound. The wound healing capabilities of silver nanoparticles were also proven to be better than the antibiotics amoxicillin and metronidazole, implying that other factors than its antibacterial activity drove their ability to improve wound healing. This is thought to derive from their ability to modulate cytokine production (particularly interleukin (IL)-6, IL-10, interferon gamma (IFN γ) and transforming growth factor beta (TGF β)), and thereby diminish the inflammatory response following burn injury, which was confirmed due to decreased neutrophil infiltration within the wound. It was demonstrated that silver nanoparticles were able to accelerate wound healing, and reduce scar appearance, which derives from their antibacterial activity and ability to reduce inflammation through cytokine modulation.

Vlachou *et al.* (2007) determined the systemic availability of silver nanoparticulates contained within Acticoat burn wound dressings following exposure. In this clinical study, 30 burn patients were treated. Acticoat dressings were changed within burn participants every 3 days, or until the wound had healed. The maximal silver serum level observed was 56.8 $\mu\text{g ml}^{-1}$, which was achieved 9 days following the initiation of treatment. As exposure to Acticoat increased, silver serum levels also increased, and this was probably related to burn size, as in general, larger burns would require a longer treatment time. Serum levels returned to normal 6 months following the cessation of treatment. No biochemical or hematological indicators of toxicity were related to silver absorption, and so the authors considered the dressing safe for use on burns, but further investigations would be required to confirm this. This study highlighted the potential for the systemic transfer of silver particles, following their administration to burns, within wound dressings, however, as also discussed in relation to uptake following pulmonary exposure this may derive from the release of silver ions from particulates.

In line with these findings, a case study conducted by Trop *et al.* (2006) investigated the absorption of nanoparticulate silver (15 nm) from a burn wound dressing. A burn patient received an Acticoat wound dressing, which was changed at day 4 and 6 post injury. The wounds healed rapidly with treatment. However, grey discoloration of the skin was observed (termed argyria), as was a change in lip colour (to pale blue), which is a common problem experienced in the use of silver containing products. Skin discoloration is suggested to result

from silver deposition within the skin (Chang et al 2006). It is known that areas exposed to sunlight (such as the hands and face) experience a more pronounced discolouration, illustrating that the condition has a photosensitive component (Wadhera and Fung (2005). The origin of this phenomenon is uncertain but is thought to derive from the stimulation of melanocytes by silver (which is enhanced by sunlight) (Chang et al. 2006). Alternatively, exposure of silver ions, contained within the skin, to UV light is anticipated to promote their photoreduction to metallic silver, which is responsible for the skin colour change observed, and similar to the reaction which occurs during the development of photographic paper (Chang et al. 2006, Luoma 2008).

The process of silver deposition involves the formation of a protein complex with sulphhydryl containing proteins, including metal specific binding proteins. Such deposits have been observed in the vicinity of peripheral nerves and the blood brain barrier, but this deposition does not appear to be associated with significant toxicity (Lansdown 2007, in Luoma 2008).

Within the study conducted by Trop et al. (2006) elevated liver enzyme levels were also evident, insinuating that liver injury had occurred, as a consequence of treatment. It was revealed, that serum and urine silver levels were elevated at 7 days post injury. As a consequence the choice of wound dressing was changed, and the discolouration of the face faded with time, and liver function tests returned to normal. It is also relevant that serum silver levels remained elevated at 7 weeks post injury, despite the alteration in treatment. This case study provided evidence that silver is able to become systemically available following dermal contact, and is able to stimulate liver toxicity and skin discolouration.

While the application of silver nanoparticles within wound dressings has obvious advantages, Hollinger (1996) (in Luoma 2008) reported that silver delayed wound healing. Hidalgo and Dominguez, (1998) (in Luoma 2008) have also reported that silver nitrate is toxic to fibroblasts and endothelial cells, both of which are required for wound healing.

Due to the fact that a number of silver nanoparticle containing products are directed at the skin, evaluating the consequences of dermal exposure is paramount. Currently, investigations have focused on the efficiency of silver particulate containing wound dressings. Following dermal exposure, it is possible that silver nanoparticles become systemically available, however this may be reliant on the skin being damaged, as has been the case in the discussed studies, so that the penetration of silver nanoparticles within 'normal' skin requires assessment within future experiments, although this has been encountered within an in vitro study (Larese et al. 2009). Further research is necessary to determine the hazards associated with dermal to silver nanoparticles, as the liver has been recognised by investigators as being a target for toxicity. The wound healing capabilities of silver nanoparticles have been demonstrated, and found to be better than traditional treatments, which further promotes their exploitation within products. However, the detrimental health outcomes associated with treatment require more assessment. It is likely that the fact that the skin is damaged on exposure to silver nanoparticles allows the particles to access the circulation and thereby exert toxicity within distal sites, due to the fact that the structure and function of the stratum corneum is compromised.

6.4.2.3 Intraperitoneal injection of metal nanoparticles

Rahman et al. (2009) investigated the effects of silver nanoparticles (25 nm) on gene expression in different regions of the mouse brain, following intraperitoneal exposure. The particles were administered to adult male mice via an intraperitoneal injection at a dose of 100, 500 or 1000 mg kg⁻¹ for 24 hours. Array data indicated changes in the expression of genes in the caudate nucleus, frontal cortex and hippocampus of mice when treated with the silver nanoparticles. Analysis of these changes lead the authors to suggest that silver nanoparticles may produce neurotoxicity by generating oxidative stress and altered gene expression, leading to apoptosis. There was no data measuring silver content of the brain tissue in this study, it is therefore not possible to ascertain whether the neural effects were associated with silver nanoparticle or ion translocation to the brain, or whether the consequences were generated via an indirect mechanism (including humoral or neural mediators).

Only one study was identified that administered silver nanoparticles via intraperitoneal injection, and focused on revealing any alterations in gene expression within the brain. The doses

administered within this study were excessively high, and therefore likely to account for the neurotoxic observations made, and therefore their relevance to humans requires consideration.

6.4.2.4 Oral Exposure to metal nanoparticles

The oral ingestion of silver particulate suspensions has been adopted as an alternative therapy for the treatment of a variety of conditions, including arthritis and cancer, which has primarily derived from Internet based claims and promotion (Wadhera and Fung, 2005). However, a number of detrimental consequences are associated with its ingestion, including intestinal ulcers and argyria (Wadhera and Fung, 2005). Luoma (2008) suggests that following ingestion, silver (not specifically nanoparticles) is likely to be converted to its ionic form due to the low pH of the stomach. Due to the large surface area of nanoparticles, this might also be true for nanoparticulate silver, but this requires investigation.

Cha *et al.* (2008) directly delivered nanoparticle (15 nm) and microparticle (2 - 3.5 μm) silver, at a dose of 2.5 mg, to the stomach, as a model of ingestion, within mice. Histopathological analysis of the liver tissue, 3 days post-exposure, demonstrated evidence of inflammation in the form of lymphocyte influx. This was further supported by changes in the gene expression of 4 genes involved in inflammation.

Kim *et al.* (2009) investigated the toxicity and tissue distribution of silver nanoparticles (60 nm), following the repeated oral exposure of rats, at low (30 $\text{mg kg}^{-1} \text{day}^{-1}$), medium (300 $\text{mg kg}^{-1} \text{day}^{-1}$) or high (1000 $\text{mg kg}^{-1} \text{day}^{-1}$) concentrations, for a total of 28 days. The doses used within this study were exceptionally high, and so it is expected that the majority of administered particles are excreted within the faeces. However, the silver content of a number of organs increased, which may be indicative of the transfer of particles into blood. Specifically, silver deposition within the brain, liver, kidneys, lungs and testes was observed. In addition, toxicity was evident within the liver, insinuating that this organ is a target site for nanoparticle toxicity.

Wadhera and Fung, (2005) determined the implications of the ingestion of colloidal silver (a nanoparticulate silver suspension in an aqueous solution (Luoma, 2008)) within an arthritic man (who was introduced to the product via the Internet). Skin discolouration (blue-grey) was observed (and more pronounced in sun exposed areas), but an improvement within the patient's arthritic symptoms was evident. Histological analysis of skin biopsies revealed that silver deposits were evident. It was therefore demonstrated that colloidal silver ingestion was able to introduce silver into the circulation, via intestinal uptake. This case study highlights the ease of availability of silver nanoparticle containing products as alternative health products on the Internet, and as a consequence perhaps more stringent regulation of its utilisation should be considered due to the health implications associated with exposure.

In line with these findings, the ingestion of colloidal silver, as an alternative therapy for diabetes has been demonstrated to cause skin discolouration (most pronounced within sun exposed areas), within a patient (Chang *et al.* 2006). Again, the results highlight the recurrence of argyria, on exposure to silver, which is likely to derive from the ability of silver nanoparticles to be absorbed by the GIT, and become distributed to the skin.

Exposure via ingestion is relevant for silver nanoparticles due to their use in the form of health remedies. In addition Luoma (2008) suggests that absorption from the gut could include uptake of silver via micelles. The review does not specify particulate or ionic silver, but this uptake mechanism could be very relevant to particulate silver rather than ionic silver. Ag^+ , has been shown, in fish, to enter cells via cationic channels/transporters used physiologically for substances such as Na^+ (Bury and Wood 1999, in Luoma 2008). It is therefore possible (but not proven) that Ag^+ could enter the body following ingestion via such pathways. The relative efficiency of silver ion, versus silver nanoparticle uptake from the gastrointestinal tract has not been studied. The relative efficiency of silver nanoparticle versus larger particle uptake from the gastrointestinal tract has not been studied either.

Following oral or pulmonary (as the mucociliary escalator allows for the swallowing of nanoparticles) exposure, it is likely that silver nanoparticles translocate from the gut into the bloodstream, and thereby become systemically available, which is responsible for symptoms

such as argyria, as well as potentially detrimental consequences such as liver damage. Absorption of silver from the GIT is therefore a realistic prospect, but again, as previously stated, it is necessary to distinguish if the systemic distribution of silver derives from the presence of silver particles or ions.

6.4.2.5 ADME profile of metal nanoparticles

Likely routes of human exposure to silver nanoparticles include ingestion, inhalation, dermal penetration and urinogenital tract exposure. It is necessary to consider whether particles are able to pass from their exposure site into the blood, and thus become distributed within the body. The biokinetics of gold nanoparticles has been a focus of a number of investigations. However, from previously mentioned studies it is apparent that silver, potentially as nanoparticles, have been observed to distribute from their site of exposure following pulmonary (Sung *et al.* 2009, Ji *et al.* 2007, Takenaka *et al.* 2001), dermal (Trop *et al.* 2006, Vlachou *et al.* 2007) or oral (Cha *et al.* 2008, Kim *et al.* 2009) exposure, with preferential localisation within the liver.

Semmler-Behnke *et al.* (2008) investigated the tissue distribution of radiolabelled gold nanoparticles (1.4 nm and 18 nm) within rats following intratracheal instillation, in order to assess the translocation of nanoparticles from the respiratory tract to the blood. At 24 hours following administration, the majority of 18 nm nanoparticles remained within the lung (99.8%). In contrast, 1.4 nm nanoparticles were observed to translocate from the deposition site, with 91.5% remaining within the lung, and 8.5% found within secondary targets, including the blood and liver. Therefore, it was evident that there was a size dependent passage of nanoparticles through the air/blood barrier of the lungs, with their subsequent distribution in the body apparent. However, it is also relevant that following intravenous injection (see later), the distribution pattern of nanoparticles was different to that of translocated particles (from the lung), implying that the tissue distribution of nanoparticles appears to be dependent on the exposure route. The authors also anticipated that following intratracheal instillation, a proportion of the particles would be removed from the lungs in the mucociliary escalator, swallowed into the GIT and then rapidly excreted from the body within the faeces. This was therefore incorporated into the study, and to determine if nanoparticle resorption from the GIT occurred, a selection of animals were orally administered nanoparticles, and it was found that there was minimal transport of nanoparticles into blood.

Savanone *et al.* (2008a) investigated the permeation of gold nanoparticles (15, 102 and 198 nm) through isolated rat skin, and intestine. It was observed that the permeation of particles was time and size dependent, so that smaller particles had a higher efficiency for penetration, which increased with time. Within skin, 15 nm particles were able to penetrate to deeper layers, whereas the larger particles were restricted to the dermis and epidermis. The permeation of gold particles within the intestine was observed to be higher than within the skin. The results suggested that gold particles are able to permeate through the skin and intestine, and so the possibility of their transfer into the blood requires confirmation within *in vivo* studies.

Elimination of metal nanoparticles within the urine and faeces has been demonstrated (Semmler-Behnke *et al.* 2008), but requires further investigation to confirm their excretion from the body.

It is apparent that metal nanoparticles are able to pass through the gastrointestinal tract, dermal and lung barriers into the blood, and thereby become distributed throughout the body. The liver appears to be the primary site of accumulation, which is likely to derive from the uptake of particles by resident liver macrophage populations. The size of particles, and the exposure route has the ability to impact on their tissue distribution, with smaller particles promoting such an observation. Elimination of particles within the faeces and urine has been encountered. The same pattern is observed following silver nanoparticles exposure, although it is still unclear whether the silver uptake, distribution and accumulation is silver nanoparticles and/or Ag⁺ ions. More extensive investigations, in the future, are required to more fully understand the tissue distribution and fate of metal particles, following exposure.

6.4.2.6 Distribution following Intravenous administration

Evaluating the tissue distribution of metal nanoparticles following injection is of relevance, as it has the ability to highlight organs that are at risk from nanoparticle toxicity. In addition, as nanoparticles appear to gain access to the circulation following exposure via the lungs, skin and GIT, a similar distribution pattern is expected.

Semmler-Behnke *et al.* (2008) observed that 24 hours following the intravenous administration of 18 nm gold particles, they were completely removed from the blood, and were preferentially located within the liver and spleen. In addition, a small fraction of the administered dose (0.5%) was eliminated via hepatobiliary clearance into the faeces, and <0.1% eliminated within urine. However, 1.4 nm nanoparticles were excreted within urine (8.6%) and faeces (5%), with a lower level of accumulation of particles within the spleen and liver, when compared to their larger counterparts, and a proportion of particles (3.7%) remained within the blood at 24 hours. Consequently, it is apparent that particle size influences the behavior of particles within the body.

Sonavane *et al.* (2008b) evaluated the tissue distribution of gold nanoparticles (15, 50, 100 and 200 nm) within mice, 24 hours following intravenous exposure (at a dose of 1g kg^{-1}). For all particle sizes, the highest accumulation of particles was observed within the liver, with a smaller fraction localised within the lung, kidney, and spleen. However, 15 nm and 50 nm nanoparticles were distributed to a greater extent, and were even observed within the brain. The size of nanoparticles also impacted on the extent of particle distribution, so that there was a higher amount of smaller particles present within organs, when compared to their larger counterparts, and so the distribution of 15 nm nanoparticles was more widespread. The translocation of 200 nm particles was observed to be minute. The dose administered within this study was high, and it is therefore necessary to evaluate any toxicity associated with this, and to evaluate its relevancy to humans.

Similarly De Jong *et al.* (2008) investigated the distribution of gold particles (10, 50, 100 and 250 nm) within rats, 24 hours following intravenous injection. As observed previously, the preferential site of particle (of all sizes) accumulation was the liver and spleen. The 10 nm particles were also observed to be wider distributed within a variety of organs, with the distribution of larger particles restricted to the liver and spleen. Particle size is therefore able to influence the distribution of particles following intravenous exposure.

Cho *et al.* (2009) administered polyethylene glycol (PEG) coated gold nanoparticles (13 nm) via intravenous injection to mice (0.17 to 4.26 mg kg^{-1}), and observations were made from 5 minutes to 7 days post injection. Nanoparticles distributed primarily to the liver, and spleen. Specifically, nanoparticles were contained within resident macrophage populations, which accounted for the accumulation of particles within these organs, and were anticipated to stimulate an inflammatory response. Inflammation within the liver was indicated by a neutrophil influx, increased cell adhesion molecule and inflammatory cytokine expression. An increased level of apoptosis was also witnessed within the liver.

Following intravenous injection, the primary sites of metal nanoparticle accumulation are the liver and spleen. The localisation of particles within the liver and spleen can most likely be accounted for by their uptake by resident macrophage populations, with the subsequent initiation of an inflammatory response of relevance. Larger particles were more restricted in their distribution so that perhaps they are more easily recognised by macrophages, and therefore less able to distribute within the body. It is relevant that following exposure via other routes, similar distribution patterns would be expected and would require their transfer into blood; however this is debatable (semmler-bundke 2008). The applicability of the findings to other particles types, including silver therefore needs to be addressed within future studies, however previously discussed studies have recognized that the liver is a target organ for silver (nanoparticles and/or ions) (Takenaka et al. 2001, Sung et al. 2009, Ji et al. 2007).

6.4.3 In vitro studies

A number of *in vitro* studies using silver nanoparticles have been identified, which have principally focussed on the penetration of nanoparticles within the skin and intestine, the pulmonary toxicity of nanoparticles, and toxicity to the liver.

6.4.3.1 Lung Models

AshaRani *et al.* (2009) studied the toxicity of starch coated silver nanoparticles to normal human lung fibroblast cells (IMR-90) and human glioblastoma cells (U251) *in vitro* (0-400 $\mu\text{g ml}^{-1}$ for up to 72 hours). The starch coated particles were found to cause increased intracellular ROS, and mitochondrial damage associated with a depletion of ATP. EC50 values are not provided in the text, but the figures indicate that for ATP content, such values are of the order of 200 $\mu\text{g ml}^{-1}$ at 48 and 72 hours. For the metabolic activity assay (MTT), the EC50 value is at least 400 $\mu\text{g ml}^{-1}$ at 72 hours and greater than 400 $\mu\text{g ml}^{-1}$ at 48 hours. TEM imaging was used to identify the presence of particles in the cytoplasm, endosomes, mitochondria and the nucleus. The composition of these particles was not analysed. DNA damage was identified by single cell gel electrophoresis (effective at 20-50 $\mu\text{g ml}^{-1}$, incubation time not stated) and the micronucleus assay (100 $\mu\text{g ml}^{-1}$, time not stated), to be dose dependent. The silver nanoparticle treatment lead to arrest of the cell cycle in the G2/M phase, which the authors suggest could be due to repair of damaged DNA. No indication of apoptosis or necrosis was identified. The authors suggested that the mechanism of toxicity involves disruption of the mitochondrial respiratory chain leading to production of ROS and interruption of ATP synthesis, which in turn cause DNA damage.

Soto *et al.* (2007) investigated the cytotoxicity of a panel of aggregated particles, including silver nanoparticles (3-100 nm) to lung macrophage and epithelial cell lines, at a concentration of 5 $\mu\text{g ml}^{-1}$ for 48 hours. In addition, the authors noted that silver nanoparticles were particularly cytotoxic to both cell types. Lung epithelial cells were more susceptible to particles than macrophages, implying that cells vary in their sensitivity to particle toxicity.

Only two studies were identified that investigated the pulmonary toxicity of metal nanoparticles in vitro, as a result it is not possible to make definite conclusions regarding their toxicity. However, the findings suggest that silver nanoparticles exhibit genotoxic, oxidative and cytotoxic behaviour, which requires further consideration in future studies.

6.4.3.2 Dermal Models

Arora *et al.* (2008) exposed both HT-1080 (human fibrosarcoma) and A431 (human skin/carcinoma) cells to silver nanoparticles (7-20 nm) at concentrations of up to 6.25 $\mu\text{g ml}^{-1}$ for 24 hours. The metabolic competence of the cells was measured using the XTT assay. IC50 values of 10.6 $\mu\text{g ml}^{-1}$ for the HT-1080 cells and 11.6 $\mu\text{g ml}^{-1}$ for the A431 cells were measured. The silver nanoparticles were found to induce oxidative stress at a concentration of approximately half the IC50 value of the nanoparticles (6.25 $\mu\text{g ml}^{-1}$), as indicated by depletion of glutathione (GSH) and increased lipid peroxidation in both cell types. They also assessed the concentrations required to illicit apoptosis (0.78 $\mu\text{g ml}^{-1}$ for the HT-1080 cells, and 1.56 $\mu\text{g ml}^{-1}$ for the A431 cells), and these were found to be much lower than the concentrations required for necrosis (12.5 $\mu\text{g ml}^{-1}$ in both cell types), which is to be expected. Again no attempt was made to attribute the effects to the particles or to soluble ions.

To determine the safety of silver nanoparticles following topical application, Larese *et al.* (2009) determined the penetration of polyvinylpyrrolidone coated silver nanoparticles (25 nm) within the intact and damaged (abraded) human skin, *in vitro*. Silver nanoparticles (70 $\mu\text{g cm}^{-2}$, for 24 hours) were able to pass completely through the skin preparation, 5 times greater within damaged skin. The penetration of silver nanoparticles within intact skin was low but detectable, and was greater within damaged skin, however the nanoparticle absorption within skin requires *in vivo* confirmation.

From the limited number of conducted studies it would appear that silver nanoparticles exhibit oxidative behaviour that has cytotoxic consequences for skin cells. However, it is relevant that in order to exert these effects *in vivo*, the nanoparticles must pass the stratum corneum to access the different skin cell populations. It is apparent that the integrity of skin is important to the penetration of nanoparticles, so that damaged skin promotes nanoparticle penetration, and therefore potentially nanoparticle toxicity, which requires further investigation.

6.4.3.3 Liver Models

A number of studies have evaluated the toxicity of metal nanoparticles to the liver, which complements evidence that this organ is a primary site of metal accumulation, following nanoparticle exposure, however at this time it is uncertain whether this represent silver nanoparticles or ions.

The earliest *in vitro* study identified was relatively recent, and was conducted by Hussain *et al.* (2005), who studied a range of metal/metal oxide in terms of their toxicity to liver cells *in vitro*. Included within the particle panel were silver nanoparticles (15 nm or 100 nm), which were exposed to BRL 3A liver epithelial cells, at concentrations ranging from 2.5 to 50 $\mu\text{g ml}^{-1}$, for 6 to 24 hours. It was identified that silver nanoparticles were one of the most toxic particles within the panel. Accordingly, silver nanoparticles induced a significant increase in cellular reactive oxygen species, and decrease in GSH was observed. However, it is relevant that silver nanoparticles are able to bind to GSH, or enzymes involved in its synthesis, and so this may account for its depletion within this experiment. However, a silver nanoparticle mediated increase in reactive oxygen species (ROS) was also observed which implies that the silver nanoparticles were able to induce oxidative stress. In addition, a dose dependent decrease in mitochondrial membrane potential, and cell viability were also associated with particle exposure. The oxidative response associated with silver nanoparticle exposure was anticipated to drive the observed toxicity. It was not clear whether the toxicity was derived from Ag^+ or nanoparticles.

More recently, Cha *et al.* (2008) investigated the effects of silver nanoparticle (15 nm) and micro particle (2 - 3.5 μm) on the human Huh-7 hepatoma cell line. This is one of the few studies to use both nano and micro forms of the silver particles *in vitro*. These are the same particles as used in the *in vivo* study by Cha *et al.* (2008) described above. The authors reported no significant effect on mitochondrial function or GSH content at any of the concentrations investigated (up to 2.4 $\mu\text{g ml}^{-1}$), when exposed for 72 hours. These concentrations are relatively low for those used in most *in vitro* studies. Given the propensity for particles to accumulate in the liver, this may actually be rather low to be relevant. In addition, measuring GSH at one time point is probably not appropriate as the depletion of this antioxidant is time dependent, and at later time points can be confounded by a recovery of the GSH levels to equal or greater than resting levels due to the induction of the production enzymes. The time point of 72 hours is relatively long and would probably therefore miss the initial depletion (likely to occur within 12 hours) and even the rebound recovery (likely to occur at 24-48 hours). Cha *et al.* (2008) also reported a decrease in the DNA content of the cells which they concluded to be indicative of apoptosis, and they suggested that the nanoparticle form was more potent than the micro particle form of Ag. DNA content of cells, however, is not an accurate analysis of apoptosis, as there are better assays available that are more sensitive and more specific. However, the *in vivo* results were in agreement with the *in vitro* interpretation, in that they identified changes in gene expression associated with apoptosis.

A recent study, conducted by Arora *et al.* (2009) investigated the effects of silver nanoparticles (7-20 nm) on primary mouse fibroblasts and liver cells. Exposures were conducted for 24 hours and resulted in IC50 values of 61 $\mu\text{g ml}^{-1}$ and 449 $\mu\text{g ml}^{-1}$ for the fibroblasts and liver cells respectively. Dark, electron dense aggregates were observed by TEM in the mitochondria and cytoplasm of the cells exposed to a dose of half the IC50. The composition of these dark aggregates was not analysed, but the authors concluded that they were likely to be the silver nanoparticles. The same dose of silver nanoparticles appeared to result in a protective effect on, or response by, the primary fibroblasts, characterised by an increase in GSH and a decrease in lipid peroxidation. The hepatocytes also demonstrated an increase in the antioxidant enzyme superoxide dismutase (SOD) and GSH, again indicative of a protective

response. Low levels of oxidative stress are considered to induce the expression of protective mechanisms, as suggested here, while larger doses of oxidative stress result in activation of pro-inflammatory mechanisms, or cell death at extreme levels. Higher concentrations of the silver nanoparticles were not investigated. Actually, relatively low concentrations of the silver nanoparticles ($3.12 \mu\text{g ml}^{-1}$ in fibroblasts and $12.5 \mu\text{g ml}^{-1}$ in hepatocytes), were shown to induce the activity of the pro-apoptotic signaling enzyme caspase-3. Arora *et al.* (2009) concluded that the concentrations of the silver nanoparticles to be used in medical gels for topical application ($20 \mu\text{g g}^{-1}$) are likely to be safe as the silver particles were shown to induce protective responses rather than toxicity. It is difficult to relate the concentration of the silver in the product to the concentrations used in the *in vitro* assay.

It is evident that metal nanoparticles have the propensity to elicit toxicity that is oxidant mediated within the liver, and culminates in genotoxic and cytotoxic consequences. However, at this time there is insufficient evidence available to make definite conclusions regarding the toxicity of metal nanoparticles to the different cell populations evident within the liver. It is necessary to now determine the impact on macrophage populations, as this liver cell population in particular has been demonstrated to be responsible for particle uptake from the circulation, and the initiation of inflammatory responses in vivo (Cho et al. 2009). Therefore the involvement on an inflammatory response may be critical to the response, and worthy of consideration within the future.

6.4.4 Biological mechanisms driving the toxicity of metal particle toxicity

6.4.4.1 Oxidative, inflammatory and genotoxic consequences of metal nanoparticle exposure

As encountered for other nanoparticles, the toxicity of metal nanoparticles appears to be driven by their oxidative, and inflammatory (Cho *et al.* 2009) nature, which drive genotoxic (AshaRani *et al.* 2009) and cytotoxic (Hussain *et al.* 2005) outcomes. Consequently, it would appear that metal nanoparticles exert their toxicity in a sequential manner, so that increases in ROS production, stimulates oxidant sensitive signaling pathways to eventually culminate in inflammatory, genotoxic and cytotoxic consequences. This pattern of events is exemplified by the findings of a number of investigators.

Carlson *et al.* (2008) determined the ability of silver nanoparticles (15, 30, 55 nm) to induce oxidative stress within NR8383 rat alveolar macrophages, at concentrations up to $100 \mu\text{g ml}^{-1}$, for a duration of up to 24 hours. Particles were internalised by cells and cell morphology was detrimentally affected by nanoparticle exposure. All particles elicited an increase in ROS production, and depletion of cellular GSH, thereby insinuating that the particles exhibited an oxidative response. An inflammatory response, observed in the increased production of tumour necrosis alpha (TNF α), IL-1 β and macrophage inflammatory protein (MIP)-2 was also exerted by silver nanoparticles. Ultimately, nanoparticles were able to decrease cell viability. An important component to the majority of responses was their size dependency, with smaller particles, in general, exhibiting the greatest response. This study therefore demonstrated that silver nanoparticles elicited an oxidant mediated response, which is likely to drive the inflammatory and cytotoxic responses that were witnessed, highlighting that the processes are inherently linked.

Hsin *et al.* (2008) exposed NIH3T3 mouse fibroblasts, A10 rat vascular smooth muscle, and HCT116 human colon cancer cells to silver nanoparticles (<100 nm) and microparticles (<250 μm) at concentrations up to $500 \mu\text{g ml}^{-1}$ for up to 72 hours. Nanoparticulate silver was able to decrease cell viability, but this was not realized for the larger particles, illustrating the size dependence of particle toxicity. The cytotoxicity observed occurred via an apoptotic mechanism. HCT116 cells were least sensitive to the observed toxicity, which was anticipated to derive from the fact that the Bcl-2 protein was upregulated within these cells, to protect against apoptosis. Intracellular ROS levels were increased by the nanoparticles, and pre-treatment of cells with the antioxidant NAC was able to protect against nanoparticle mediated cytotoxicity implying that ROS were fundamental to the response. A series of events were therefore postulated to be integral to the cytotoxicity of silver nanoparticles, specifically an

increase in ROS production, which stimulates Jun N-terminal Kinase (JNK) and p53 (apoptosis regulators) to subsequently induce mitochondria mediated apoptosis (involving cytochrome c release and bax translocation). It is also important that size and cell dependent effects were observed, and must be considered within particle toxicity.

Ahamed *et al.* (2008) investigated the ability of uncoated or polysaccharide coated silver nanoparticles (25 nm) to elicit DNA damage within mouse embryonic stem cells and fibroblasts (at a concentration of 50 $\mu\text{g ml}^{-1}$, for a duration of up to 72 hours). The uncoated nanoparticles were observed to agglomerate, and the authors suggested that this restricted their cellular distribution, and as a consequence they were not apparent within the nucleus and mitochondria. In contrast, coated nanoparticles were distributed throughout the cell. The proteins p53 (whose activation is related to DNA damage), and Rad51 (responsible for DNA double strand repair) were upregulated, and H2AX phosphorylation was witnessed (which is ordinarily induced by DNA double strand breakage) were induced by silver nanoparticles, thus suggesting that the nanoparticles were capable of stimulating DNA damage. A decrease in cell viability was also associated with nanoparticle exposure, and was suggested to occur via apoptosis, in response to DNA damage. Coated nanoparticles elicited a greater extent of damage than their uncoated counterparts, implying that the toxicity of nanoparticles is related to their surface chemistry.

Chi *et al.* (2009) also examined the genotoxicity of silver nanoparticles using an acellular biochemical system. They concluded that silver nanoparticles are only weakly genotoxic, but the relevance of such a model system is questionable. Such a system only relates to direct genotoxicity and does not allow for the identification of the consequences of cell derived ROS or inflammation on gene integrity.

It would appear that the toxicity of silver nanoparticles is mediated by an increase in ROS production, which stimulates oxidant sensitive signaling pathways, and stimulates inflammation and genotoxic events, to eventually culminate in cell death. It is also relevant that the concentration of nanoparticles administered is able to influence the toxicity that is stimulated; specifically, at low levels of oxidative stress a protective response is initiated (Arora et al. 2009) which progresses to a damaging response (Hussain et al. 2005) with increasing particle concentration, and therefore oxidant levels (Nel et al. 2005).

6.4.4.2 Cellular uptake of metal nanoparticles

The uptake of gold nanoparticles by a number of cell types has been investigated to determine the intracellular fate of particles, as gold nanoparticles can be easily identified using electron microscopy. The uptake of silver nanoparticles by cells is also worthy of consideration due to their accumulation within particular tissue sites (such as the skin), following exposure, however the contribution of silver ions to this phenomenon requires assessment. Investigating the uptake of particles is necessary to consider due to its ability to influence cell physiology and function.

The shape and size of metal nanoparticles has been demonstrated to impact on nanoparticle uptake. Chithrani *et al.* (2006) used negatively charged spherical (14, 30, 50, 74, and 100 nm diameter) and rod (14x40 nm, and 14x74 nm) shaped gold nanoparticles and investigated their uptake by HeLa epithelial cells after a 6 hour exposure (in cell culture medium containing serum). The uptake of spherical particles was greatest and it was observed that they were contained within vesicles in the cytoplasm of the cells, and it was suggested that nanoparticle uptake was mediated by proteins bound to the particle surface. This derived from the knowledge that nanoparticle uptake increased with time until it plateaued, suggesting that the uptake process reached saturation. Consequently it was also speculated that the saturation of nanoparticle uptake may be a result of the extent of protein binding, due to the fact that protein that is not adsorbed onto the particle surface and therefore 'unbound' protein can compete for receptor sites on the site against the protein-adsorbed nanoparticles. The uptake of 50 nm particles was greatest, so that it was suggested that larger particles, which have a smaller surface area, have less protein adsorption to the particle surface, which explains their more limited uptake. It was therefore hypothesised that the uptake of gold nanoparticles was mediated by the adsorption of serum proteins onto the particle surface, and these proteins enable nanoparticle entry via clathrin mediated endocytosis. However, the uptake of smaller

particles was less than that of their 50 nm counterparts; this could be explained by the fact that there are size restrictions to endocytosis, so that these particles are too small to be recognised. To confirm uptake by endocytosis, particle uptake was compared at 37°C versus 4°C (as at low temperature ATP generation is diminished, and therefore active uptake mechanisms cannot function), and it was found that uptake was reduced by the low temperature. This study therefore makes a number of important observations, specifically that nanoparticle uptake occurs by clathrin mediated endocytosis, but that uptake is shape, size, charge, and serum dependent. The results of this study were expanded upon by Chithrani and Chan, (2007) where the spherical and rod shaped gold nanoparticles were coated with the serum protein transferrin and uptake by HeLa (epithelial), STO (fibroblasts) and SNB19 (neuronal) cell lines was investigated. Uptake of these nanoparticles was confirmed via confocal microscopy (through the attachment of a fluorescent tag to the transferrin protein adsorbed onto the nanoparticle surface) and then low temperature, and ATP depleted environment used to confirm the involvement of endocytosis. To determine if a clathrin mediated mechanism was involved, cells were pre-treated with sucrose or potassium depletion (which is assumed to promote the dissociation of the clathrin coat, so that clathrin mediated endocytosis cannot occur), and uptake of the gold nanoparticles was reduced under these circumstances. The findings were found to be similar for both gold nanoparticle shapes, and so the mechanism of uptake was assumed to be identical. These findings therefore demonstrated the ability of a variety of cell types to internalise nanoparticles by endocytosis, and highlighted the importance of particle shape, in addition to highlighting that external factors such as protein adsorption are able to influence nanoparticle uptake.

Rothen-Rutishauser *et al.* (2006) investigated the uptake of fluorescent particles (1µm, 0.2µm and 78 nm, positively, negatively and non-charged) by red blood cells (RBCs). Nanoparticle uptake was identified using confocal microscopy, and it was observed that 1µm polystyrene particles were attached to the cell surface but were not internalised by cells during any of the incubation times (4-24 hours). Negatively and non charged 200 nm particles were found within cells, as were positively charged particles, however these particles were also apparent at the cell surface. The 78 nm particles (non charged and negatively charged) were also found within cells. Positive polystyrene fluorescent particles were not available in the nano size range, so positively charged gold particles were used to investigate the impact of nanoparticle charge on uptake by RBCs (as they are non-phagocytic and allowed other mechanisms of nanoparticle uptake to be revealed). TEM analysis of the cells was conducted using gold nanoparticles to investigate the influence of nanoparticle charge (positive, negative and neutral) to their uptake and it was found that uptake was comparable. It was highlighted that identifying particles within the nano size range was difficult due to their similarity in size (and appearance) to cellular structures. Therefore confocal visualisation, using fluorescent polystyrene particles, allowed the size dependence of particle uptake to be compared, with TEM analysis using gold particles was utilised to further investigate the intracellular fate of particles. It was observed that the gold nanoparticles were not membrane bound within the RBCs, and it was therefore concluded that endocytosis was unlikely to contribute to their internalisation. This study therefore highlights that the usefulness of using gold to assess the uptake of particles within cells.

Silver accumulation by macrophages has also been identified *in vivo* (Takenaka *et al.* 2001, Cho *et al.* 2009), and therefore they fulfill their role within host defense, through the removal of particles from the site of deposition or circulation. However, this has been demonstrated to have detrimental consequences, due to the initiation of an inflammatory response (Cho *et al.* 2009). The consequences of this accumulation are therefore not fully known at this time but are likely to play a role in particle clearance, but also the initiation of inflammatory responses.

The uptake of silver nanoparticles by cells, may also contribute to symptoms associated with silver. In addition, argyria development, is commonly associated with silver exposure, and the discolouration of the skin is related to the deposition of silver within the skin and so the ability of silver to localise within particular skin regions has been encountered, and has been observed in fibroblasts, macrophages, nerves, capillary walls (Wabgdera and Fung 2005). Silver induced increases in melanin can also contribute to skin discolouration.

The uptake of metal particles by cells has been demonstrated, which is likely to be driven by particle size, charge, shape and protein adsorption. The uptake of particles may be able to impact on cell function, although interactions at the cell surface may also impact on the toxicity.

6.4.4.3 Reproductive Toxicology of metal nanoparticles

Evaluation of the effects of metal nanoparticles on the reproductive system is limited to a small number of *in vivo* (restricted to fish) and *in vitro* studies. Bar-Ilan *et al.* (2009) exposed transparent zebrafish embryos to colloidal silver and gold nanoparticles at various sizes (3, 10, 50, and 100 nm), at a concentration of 100 or 250 μM , from 4 to 120 hours post fertilisation (hpf). Silver nanoparticles produced almost 100% mortality at 120 hpf, while Au nanoparticles produced less than 3% mortality at the same time point. Furthermore, while Au nanoparticles induced minimal sub-lethal toxic effects, Ag nanoparticle treatments generate a variety of embryonic morphological malformations. The group reports both Ag and Au were shown to be taken up by the embryos, suggesting that Ag toxicity is caused by the nanoparticles themselves or Ag ions that are formed during *in vivo* nanoparticle destabilisation. The group suggest that Ag nanoparticle toxicity was slightly size dependent at certain concentrations and time points, with the most important result showing parallel sizes of Ag and Au nanoparticles induced significantly different toxic profiles, with Ag being toxic and Au being inert in all exposed sizes. The paper therefore concludes and proposes that nanoparticle chemistry is as, if not more important than specific nanosizes at inducing toxicity *in vivo*.

Lee *et al.* (2007) conducted a similar study, and characterised the transport of single silver nanoparticles (5–46 nm) into zebrafish embryos and investigated their effects on early embryonic development. A dilution series of washed Ag nanoparticle solutions (ranging from 0.04, to 0.71 nM) was incubated chronically with cleavage-stage (8-cell) embryos in egg water for 120 hpf. The group reports that single Ag nanoparticles were transported into and out of embryos through chorion pore canals (CPCs). Lee *et al.* (2007) further report that the nanoparticles become trapped inside CPCs and the inner mass of the embryos, showing restricted diffusion. The groups' data highlights individual Ag nanoparticles observed inside embryos at each developmental stage and in normally developed, deformed, and dead zebrafish. The group concludes that toxicity and biocompatibility of Ag nanoparticles and the types of abnormalities observed in zebrafish are highly dependent on the dose of Ag nanoparticles, with a critical concentration of 0.19 nM.

A study conducted by Bradyich-Stolle *et al.* (2005) examined the suitability of a mouse spermatogonial stem cell line as a model to assess nanotoxicity, via the LDH and MTT assay, in the male germline, *in vitro*. Silver (15 nm), molybdenum trioxide (MoO_3 , 30 nm), and aluminum (Al, 30 nm) nanoparticles were obtained commercially, and made and in a pulsed-plasma reactor, which forms the particles in a gas phase process. Control soluble salts were used as well as a positive control, ~1000 nm cadmium oxide, known for its cytotoxic properties. Each particle was added to cells at concentration $10 \mu\text{g ml}^{-1}$ for 48 hours. Findings demonstrate a concentration-dependent toxicity for all types of particles tested, whereas the corresponding soluble salts had no significant effect. Silver nanoparticles were the most toxic while molybdenum trioxide nanoparticles were the least toxic. The findings of the paper suggest that this cell line provides a valuable model with which to assess the cytotoxicity of nanoparticles in the germ line *in vitro*.

Wiwanitkit *et al.* (2009) highlighted the direct *in vitro* toxicity of gold nanoparticles on mature male germ cells, spermatozoa. The study exposed fresh donor semen samples from healthy individuals to gold nanoparticle (9 nm) solution (produced by the Turkevich citrate reduction method, at a concentration of 44 ppm) at a volume of 500 μL . After mixing the semen with a gold nanoparticle solution, 25% of sperm were not motile. Penetration of gold nanoparticle into the sperm heads and tails was observed.

Not all studies examining nanoparticle interaction with the reproductive system have looked at toxic effects. Liu *et al.* (2007) examined possible improvements to the method of using copper-containing intrauterine device (Cu-IUD), as a widely accepted reversible female contraceptive that it is both cost effective and efficient. Cu-IUD has been linked to side effects, such as intermenstrual bleeding, spotting and pain within the first few months of Cu-IUD insertion. This

study assessed the effects of a new version containing nano copper/low-density polyethylene nanocomposite (nano-Cu/LDPE) as an alternative. The study used mature female Sprague–Dawley rats randomly divided into 4 groups: sham-operated control group (SO group, n =10), bulk copper group (Cu group, n =40), LDPE group (n =40), and nano-Cu/LDPE groups (n =80). Twenty rats in each group except for the control group were mated with male rats of proven fertility, from 30 days after insertion, and the antifertility rates were observed at day 11 of pregnancy. Liu *et al.* (2007) report the effects of nano-Cu/LDPE on the corresponding factors involved in irregular bleeding and pain, after insertion are lower than those of bulk copper, and its mechanism of reducing the side effects of IUD might be related to inhibition of local prostaglandins and tPA level, measured by using ELISA at the 30th and 60th day after insertion. The group concluded that nano-Cu/LDPE, may be a potential substitute for conventional materials for IUDs in the future due to its satisfactory contraceptive efficacy and decreased side effect. There was however no mention of toxicity testing in relation to the use of this new nano-copper composite.

The reviewed literature examines the effects of metal nanoparticles, primarily silver and gold, and their effects during embryonic development and resultant toxicity. The literature highlights toxicity of male germ cells and defects in sperm function; however studies are limited in number and in sample size. Only one study identified examined the positive effects of a nano copper device on female contraception however there was no focus on potential side effects such as toxicity to other organs or cell types in the female reproductive system.

6.4.5 Linking the physico-chemical attributes of metals to their pathogenicity or toxicity

6.4.5.1 The importance of size to metal particle toxicity

Particle size has been demonstrated to influence the tissue distribution (Savanone *et al.* 2008b, De Jong *et al.* 2008), intestine and dermal penetration (Savanone *et al.* 2008a), cellular uptake (Chithrani *et al.* 2006) and toxicity (Cha *et al.* 2008, Hsin *et al.* 2008) of metal particles. Therefore, in general, greater effects are observed for smaller particles.

In addition, Pan *et al.* (2007) investigated the size dependent cytotoxicity exhibited by gold nanoparticles (stabilised with triphenylphosphine derivatives) to cells which represent barriers to nanoparticle entry into, or movement within the body (namely; epithelial cells (HeLa), phagocytes (J774A1), melanoma cells (SK-Mel-28) and fibroblasts (L929)). The nanoparticles ranged in size from 0.8 to 15 nm. All cell types internalised gold nanoparticles, and showed signs of stress, such as membrane blebbing. Particle exposure resulted in cell death that was size dependent in nature. Specifically, smaller particles (<1.4 nm), were more toxic than their larger equivalents, so that the 15 nm nanoparticles were relatively non-toxic (despite their internalisation by cells). Therefore, despite all particles having nano dimensions, they were not equally toxic to cells. However, it is relevant that 1.4 nm nanoparticles were the most toxic particle tested, and drove death by necrosis, but 1.2 nm nanoparticles mediated an apoptotic driven cell death. Therefore the size of particles was able to influence the mode of cell death.

The size of particles has been proven to be very influential to their behaviour. Accordingly, smaller particles have a wider tissue distribution, penetrate further within the skin and intestine, are internalised to a greater extent, and have a larger toxic potency. The novel properties associated with smaller particles drive the exploitation of nanoparticles, but also fuel the skepticism surrounding their exploitation, due to their apparently greater toxicity than their larger counterparts. However, limited studies have directly compared nanoparticulate and microparticulate forms of metals, and often comparisons are made between different particles that all have nano dimensions. In addition, perhaps the toxicity of smaller silver particles is a consequence of their greater capacity to release silver ions which mediate the observed toxicity, but this is uncertain at this time.

6.4.5.2 The impact on particle solubility on silver nanoparticle toxicity

The release of silver ions from particles warrants further investigation in the future, as in a number of cases it is difficult to interpret if toxicity emanates from the particles themselves or due to the release of ions from the particles which mediate the observed toxicity. Limited studies have been conducted to investigate this.

Lok *et al.* (2007) investigated which physico-chemical properties of silver nanoparticles (specifically their size or ion release) were responsible for their antibacterial behaviour. Silver nanoparticles (9 nm) exhibited antibacterial behaviour, which was greater than that exhibited by their larger counterparts (62 nm), illustrating that the phenomenon may be size related. It was suggested that their antibacterial behaviour was related to their ability to release Ag^+ ions (due to particle oxidation), with smaller particles having a greater surface area, and therefore greater capacity to release Ag^+ from their surface, which mediate the antibacterial effects. The response of oxidised nanoparticles (to cause the release of silver ions) and reduced nanoparticles was also considered, and found that only oxidised exhibited antibacterial behaviour, supporting the concept that Ag^+ was mediating the antibacterial properties of silver nanoparticles. The aggregation of particles was also observed to detrimentally affect the antibacterial properties of silver nanoparticles. However, at this time it is unknown how silver nanoparticles deliver Ag^+ to bacteria, as levels of extracellular Ag^+ were low, so a direct association between the particles and bacteria is thought to be necessary, but further investigations are necessary.

Luoma (2008) suggests that following ingestion, silver (not specifically nanoparticles) is likely to be converted to its ionic form due to the low pH of the stomach. Due to the large surface area of nanoparticles this might also be true for nano silver, but this requires investigation.

It is anticipated that the release of silver ions from nanoparticulate silver is a realistic prospect, and responsible for their antibacterial properties. However although Ag^+ is postulated to mediate these effects, but the mechanism by which this occurs is unknown at this time, but is likely to involve particle oxidation to enable their release. However, further investigations are necessary to confirm the contribution of particles and/or ion release to their toxicity.

6.4.5.3 Interactions of metal particles with biological moieties

The ability of metal particles to interact with biological molecules, such as proteins and DNA is of concern, as it has the propensity to alter the normal structure and function of these biological macromolecules, but also is able to modify the behaviour of the particles.

On entering the body, particles immediately become coated in biological molecules, including proteins. It is hypothesized that this coating can influence particle behavior and toxicity, with different particles having different capacities to bind different molecules. Furthermore, there is a possibility that the particles can alter the protein structure and function; again this may contribute to toxicity. Liu *et al.* (2009) have investigated the consequences of bovine serum albumin (BSA) interaction with silver nanoparticles using a combination of techniques. They conclude that the BSA and silver nanoparticles formed a complex held together by van der Waals and electrostatic forces. They indicated that silver nanoparticles increased the amount of helix and decrease the beta sheet structure, leading to a loosening of the protein skeleton. The authors explain that in the loose structure, internal hydrophobic amino acids are exposed. Such changes in structure could lead to functional changes or inhibition of proteins.

It is also acknowledged that silver nanoparticles are able to bind to thiol ($-\text{SH}$) groups within proteins, with high affinity, and thereby promote their denaturation (Chen *et al.* 2008, Wadhwa and Fung 2005). This is of particular concern, due to the known high frequency of such functional groups within antioxidants (such as glutathione, and SOD), which are key to the cell's antioxidant defense system. Therefore, silver nanoparticles may deplete cellular antioxidants and thereby promote the development of an oxidant mediated response due to the accumulation of ROS within cells (Chen *et al.* 2008). Silver nanoparticles have also been demonstrated to promote ROS production within cells (Hussain *et al.* 2004), which would further promote oxidative stress development.

In addition, within blood silver binds to albumin, and this enables its transportation throughout the body (Wadhera and Fung, 2005).

Gold nanoparticles are also recognised in their ability to bind to DNA, which may be exploited within the treatment of disease (such as anticancer agents or within gene therapy), but may also contribute to genotoxicity, or block transcription (Pan *et al.* 2007). Liu *et al.* (2003) used molecular simulations and TEM analysis to demonstrate that gold nanoparticles (Au₅₅ clusters; 1.4 nm diameter) were able to interact with DNA. To understand the implications of this finding, the ability of (the same) gold nanoparticles to traverse the plasma membrane, and their subsequent sub-cellular distribution (using radiolabelled particles) was determined by Tsoli *et al.* (2005). Eleven cell lines were utilised (including melanoma, osteosarcoma, bone cells, fibroblasts, epithelial, and lung cancer cells), and it was observed that the cells varied in their sensitivity to the toxicity exerted by gold nanoparticles. Specifically, melanoma derived cells were most sensitive to nanoparticle toxicity. It was revealed that (radiolabelled) nanoparticles were distributed within the cytoplasm and nucleus, where they were capable of binding to DNA. This finding therefore confirmed that of the previous observations, where nanoparticles were strongly bound to DNA, but also demonstrated their cytotoxic potential. In addition, Goodman *et al.* (2006) illustrated the ability of gold nanoparticles (~10 nm) to bind to DNA, which caused a conformational change within the structure of DNA. This was suggested to impact on DNA transcription, and was exemplified by the finding that RNA polymerase activity was inhibited. This may derive from the fact that polymerase could no longer recognise DNA, or due to the fact that polymerase had a smaller binding affinity for DNA than the gold nanoparticles.

However, Connor *et al.* (2005) demonstrated that gold nanoparticles (4, 12, 18 nm) are not internalised by a human leukemia K562 cell line, illustrating that perhaps this is a size dependent observation, or due to surface modifications. In addition, as demonstrated, the cell type used is also able to influence the findings.

The interaction of silver nanoparticles with serum proteins is likely to enable their transportation within the body. In addition, interactions with proteins may have detrimental consequences for particle behavior or normal protein structure and function, and therefore impact on normal cell function, especially antioxidant defense. Furthermore, the ability of gold nanoparticles to bind to DNA is of concern, due to their potential cytotoxic or genotoxic consequences, which may be exploited within anticancer drugs, or gene therapy, which warrants further investigation. In addition, the ability of gold nanoparticles to interrupt transcription is of concern. The gold nanoparticles (size, surface modification) and cell types used are likely to influence the uptake, sub-cellular distribution, and toxicity of gold nanoparticles.

6.4.6 Summary

It is evident that silver and gold metal nanoparticles represent a potential hazard to human health. However, investigations purporting to study the toxicity of metal particulates are still in their infancy at this time, and have concentrated on revealing the toxicity, tissue distribution and antibacterial properties of silver nanoparticles, and in addition the tissue distribution and cellular uptake of gold nanoparticles. Consequently, more comprehensive studies are required to more fully understand the toxicity associated with metal particulate exposure. Silver and gold nanoparticles can become systemically available following exposure, which is evidenced by their preferential accumulation within the liver. The appearance of argyria, which is reliant on the accumulation of silver within the skin, and has transpired following silver nanoparticulate ingestion, which further emphasises its propensity to become systemically available. The liver appears to be the primary site of particle accumulation, and so the consequences of this require investigation, as this organ may enable particle elimination from the body, within bile, or particle presence within the liver may be associated with toxicity, and inflammatory responses. It is apparent that the toxicity of metal particulates is reliant on their internalisation and oxidative nature, which drives inflammatory, genotoxic and cytotoxic events. However, the specific properties of particles driving the observed toxicity is uncertain. Specifically, it is of relevance to determine where the toxicity derives from the small size of nanoparticles, or whether toxicity is mediated through the release of ions from particles, or perhaps a combination of both concepts is responsible, as smaller particles are likely to allow for the greater release of Ag⁺ ions.

6.5 METAL OXIDES

6.5.1 Introduction

Metal oxide nanoparticles are based on a number of diverse materials, with the most widely exploited including; titanium, zinc, aluminium, cerium, and iron oxides. Due to its widespread exploitation, the greatest number of studies have been focussed on revealing the toxicity of titanium dioxide (TiO₂), and these studies will therefore form the major part of this review.

Such studies have addressed the consequences of exposure via a number of routes. Pulmonary exposure to metal oxides has been studied for a number of the metal oxides. TiO₂ exposure via the pulmonary route has included the use as a control particulate compared to pathogenic materials such as alpha-quartz (see below), but has also been studied in relation to occupational exposures. Cerium dioxide, due to its supplementation within fuel, has also been studied in relation to pulmonary exposure.

Dermal penetration and toxicity of TiO₂ and zinc oxide (ZnO) particles have also formed the focus of a number of studies due to their inclusion within sunscreens and cosmetic products.

Intravenous exposure is primarily associated with magnetic particles such as iron oxide (as they are expected to be injected into the body for imaging purposes), and so their tissue distribution and longevity within the body has been concentrated on within a number of studies, while, the ingestion of TiO₂ is also of relevance due to its incorporation into foods and medicines.

Concern about the potential toxicity of nanoparticles in general, originally emanates from the studies conducted by Ferin *et al.* (1990 and 1992) and Oberdorster *et al.* (1990), which first demonstrated that the pulmonary inflammation, retention and translocation of ultrafine TiO₂ (equivalent to nanoparticles, in terms of size; 21 nm diameter) was enhanced, compared to that of its larger fine equivalent (250 nm diameter). Previously TiO₂ has been largely used in pulmonary toxicology studies as a negative control when assessing the toxicity of pathogenic particulates such as alpha-quartz. These studies paved the way for the consideration of size dependent particle toxicity. More recently studies have continued to focus on the importance of size, but have now expanded considerations to include surface area, the contribution of the particle crystal phase, photoactivity and particle dissolution to toxicity.

6.5.2 *In vivo* assessment of metal oxide toxicity

In general, investigations focus on a particular route of exposure due to the anticipated application of the metal oxide in question.

6.5.2.1 Pulmonary exposure to metal oxides

The greatest number of studies, by far address the consequences of the exposure of the lungs to metal oxides, and in particular the size dependence of any effects.

As mentioned previously, a study conducted by Ferin *et al.* (1992) was one of the first to demonstrate that particle size was fundamental to the pulmonary toxicity of TiO₂ particle. In this study, rats were exposed via inhalation, to TiO₂ (21 nm; 23.5 mg m⁻³) and 250 nm; 23.0 mg m⁻³) for 12 weeks, and examination of the consequences of TiO₂ exposure were evaluated over a 64 week post-exposure period. Alternatively, rats were administered TiO₂ of various sizes (12, 21, 230 and 250 nm in diameter) via a single intratracheal instillation (up to 1000 µg per rat) and toxicological investigations were made 24 hours post exposure. Following intratracheal or inhalation exposure, nanoparticulate TiO₂ induced a greater pulmonary inflammatory response (characterised by neutrophil infiltration), than its microparticulate (250 nm) counterpart, which did not elicit any changes in the inflammatory status of the lung. The smaller sized, TiO₂ particles were also found to remain within the lung for longer (501 days) periods, following inhalation, than the larger TiO₂ particles (174 days), thus highlighting that the clearance of smaller particles from the lung was slower. In fact, the prolonged retention of smaller TiO₂ particles in the lung was suggested to derive from the finding that they were able to translocate

to the pulmonary interstitium more efficiently than the larger TiO₂ particles. The authors proposed that this phenomenon was facilitated by the fact that smaller particles were not efficiently taken up by alveolar macrophages, which thereby allowed for their prolonged interaction with alveolar epithelial cells. In addition, it was found that an increased mass dose (which translates to an increased number of particles, with smaller particles) promoted the movement of particles within the pulmonary system. It was therefore observed that particle size, the number of particles administered (which was related to the delivered dose) impacted on the translocation process, and therefore TiO₂ toxicity. These findings prompted the concern regarding nanoparticle use, due to the apparent size dependency of particle toxicity.

The size dependency of metal oxide toxicity was also investigated by Ogami *et al.* (2009), who investigated the influence of particle size on the pulmonary toxicity of nickel oxide (NiO). Nano (27 nm) and micro (4.8 µm) forms of NiO particles were exposed to rats via intratracheal instillation (2 mg per rat), with inflammatory and morphological assessments made from 3 days to 6 months post exposure. Nanoparticulate NiO (nanoNiO) stimulated the infiltration of neutrophils into bronchoalveolar lavage fluid ((BALF) that was maximal at 3 days, but still apparent at 6 months), which was apparent to a greater extent than that elicited by their larger counterparts. In addition, histological analysis confirmed that neutrophil infiltration was associated with nanoNiO, and also demonstrated that a macrophage influx, alveolitis, and alveolar wall thickening, were components of the inflammatory response, which peaked at 3 months. Increased collagen deposition within the lung tissue was also associated with nanoNiO. In contrast, normal pulmonary structure and only slight inflammatory changes were observed with microparticulate NiO. As a result, it was suggested that the inflammogenicity of NiO particles was size (and therefore surface area) dependent. The toxicity of NiO was also compared to that of the positive control, silica and the negative control, TiO₂ (1.5 µm). It was observed that crystalline silica (1.6 µm) and nanoNiO behaved similarly, and that TiO₂ and microparticulate NiO were substantially less inflammogenic.

Renwick *et al.* (2004) also investigated whether the size of TiO₂ (or carbon black (CB)) particles were able to impact on their ability to elicit an inflammatory response, or impact on clearance mechanisms within the rat lung. Rats were exposed to particles via intratracheal instillation, at 125 µg per rat or the relatively high dose of 500 µg per rat, and toxicological investigations conducted at 24 hours post exposure. At the highest dose only, nanoparticulate (29 nm), but not microparticulate (250 nm) TiO₂ stimulated the recruitment of neutrophils into the lungs, epithelial damage, increased permeability of the lung epithelium, and cytotoxicity, which were measured within the bronchoalveolar lavage fluid (BALF). The nanoparticles were also able to diminish the phagocytic ability of isolated rat alveolar macrophages (from exposed animals), and increase the ability of these macrophages to migrate towards the chemotactic signal, C5a. The authors suggested that the consequences of such a stimulation would be promotion of the retention of macrophages, and therefore their particle burden, within the lung. The nanoparticles were therefore demonstrated to elicit a greater pulmonary inflammatory response than their larger counterparts.

Chen *et al.* (2006) exposed mice, via intratracheal instillation (0.1 and 0.5 mg per mouse), to nano (19-21 nm) and micro (180-250 nm) forms of TiO₂, and determined their pulmonary toxicity 3 days, 1 week or 2 weeks post exposure. Histological assessments illustrated that morphological alterations within the lung were evident on exposure to TiO₂ nanoparticles, which were emphysema-like in nature (including, for example alveolar enlargement) and were more pronounced in areas where particles preferentially accumulated, and increased in severity, with increasing time and dose. An inflammatory response, recognised by the infiltration of macrophages (that were particle laden), upregulation of cytokines (including monocyte chemotactic protein (MCP)-1, interleukin (IL)-1, tumour necrosis alpha (TNFα) and several neutrophil chemoattractants) and complement activation was also observed. Despite the huge doses used, no pathological lesions were found in response to microparticulate TiO₂.

Bermudez *et al.* (2004) determined if choice of species was able to influence the pulmonary response to nanoparticulate TiO₂ (21 nm). This was achieved through the exposure of rats, mice and hamsters, via inhalation, to TiO₂ nanoparticles for 13 weeks (6 hours per day, 5 days per week, at concentrations of 0.5, 2, or 10 mg m⁻³) and the pulmonary response was assessed up to 52 weeks post exposure. It was demonstrated that a pulmonary inflammatory response

was stimulated by TiO₂ within mice and rats, but was absent in hamsters. The nature of the response was also observed to vary within the different species, so that a greater neutrophilic response, which decreased with time, was apparent in rats with progressive epithelial fibroproliferative changes also apparent. However, within mice, the neutrophil and macrophage component of the response remained elevated throughout the observation time. The severity of the response was ranked in the following order, by the authors; rat > mouse > hamster. Consequently, the rat was found to be the most sensitive species to the effects of TiO₂, with the limited toxicity apparent within hamsters thought to derive from the low lung burden of particles, which was apparent due to the rapid clearance of particles associated with this particular species. Hamsters retained TiO₂ within the lungs to the lowest extent, indicating that hamsters had the greatest propensity to efficiently clear particles from the lung. However, for mice and rats, the initial retention of particles was similar, and decreased with time, highlighting that the pulmonary clearance kinetics differed amongst the different species. The study highlights that differences in the response, and therefore sensitivity of different species are worthy of consideration within investigations, and the model which mimics the human situation most accurately requires assessment.

Warheit *et al.* (2005) determined if the exposure scenario, or TiO₂ formulation (specifically concentrating on surface coating modification, with the diameter of particles ranging from 290 to 440 nm) were able to impact on their pulmonary toxicity, within rats. In general, following inhalation of TiO₂ (up to 1300 mg m⁻³, for 4 weeks), there was an accumulation of particle containing macrophages. Following intratracheal instillation (up to 10 mg kg⁻¹) it was observed that some TiO₂ preparations were able to stimulate a transient, pulmonary inflammatory response that was typified by the infiltration of neutrophils, and LDH release, but this resolved within one week post exposure. It is relevant that the response was very much dependent on the TiO₂ formulation in question, so that the samples that contained the highest alumina or amorphous silica content elicited the greatest adverse pulmonary response. Overall, both exposure scenarios are associated with minimal adverse effects, but this may be derived from their size, as all samples were out-with nano dimensions (i.e. >100 nm).

Similarly, Grassian *et al.* (2007) investigated the toxicity of TiO₂ nanoparticles (5 and 21 nm) within mice, subsequent to inhalation (0.7 or 7 mg m⁻³, for 4 hours) or nasal instillation (up to 150 µg per 50 µl), and aimed to identify the properties of particles that drove any toxicological observations. An elevated macrophage population was associated with the inhalation of particles (4 and 24 hours post exposure), and were observed to internalise particles. An infiltration of neutrophils, was associated with the nasal instillation of TiO₂. In both exposure scenarios, the response induced by the 21 nm particles was greater than that exerted by their 5 nm counterparts, and so surface area was not deemed to be the sole determinant of TiO₂ toxicity, which was unexpected. Consequently the crystallinity of the samples was suggested to influence the observed toxicity, as 5 nm particles were pure anatase, whereas the 21 nm particles were a mixture of rutile and anatase forms of TiO₂. It is also relevant that the nature of the response varied for the different exposure scenarios, specifically inhalation exposure promoted a macrophage driven response, and instillation exposure triggered the development of a neutrophil driven response, which insinuates that the experimental set up influences the results of toxicity tests, and is thought to relate to differences within their distribution within the lung following exposure.

In line with these findings, Warheit *et al.* (2007) aimed to determine if the crystalline form of TiO₂ was able to influence its pulmonary toxicity within rats, subsequent to intratracheal administration (1 or 5 mg kg⁻¹). Two rutile nanoparticle samples were investigated, a 'mixed' nanoparticle TiO₂ (80% anatase, 20% rutile), and a microparticulate rutile TiO₂ sample (as a negative particulate control), in addition to α-quartz (included as a positive particulate control). The pulmonary response was evaluated for up to 3 months post exposure. Although quartz had the greatest inflammogenicity, it was evident that several particle types elicited a short-term, pulmonary inflammatory response that was characterised by the infiltration of neutrophils, and was greatest, and more sustained for the mixed crystallinity nanoparticle sample. Furthermore the only samples capable of eliciting a release of lactate dehydrogenase (LDH, a measure of cytotoxicity) and protein (indicative of increased vascular permeability) into BALF were quartz and the mixed sample. The properties responsible for driving TiO₂ toxicity were then considered and it was suggested that differences in particle surface area accounted, in part, for

the responses exhibited by particles. Accordingly, the mixed sample had the greatest surface area ($53 \text{ m}^2 \text{ g}^{-1}$), and therefore greatest reactivity, so that surface properties were suggested to drive TiO_2 toxicity. However as the rutile nanoparticle samples had surface areas of $18.2 \text{ m}^2 \text{ g}^{-1}$ and $35.7 \text{ m}^2 \text{ g}^{-1}$, and the TiO_2 microparticles had a surface area of $5.6 \text{ m}^2 \text{ g}^{-1}$, it was expected that the rutile nanoparticle particle samples would exhibit an intermediate level of toxicity (between the mixed, and microparticulate samples), but this did not transpire within the findings. However, this supported the notion that additional factors may contribute to the toxicity of particulates, besides their surface area. Therefore, it was suggested that the rutile nanoparticle samples were less toxic than those that were predominantly anatase. In addition, the authors suggested that post production processing, that removed chloride from the particle surface, and enhanced agglomeration of particles, may also contribute to the reduced toxicity of the rutile nanoparticle particles.

Ahn *et al.* (2005) investigated what processes were responsible for particulate mediated stimulation of excessive mucus secretion within humans. To achieve this, TiO_2 (4 mg kg^{-1}) was exposed to rats via intratracheal instillation, and toxicological observations made from 4 to 72 hours post exposure. TiO_2 exposure stimulated an increase in goblet cell hyperplasia, which is, in part, attributed to an increase in muc5 gene expression and IL-13 production. Therefore, it could be speculated that particle mediated increases in mucus secretion contributed to the aggravation of chronic airway disease symptoms within humans, and therefore warrants further investigation.

Heinrich *et al.* (1995) exposed rats to TiO_2 nanoparticles via inhalation for 2 years, at an average exposure concentration of 10 mg m^{-3} , which was followed by exposure to clean air for 6 months. Mice were exposed to TiO_2 for 13.5 months (at 10 mg m^{-3}) then clean air for 9.5 months. TiO_2 was able to increase mortality within rats and mice, so that their lifetime was significantly shortened. Lung tumours were observed in both species, as a consequence of the chronic exposure to TiO_2 .

Inoue *et al.* (2008) determined the impact of TiO_2 (15, 50, 100 nm) intratracheal instillation (8 mg kg^{-1}) on LPS mediated pulmonary inflammation. Combined TiO_2 and LPS treatment was able to exacerbate LPS mediated inflammation (indicated by keratinocyte chemoattractant (KC), IL-1 β and MCP-1 production). Circulating cytokines were also increased, as well as coagulatory factors such as fibrinogen. A size dependency to the findings was also observed, whereby smaller particles were most toxic.

In a different approach, Nurkiewicz *et al.* (2006) determined the impact of TiO_2 particles ($1 \mu\text{m}$) or ROFA particulate matter on the systemic microvascular, following intratracheal instillation of rats (0.1 or 0.25 mg per rat), 24 hours post exposure. Therefore, the authors encompassed the possibility that systemic effects were a component of the pulmonary response to particulates. Particle exposure stimulated a neutrophil influx into the lungs, but no cytotoxic response was evident. Particles were able to induce an impairment of endothelium dependent arteriolar dilation. The response was suggested to be related to increased neutrophil adhesion to the vessels, and myeloperoxidase (MPO) deposition and oxidative stress within the vessel wall. These findings are of concern, as they indicate that an inflammatory response may be stimulated within the vessel. However the response was independent of the level of pulmonary inflammation, and was not thought to be reliant on the migration of particles from the lung. This study highlighted the systemic (inflammatory mediated) responses that are associated with particle exposure.

Wang *et al.* (2008a) investigated the distribution of rutile (80 nm) and anatase (155 nm) TiO_2 particles within the mouse brain, following nasal instillation exposure ($500 \mu\text{g}$ per mouse, every other day for a total of 30 days) and determined if any neurotoxicity associated with exposure. Both forms of TiO_2 were able to access the brain, with accumulation within the cerebral cortex, thalamus and hippocampus evident, and was postulated to occur via the olfactory bulb. This route of uptake however, was unlikely to be mediated via penetration into the cardiovascular system and via the blood. Instead, TiO_2 delivery to the brain occurred via neuronal transport, with preferential localisation evident within the hippocampus and olfactory bulb. Accumulation of TiO_2 resulted in morphological alterations and loss of neurones in the hippocampus, which was accounted for by the higher distribution of TiO_2 within this brain region. In addition it was

suggested that TiO₂ elicited oxidative stress within the brain due to the elevation of superoxide dismutase (SOD), and catalase activity, and evidence of increased lipid peroxidation and protein oxidation. Therefore neuronal mediated translocation of TiO₂ to the brain, following nasal instillation, was observed, with the hippocampus illustrated as being the main target of accumulation and toxicity. Wang *et al.* (2008b) expanded upon these findings and found that the phenomenon was time dependent (was maximal at 30 days), and that an inflammatory response (indicated by IL-1 β , and TNF α) within the brain was also stimulated by TiO₂ exposure. The response was measured at day 2, 10, 20, and 30. It was apparent that repeated exposures, over a period of 30 days, were required to enable the accumulation of TiO₂ within the brain. It is therefore of interest that the neuronal transport of nanoparticle containing substances between the nose and CNS could be exploited, in order to bypass the blood brain barrier.

Similarly, Elder *et al.* (2006) demonstrated that manganese oxide nanoparticles (50 nm) were able to accumulate within several brain regions (and was highest in the olfactory bulb), the lung and liver, subsequent to intranasal exposure (6 hours per day, for up to 12 days). They also observed that the translocation of nanoparticles to the brain could occur via the olfactory nerve. However, the accumulation of nanoparticles within the brain, via their transportation within blood was not excluded as a possible mechanism of delivery of nanoparticles to the olfactory bulb, and then to other brain regions.

One study was identified that investigated the *in vivo* pulmonary effects of ZnO nanoparticles. Sayes *et al.* (2007) determined the predictive nature of *in vitro* testing, for assessing *in vivo* toxicity, with regards to ZnO toxicity in nanoparticulate (50-70 nm) or microparticulate (<1000 nm) forms. *In vivo*, rats were exposed, via a single intratracheal instillation (1 or 5 mg kg⁻¹), and from 24 hours to 3 months post exposure BALF was collected, in order to assess the cytotoxic and inflammatory effects of ZnO. A cytotoxic response was apparent, for both particle sizes at 24 hours, and one week post exposure. Both sizes of particles were capable of eliciting a short term, potent pulmonary inflammatory response that was maximal at 24 hours, was characterised by a neutrophil infiltration and resolved by one month post exposure. The study therefore highlighted the cytotoxic and inflammatory potential of ZnO particles, that was replicated within both nano and micro particulate forms.

The results of the studies outlined imply that the toxic potential of TiO₂ was primarily dictated by particle size, and crystallinity; whereby decreasing particle size, and anatase forms of TiO₂ enhanced particle toxicity. In addition, it is suggested that the exposure scenario (including species used, exposure method and dose administered) was able to impact on the toxicity of metal oxides, which complicates making comparisons between investigations. The pulmonary response to metal oxides in general, but TiO₂ in particular has been demonstrated to be inflammogenic in nature, with a contribution from neutrophils and macrophages (which are elevated and thought to contribute to particle removal), with epithelial damage, oxidative stress and cytotoxicity also being common findings. In addition, chronic exposure to TiO₂ also has the potential to promote tumour development, illustrating their carcinogenic behaviour. The single study investigating ZnO did not demonstrate a difference in the inflammogenicity of particles of different sizes. It is difficult to draw firm conclusions from a single study of this nature, but this would suggest that for this particular metal oxide size might not be as important as for TiO₂. This could potentially be explained by the relatively higher solubility of ZnO, but such a hypothesis requires further investigation. The responses observed within a few studies were measured following relatively high exposure doses. For the TiO₂ samples, variations in crystallinity suggest that it might be difficult to compare across studies and to generate an overall conclusion. Clearer characterisation of the particles used is required within studies in order to identify the attributes of TiO₂, ZnO and NiO that are most influential in driving toxicity. It can also be suggested that systemic responses, following pulmonary exposure are possible. However, this would require further investigations, which could also focus on determining the factors responsible for such a response, if the particles cannot directly access the circulation. In addition, the neuronal transport of nanoparticles has been demonstrated, which offers a potential route for nanoparticle distribution within the body, and enables them to bypass the blood brain barrier, and thereby access the brain.

6.5.2.2 Intraperitoneal exposure to metal oxides

Chen *et al.* (2008) investigated the acute toxicity of TiO₂ nanoparticles (80-100 nm) subsequent to intraperitoneal injection, of mice. The doses used were exceptionally high (ranging from 324 to 2592 mg kg⁻¹), and it is therefore unsurprising that mortality was associated with exposure. Furthermore, TiO₂ was observed to block pulmonary vessels, leading to thrombosis, with pathology also evident within the liver, spleen and kidneys.

The only study that used intraperitoneal injection as a means of particle delivery, highlighted the need to consider the use of relevant doses of particles, as the observed effects derived from the high dose and not the inherent toxicity of particles.

6.5.2.3 Dermal exposure to metal oxides

The impact of metal oxide exposure on the skin is especially relevant due to the inclusion of TiO₂ and ZnO particles within sunscreens and cosmetics that are directly applied to skin. As a consequence, the penetration of particles within the skin, and their dermal toxicity has been a focus of a number of investigations. The skin, and specifically the stratum corneum, is a primary barrier against the penetration of particles contained within dermally exposed substances, and therefore its efficiency will also be discussed.

Mavon *et al.* (2007) determined the distribution of TiO₂ (20 nm) within the skin *in vitro* and *in vivo*. Five hours following the direct topical application of TiO₂ (2 mg cm⁻²) to human skin or to human skin explants, tape stripping (placing and removing adhesive tape onto and from the skin repeatedly and subsequently analysing the components striped from the skin surface) was used to determine the dermal penetration of TiO₂. *In vivo*, and *in vitro*, the majority of the TiO₂ was contained within the stratum corneum, with minimal distribution within the epidermis. Therefore in both preparations, TiO₂ presence decreased with increasing depth of the skin, so that the penetration of TiO₂ within skin, past the stratum corneum into viable skin layers, was minimal.

Schulz *et al.* (2002) also determined the influence of particle size, coating and shape on TiO₂ skin penetration. A number of TiO₂ containing sunscreen formulations were tested that had different particle surface characteristics; trimethyloctylsilane coated (20 nm), or aluminium oxide (Al₂O₃) and silica (SiO₂) coated (10-15 nm). Formulations were topically exposed to human skin at a concentration of 4 mg cm⁻², for 6 hours, and skin biopsies taken. All particle types were solely located on the outermost surface of the stratum corneum, and did not penetrate deeper to subcutaneous, epidermis or dermis layers.

Kiss *et al.* (2008) evaluated the barrier function of skin, within human foreskin grafts transplanted onto severe immunodeficient mice. TiO₂ particulate containing sunscreen was administered (2 mg cm⁻²), via an occlusive bandage, to skin grafts for 24 hours, and the penetration of TiO₂ determined within skin biopsies. It was found that TiO₂ particles did not penetrate through the stratum corneum of human skin transplants. The stratum corneum was therefore accepted as being an adequate, effective barrier against TiO₂ penetration in intact human skin. However, the authors noted that as it is likely that TiO₂ exposure occurs when skin barrier functions may be impaired (such as sunburn), and as a consequence, TiO₂ may come into direct contact with underlying skin cells, a concept which has yet to be investigated.

*The findings from the available studies demonstrated that the penetration of TiO₂ is negligible within the skin. This is important, as nanoparticles appear to be unlikely to reach the living cells present within the deeper skin layers, and thus their propensity for toxicity is anticipated to be minimal. Therefore, it is relevant, within future studies, to consider the fate of particles within skin models that take into consideration that sunscreens are often applied to burnt, damaged and diseased skin, where the barrier function of the stratum corneum will inevitably be impaired (Borm *et al.* 2006; Kiss *et al.* 2008). This has been discussed within several investigations but not actually studied.*

6.5.2.4 Oral exposure to metal oxides

Only one study could be identified that addressed the oral exposure of TiO₂. Wang *et al.* (2007) investigated the distribution and acute toxicity of nanoparticulate (25 and 80 nm) and microparticulate (155 nm) forms of TiO₂, following the oral exposure (5 g kg⁻¹) of mice. TiO₂ particles of all sizes, 2 weeks post exposure, were distributed to the liver, spleen, lungs, kidneys, thus providing evidence that they could be transported to other sites subsequent to exposure, due to their translocation into the blood. Within the liver, nanoparticles initiated an inflammatory response, with liver damage also indicated by a rise in serum transaminases, and hepatic necrosis was revealed in histopathological investigations. Markers of cardiac damage were also observed to be elevated by TiO₂ nanoparticles, within the serum. Limited toxicity was associated with microparticle exposure.

The only study identified that evaluated the consequences of oral exposure to metal oxides provided interesting results. Specifically the potential for metal oxide particle transfer into the blood from the GIT was demonstrated, which would necessitate that they are able to pass through the gut wall. It also revealed that the liver was a primary target for the toxicity of metal oxides. However, the applicability of the finding to metal oxides as a whole, or to other exposure sites requires consideration in future experiments, as the transfer of particles to the circulation is likely to impact on their toxicity, and a requirement to drive appropriate investigations that determine the impact of particle exposure on relevant target organs. However, the use of exceptionally high dose used within the study, undermines the relevancy of the results, specifically in terms of their applicability to humans, as such exposure levels are unexpected.

6.5.2.5 Cardiovascular consequences of metal oxide exposure following intravenous exposure

The toxicity of iron oxide nanoparticles requires investigation following intravenous exposure, due to their development within medical applications including contrast agents for magnetic resonance imaging (MRI) (reviewed in Sosnovik *et al.* 2008). This is exemplified by the findings of Reynolds *et al.* (2006) who found that iron oxide nanoparticles (that were surface coated with dextran and modified with anti-E-selectin antibodies) could be used to detect E-selectin on activated vascular endothelium, using MRI, within an *in vivo* mouse model (oxazolone-induced) of ear inflammation. This binding was selective, as evidenced by the fact that the nanoparticles only accumulated within inflamed tissues, following intravenous administration. This finding was investigated *in vitro*, whereby the iron oxide nanoparticles were observed to bind to CHO cells, expressing E-selectin. Therefore, it was suggested that the expression of E-selectin during inflammatory responses could be imaged using iron oxide nanoparticles. Similarly, Manninger *et al.* (2005) demonstrated that iron oxide nanoparticles could be used as imaging agents to detect inflammation within the brain, in humans, which is driven by transport across the blood-brain barrier (BBB), and their accumulation within macrophages. Iron oxide nanoparticles may therefore be useful when diagnosing and monitoring inflammatory responses. However, the detrimental consequences associated with the exploitation of iron oxide nanoparticles as imaging agents requires consideration in the future. Only one study purporting to study the cardiovascular toxicity of metal oxide particulates could be identified, and related to their ability to inflict systemic effects, following pulmonary exposure.

There is a lack of studies available that investigate the detrimental consequences of metal oxide exposure on the cardiovascular system. This is particularly concerning due to the potential exploitation of iron oxide nanoparticles as imaging agents. Consequently, this property may be negated by their ability to elicit toxicity, following exposure. This is of particular relevance as TiO₂ nanoparticles may promote an inflammatory and oxidative driven response within vessels, and thereby contribute to vascular disease, however this requires further investigation.

6.5.2.6 ADME Profile of metal oxides

Determining the kinetics of metal oxides within the body, subsequent to exposure (via the lungs, gut and skin) is necessary to identify potential targets of toxicity. This is necessary, as the

delivery of metal oxide particles to target organs, such as the liver or brain, requires their transfer into blood, or within neurones from their exposure site. Therefore their likelihood of accessing different organs and tissues within the body is of relevance, and necessary to determine in order to assess the relevancy of *in vitro* investigations that assess toxicity at various target sites. However, a number of barriers (at the exposure site and those apparent within secondary targets) are in place to prevent against uptake, and it is necessary to determine if they are overcome by metal oxides, to determine their systemic uptake and therefore availability.

It is necessary to consider the potential for particle adsorption from the site of exposure into the blood, as this will dictate their systemic availability and distribution within the body. Only one example of particle translocation into the blood from the gut could be identified. Specifically, TiO₂ nanoparticles were observed to translocate into the blood, following oral exposure, and thereafter distribute to secondary targets, including the liver, spleen, lungs, and kidneys (Wang *et al.* 2007).

Studies investigating dermal exposure of TiO₂ suggest that penetration of nanoparticles past the stratum corneum is negligible, and therefore it is unlikely that the particles will access the circulation via this route.

Wang *et al.* (2008a and 2008b) illustrated the transfer of TiO₂ nanoparticles to the brain, following intranasal exposure, which occurred via their transport within neurones. In line with these findings, Elder *et al.* (2006) demonstrated that manganese oxide nanoparticles were able to accumulate within the brain, and liver following intranasal exposure, which may occur via blood or neuronal transport.

No evidence of metal oxide metabolism, or elimination from the body could be identified in the literature at this time.

The translocation of particles, subsequent to pulmonary and oral exposure should encompass the possibility that distal sites are affected; including the CNS, liver, and cardiovascular system. The translocation is likely to be due to their passage into blood, but may also be mediated by neuronal transport. Both these processes would allow for their transport to targets sites (such as the brain), and within different regions of a particular target (including different brain regions). Applicability of the findings to other metal oxides, and other species requires assessment. Further investigations would therefore be required to confirm the findings, as the distribution profile of metal oxides is currently based on a limited number of studies. In contrast the penetration of particles within the skin is likely to be negligible, and so particles are unlikely to become systemically available, following exposure via this route. The elimination of particles from the body is also a necessary consideration, which would provide information regarding the longevity of particles within the body, and therefore their propensity for damage.

6.5.2.7 Distribution of metal oxides particles following intravenous exposure

Following injection, it is necessary to determine particle distribution, and therefore targets for toxicity. There are limited available studies that purport to investigate this.

Fabian *et al.* (2008) determined the tissue distribution of TiO₂ nanoparticles (20-30 nm) within rats, at 1, 14 and 28 days post exposure, via intravenous injection (5 mg kg⁻¹). TiO₂ was cleared from the blood and primarily accumulated within the liver, but was also apparent within the spleen, lungs and kidneys. The level of TiO₂ was retained over the observation time within the liver, however levels decreased with time within the other organs. No serum cytokine or enzyme changes, which insinuated that no toxicity was associated with TiO₂ exposure, however further investigations, including histopathological analysis would be necessary to confirm this.

Muldoon *et al.* (2005) exposed rats to numerous commercial preparations of iron oxide, which were either injected directly into the cerebellum, into the blood of rats with an incomplete BBB, or into rats with intracerebral tumour xenografts, in order to assess the distribution and neurotoxicity of the particles. MRI imaging revealed that the MRI signal declined over weeks to months following the intracerebral injection, suggesting that the particles were cleared from the

CNS. For the nanoparticles injected into rats with a defective BBB, a CNS MRI signal could be detected transiently for some samples (3 days) and more long term for others (28 days). These exposures were not associated with pathological changes to the CNS of normal rats. However, in one of the tumour models investigated, some tumour enhancement was observed in one animal. The authors suggested that these results demonstrate the safety of the commercially available iron oxide preparations used for MRI. Further work is required to verify this result.

The studies identified suggest that following intravenous administration, metal oxide particles were cleared from the blood and were able to accumulate primarily within the liver, but also the lungs and spleen, which is likely to derive from their uptake by the resident macrophage populations. Further investigations are necessary to determine if toxicity is associated with such an accumulation. In the one study identified, where nanoparticles of metal oxides were injected directly into the brain, minimal toxicity was observed, along with their clearance from the body. Again this requires verification with a wider variety of particles.

6.5.3 *In vitro* investigations of metal oxide toxicity

6.5.3.1 Lung models

Investigations that assess the pulmonary toxicity of metal oxide particles *in vitro* have used a variety of models including macrophages (due to their contribution to clearance), epithelial cells (due to their abundance, and expected interaction with particles) and explanted tissue.

Park *et al.* (2008b) investigated the cytotoxicity of TiO₂ nanoparticles (21 nm) to BEAS-2B lung epithelial cells, at concentrations ranging from 5 to 40 µg ml⁻¹, for up to 96 hours. A dose and time dependent decrease in cell viability was observed. Caspase-3 was activated by TiO₂, with chromosome condensation also observed, which was suggestive that an apoptotic mechanism of cell death was involved. Reactive oxygen species (ROS) production, glutathione (GSH) depletion, and increased hemeoxygenase (HO-1) expression was evident, thereby implying that an oxidative mechanism drove the cytotoxic response. IL-8, IL-1, IL-6, TNFα, and CXCL2 (neutrophil chemoattractant) cytokine gene expression were increased, insinuating that an inflammatory response was also stimulated by TiO₂ exposure.

Churg *et al.* (1999) determined the impact of TiO₂ exposure (up to 7 days), in microparticulate (0.12µm) or nanoparticulate (0.021µm) forms, on the development of a fibrotic response, within the airway wall on rat tracheal explants. It was observed that particles were internalised by epithelial cells, and were then able to pass into the interstitium. Nanoparticles stimulated growth factor release (such as platelet-derived growth factor (PDGF)) and enhanced pro-collagen expression. The results, suggested by the authors, were that the nanoparticles were more fibrogenic in nature, which is anticipated to contribute to airway obstruction *in vivo*. However, TGFα and TGFβ expression was increased for microparticles only, but no fibrotic-like morphological changes occurred, and so it was suggested that these mediators were not driving the fibrotic response mediated by nanoparticles.

Gurr *et al.* (2005) investigated the oxidative damage exhibited by TiO₂ (10, 20 or >200 nm) within bronchial BEAS-2B cells. The oxidative potential of TiO₂ was supported by the findings that lipid peroxidation (indicated by increased MDA) and ROS (hydrogen peroxide (H₂O₂)) and reactive nitrogen species (nitric oxide (NO)) production were only enhanced by nanoparticulate forms of TiO₂. DNA adducts were only formed following exposure of cells to TiO₂ nanoparticles. Therefore, the oxidative and genotoxic potential of nanoparticulate forms of TiO₂ was superior to that of their larger counterparts.

Simon-Deckers *et al.* (2008) investigated the toxicity of a panel of particles, including TiO₂ (in anatase and rutile forms, and a variety of sizes), and Al₂O₃, to A549 cells. In general, the cytotoxicity of the particles was low, despite the fact that they were all internalised by cells, and contained within cytoplasmic vacuoles or lysosomes. However, the crystal phase of TiO₂ influenced their cytotoxicity, so that the greater the anatase content, the greater the ability to induce cell death. In addition, the size of nanoparticles was suggested to contribute to their toxicity, as TiO₂ nanoparticles were more toxic than their larger counterparts. However, the chemical composition of nanoparticles was also thought to impact on their toxicity, as 12 nm

TiO₂ nanoparticles were more toxic than 11 nm Al₂O₃, despite their similarity in size. The size, composition and crystal phase of particles were all suggested to influence nanoparticle toxicity, despite the fact that the toxicity of nanoparticles used was generally low. However it is acknowledged that investigating size dependent effects of particles are confounded by changes to other attributes such as the sample phase, consequently determining the properties of TiO₂ that drive toxicity is difficult.

Kim *et al.* (1999) assessed TiO₂ (1µm) mediated cytotoxicity to alveolar macrophages, when exposed to concentrations ranging from 0.5 to 5 mg ml⁻¹, for up to 5 hours. TiO₂ elicited a dose dependent decrease in cell viability, which was suggested to be mediated by suppressed ATP generation, and to be reliant on the interaction of TiO₂ with scavenger receptors on the cell surface. It is relevant that the response of silica particles (1.6µm) was also considered. Silica had a greater cytotoxic potential than TiO₂ and the mechanisms underlying the response were different.

Sayes *et al.* (2007) investigated the toxicity of ZnO, and determined the predictive nature of *in vitro* testing. *In vitro* studies determined the impact of ZnO on the L2 epithelial cell line, primary rat alveolar macrophages and macrophage/epithelial co-cultures (1-30 mg ml⁻¹) for 1 to 48 hours. A cytotoxic response was observed in all cell models investigated, although the epithelial cells were most sensitive. No increases in MIP-2, TNFα or IL-6 were observed, and so these markers were not considered relevant for predicting inflammatory responses *in vivo*. It was concluded by the authors that *in vitro* experimentation could provide useful mechanistic insights into what drives ZnO toxicity, but they suggested that the results do not accurately reflect *in vivo* toxicity.

As responses of organs and tissues are likely to involve interactions between different cell types, it is necessary to incorporate this within *in vitro* tests. Barlow *et al.* (2005) investigated the ability of L2 epithelial cells to modulate macrophage migration *in vitro*, on exposure to nano and microparticulate forms of TiO₂ and CB. TiO₂ and CB induced a dose dependent decrease in epithelial cell viability, which was greatest for nanoparticles (when exposed at concentrations ranging from 62.5 to 2000 µg ml⁻¹ for 24 hours). Conditioned medium (obtained from epithelial cells treated with particles) was able to increase macrophage migration, but only for ultrafine carbon black particles. Therefore, TiO₂ nanoparticles were unable to stimulate the release of chemotaxins from epithelial cells, which the authors suggest could be due to their smaller surface area, when compared to that of ultrafine carbon black.

Cerium dioxide (CeO₂) has been developed as a fuel additive to reduce particulate matter emissions from diesel engines, in an attempt to reduce the adverse health effects ordinarily associated with particulate exposure (Fall *et al.* 2007). Fall *et al.* (2007) demonstrated that the CeO₂ content of diesel exhaust fumes was unable to impact on the toxicity of CeO₂ supplemented diesel, within rat lung slices. On exposure of lung slices to freshly generated fumes, no alterations in cell viability, pro-inflammatory mediator expression (TNFα) and antioxidant enzyme activity (glutathione peroxidase, superoxide dismutase) were identified. However, it was demonstrated that the antioxidant enzyme catalase, and GSH were elevated, at low concentrations of CeO₂, which is likely to derive as a defence response. Overall, it was concluded that CeO₂ could be tolerated at a high dose. In contrast, exposure of lung slices to non-supplemented fuel was able to moderately decrease ATP content, and decrease GSH levels. Therefore, it is implied that the addition of CeO₂ introduces a protective effect. As a result, the authors concluded, that there is little risk associated with exposure to diesel fume generated from CeO₂ supplemented diesel fuel.

Lin *et al.* (2006) investigated the effects of CeO₂ (20 nm diameter) on the A549 epithelial cell line. Cells were exposed to CeO₂ at concentrations of up to 23 µg ml⁻¹ for up to 72 hours. A dose and time dependent increase in cytotoxicity and ROS production, antioxidant (GSH and α-tocopherol) depletion and lipid peroxidation (indicated by malondialdehyde) were observed. This study therefore highlighted the oxidative and cytotoxic behaviour of CeO₂. Similarly, in a study by Park *et al.* 2008a the cytotoxicity and oxidative potential of CeO₂ nanoparticles (15, 25, 35, 40 nm) to BEAS-2B epithelial cells was investigated, at concentrations up to 40 µg ml⁻¹, for a period of up to 96 hours. CeO₂ elicited a dose and time dependent decrease in cell viability within BEAS-2B cells, with all particle sizes eliciting a similar response. Cytosolic caspase 3

activation and chromosome condensation were also observed, suggesting that an apoptotic mode of cell death was involved. CeO₂ exposure was also associated with enhanced cellular ROS production, and a decrease in cellular GSH, so that the cytotoxic response was assumed to be driven by oxidative stress. No cytotoxic response was observed within H9C2 cardiomyocyte or T98G brain fibroblast cells, indicating that some cell types may be more sensitive to the effects of CeO₂.

From the investigations discussed, it is evident that metal oxide particles are able to detrimentally affect both lung derived macrophage and epithelial cells; so that in general particle mediated oxidative, inflammatory and genotoxic effects eventually culminate in a cytotoxic response. However, in some situations there was no evidence of toxicity. These differences may be derived from the model used, or the particle under investigation. From the results obtained it is not possible to rank the metal oxide particles in terms of their pulmonary toxicity in vitro. Although, it is of interest that the mechanisms driving the toxicity of metal oxides in vitro (particularly inflammation and oxidative stress) are also witnessed in vivo.

6.5.3.2 Dermal models

The penetration of particles within the skin has been a focus of a number of *in vitro* investigations. Gamer *et al.* (2006) evaluated the penetration of sunscreen formulations containing μm -sized TiO₂ and ZnO (up to 400 $\mu\text{g cm}^{-2}$, for 3 to 24 hours) within porcine skin explants. It was observed that both particle types were limited to the stratum corneum, following topical application. Specifically, the particles were deposited on the skin surface, within the outmost layer of the stratum corneum, as evidenced by removal via washing or tape stripping analysis. Particles were therefore not detected to penetrate into the deeper stratum corneum layers, epidermis or dermis.

Similarly, Pflucker *et al.* (1999) illustrated that TiO₂ nanoparticles (20-50 nm) did not penetrate porcine skin *in vitro*. A TiO₂ emulsion (4 mg cm^{-2}) was topically applied to excised pig skin for 24 hours. Again TiO₂ was exclusively located within the outermost layer of the stratum corneum, with no distribution to the underlying living cell layers, within skin biopsies, following tape stripping. Consequently, this study suggests that the intact stratum corneum is considered to be an effective barrier to TiO₂, and that the penetration of particles within the skin is negligible.

In line with these findings, Cross *et al.* (2007) investigated the ability of ZnO nanoparticles (26-30 nm) to penetrate the stratum corneum of human skin explants *in vitro*, following topical administration of the ZnO containing formulations (10 $\mu\text{l cm}^{-2}$) for 24 hours. The presence of ZnO was limited to the outer surface of the stratum corneum, and so did not distribute to underlying structures. Consequently, the epidermal penetration of ZnO was negligible. However, Zn²⁺ ions were able to penetrate past the stratum corneum, subsequent to their release from a small proportion of ZnO particles, so that particle dissolution may be an important component of their toxicity.

In addition, Dussert *et al.* (1997) investigated the distribution of ZnO and TiO₂ nanoparticle containing sunscreen formulations within human skin explants. Again, sunscreens were topically applied to skin but no penetration of particles, or intracellular delivery was observed past the stratum corneum skin surface.

Park *et al.* (2007) investigated the toxicity of nano (9 nm) and micro (320 nm) forms of CeO₂, to the skin, *in vitro*. The EpiDerm skin model revealed that both forms of CeO₂ tested were potential skin irritants. No cytotoxicity or mutagenicity (within the Ames test) was evident. No size dependent toxicity was observed within this study.

The functional implications of the exposure of the various skin cell populations have also been determined. From the data available for TiO₂ and ZnO particles, such studies are potentially more relevant to compromised/damaged/diseased skin, than normal healthy skin. There is a need to consider the distribution of particles within damaged or sunburnt skin, which would be likely to promote the penetration of particles, due to damage to the stratum corneum. There is

little or no data currently available to assess the impact of such damage on penetration or hazard.

HaCaT keratinocytes, human dermal fibroblast cells, SZ95 sebaceous gland cells and primary human melanocytes were exposed by Kiss *et al.* (2008) to TiO₂ at concentrations of 0.15 to 15 µg cm⁻², for up to 4 days. Particles were internalised into the cytoplasm, and perinuclear region of fibroblasts and melanocytes, and this uptake was associated within an increase in intracellular calcium. However no particle uptake, or alterations in calcium signalling were observed within keratinocytes or sebocytes. A dose and time dependent decrease in cell proliferation was evident within all cell types, and an increase in cell death (via apoptosis) within fibroblasts was apparent. The direct contact of cells with TiO₂ particles is therefore of concern, as it can disturb normal cell functions within the different skin cell populations, but it is noteworthy that all cells were not affected similarly.

Jin *et al.* (2008) determined the cytotoxicity of TiO₂ (20-100 nm) to L929 fibroblasts at concentrations of 3-600 µg ml⁻¹, for up to 48 hours. There was a time and dose dependent decrease in cell viability induced by TiO₂. An increase in ROS production and decreases in GSH and SOD activity were observed, thereby implying that oxidative stress was a feature of the response. TiO₂ was suggested to be internalised by phagocytosis, and were contained within lysosomes. Analysis of cell morphology illustrated that cell morphology was detrimentally affected by TiO₂, and that cell adhesion and proliferation was prevented, confirming the decrease in cell survival.

Sharma *et al.* (2009) investigated ZnO nanoparticle (30 nm) toxicity to the skin *in vitro*. This was achieved through the exposure of A431 skin epithelial cells to ZnO nanoparticles at concentrations of 0.008-20 µg ml⁻¹, for up to 48 hours. A dose and time dependent decrease in cell viability was observed within 3 separate cytotoxicity tests. Alterations to cell morphology were also evident, so that cells retracted into a spherical shape, detached from the surface, and formed clusters. The Comet assay revealed that ZnO elicited DNA damage. The toxicity of ZnO was anticipated to be driven by oxidative stress due its ability to elicit a depletion in glutathione, SOD and catalase from cells, and ability to stimulate an increase in lipid peroxidation (using increased hydroperoxide as a marker).

The studies conducted demonstrated that the toxicity of metal oxides to skin cells is of concern, with oxidative, genotoxic and cytotoxic consequences evident, and likely to act in concert. However, in order to execute these effects, particles would be required to penetrate past the stratum corneum, to reach the underlying cell populations, which is unlikely to occur in healthy skin. As noted previously, the penetration of particles within damaged skin should therefore be considered in future experiments.

6.5.3.3 GIT models

Only one study could be identified that evaluated the impact of metal oxide exposure on the GIT, *in vitro*. Zhang *et al.* (2004) investigated the contribution of photoexcitation, to the cytotoxicity of TiO₂ (21 nm) within human colon carcinoma Ls-174-t cells. Limited cytotoxicity of cells was observed, following TiO₂ exposure (up to 1000 µg ml⁻¹) for 24 hours, in the absence of ultraviolet (UV)A irradiation. In contrast, a high level of TiO₂ induced cell death was observed in the presence of UVA light, which was a dose and time dependent phenomenon. Therefore, TiO₂ particles and light irradiation therapy was suggested to be a suitable candidate for the treatment of cancer, and warrants further investigation. However, as UV light cannot penetrate the human body, the exploitation of this for tumour treatment will be restricted.

Investigation into the consequences of metal oxide exposure within the gastrointestinal tract is severely lacking. The only study that was identified suggested that a photocatalytic component of the response was integral to the toxicity of TiO₂. However, as this would not occur in normal conditions, but could be exploited for a specific purpose such as the treatment of cancer, but the specificity of the response would require investigation.

6.5.3.4 Liver models

Hussain *et al.* (2005) compared the impact of a range of nanoparticles, including iron oxide (Fe_3O_4 , 30 nm), and TiO_2 (40 nm) on BRL 3A liver cells, at concentrations up to $250 \mu\text{g ml}^{-1}$, following a 24 hour exposure. Both particle types were able to decrease cell viability, but only at the highest concentration tested.

Linnainmaa *et al.* (1997) assessed the toxicity of TiO_2 , in nanoparticle (rutile and anatase (20 nm)), and microparticulate (170 nm) forms) to rat liver epithelial cells, in the presence and absence of UVA light. No cytotoxicity or chromosomal damage was observed within cells exposed to all TiO_2 particle types in the presence or absence of UVA.

The two studies that investigated the toxicity of metal oxide particles to the liver, suggest that metal oxides exhibit a low level of toxicity to liver cells. However, the liver is formed from a collection of cells, and so the response of the different cell populations requires assessment. The consequence of liver exposure to metal oxides require further investigation due to evidence of preferential nanoparticle accumulation within this organ.

6.5.3.5 Cardiovascular models

The interaction of metal oxides with endothelial cells that line blood vessels has been a focus of a number of investigations, to determine the consequences of particle transport with the blood. In addition, the impact of metal oxides on cardiomyocyte function has been investigated.

Gojova *et al.* (2007) exposed HAEC endothelial cells to Fe_2O_3 (47 nm), ZnO (20-70 nm) or yttrium oxide (Y_2O_3 , 20-60 nm) nanoparticles *in vitro* (up to $50 \mu\text{g ml}^{-1}$, for 1 to 8 hours). Fe_2O_3 particles had no effect on endothelial cell pro-inflammatory (ICAM-1, IL-8 and MCP-1) gene expression. In contrast, ZnO and Y_2O_3 stimulated a pro-inflammatory response, that was most pronounced for ZnO. ZnO exposure was also associated with cytotoxicity, which was not observed with the other particle types. However, this was concluded from the trypan blue assay, and so more comprehensive tests (such as the MTT or LDH assays) would be useful to confirm this. Differences in the internalisation of the different particle types were not thought to be attributable for the varied toxicity evident, as all nanoparticle types were internalised by the cells, and were predominantly located within cytoplasmic vesicles. Therefore, further studies are required to address what properties of metal oxide particles are responsible for determining their inflammogenicity within endothelial cells, including the contribution of composition, and surface area. The findings are of concern, as exposure of humans to nanoparticles contained within the environment is known to increased CV disease (Schwartz, 1994), and as endothelial inflammation is a critical, early event within the progression of CV disease.

Oesterling *et al.* (2008) exposed primary vascular porcine endothelial cells and endothelial HUVEC cells, to aluminium oxide nanoparticles (10-20 nm) to determine their impact on endothelial function (at concentrations up to $250 \mu\text{g ml}^{-1}$ for a period of up to 40 hours). It was observed that the nanoparticles were able to increase the expression of cell adhesion molecules (including VCAM-1 and ICAM-1) in cells, which caused the increased adhesion of activated monocytes. These findings demonstrate that exposure of blood vessels to nanoparticles encourages the recruitment of inflammatory cells from the blood that may favour the development of a pro-inflammatory response within the tissue supplied by the affected blood vessel. However there is also the potential for nanoparticle exposure to contribute to the progression of inflammatory diseases within blood vessels, including conditions such as atherosclerosis (Oesterling *et al.* 2008). These endpoints therefore require further consideration in the future.

Helpenstein *et al.* (2008) observed that TiO_2 (up to $125 \mu\text{g ml}^{-1}$) was able to affect cardiomyocyte electrophysiology, enhance ROS production, and reduce myofibril organisation. A panel of particles was tested, of which diesel exhaust particles (DEPs) were demonstrated to exhibit the greatest toxic potency, and SWCNT were found to have limited toxicity in comparison. The findings from this study suggest that cardiac cells may be damaged by TiO_2 or DEP exposure, and so were unable to function normally *in vitro*.

Courtois *et al.* (2008) determined the impact of TiO₂ (15 nm or 0.4µm diameter) exposure on NO mediated relaxation of pulmonary arteries *in vitro*. Pulmonary arteries were exposed to particles for 24 hours (at a concentration of up to 200 µg ml⁻¹), in the presence or absence of vascular relaxants. It was found that urban particulate matter was able to impair NO-dependent relaxation within intrapulmonary arteries *in vitro* and *in vivo* (following intratracheal exposure), but in contrast, manufactured nanoparticles, including TiO₂ did not exhibit this effect. Therefore this study suggested that the pulmonary circulation was not affected by TiO₂ exposure, and that cells exhibited differed in their sensitivity to particles.

Peters *et al.* (2004) also evaluated the impact of a nanoparticle panel (including TiO₂, 70 nm) on HDMEC endothelial microvascular cell viability and function, following an exposure time of up to 72 hours, at concentrations ranging from 0.5 to 50 µg ml⁻¹. TiO₂ were relatively non-toxic, with no cytotoxicity, and minimal IL-8 release stimulated by exposure. However nanoparticles were internalised by cells into cytoplasmic vacuoles.

A limited number of available studies were conducted to determine the impact of metal oxide particles on in vitro models of the cardiovascular system. However, the findings suggest that metal oxides are able to detrimentally affect endothelial function and promote an inflammatory response, which has the propensity to stimulate cardiovascular disease. Consequently, the ability of TiO₂ to contribute to inflammatory mediated disease requires further consideration in vivo. In addition, the ability of metal oxides to negatively impact of cardiomyocyte function has the ability to disturb normal cardiac electrophysiology. Again, further studies, in vivo, would be required to verify this observation.

6.5.3.6 CNS models

Investigations regarding the neurotoxicity of metal oxides are limited, with some concentrating on the oxidative properties of TiO₂, on exposure to microglia and neurones, *in vitro*.

Long *et al.* (2006) established the contribution of oxidative stress to the neurotoxicity of TiO₂. BV2 microglia cells were exposed to TiO₂ for periods of up to 120 minutes, at concentrations ranging from 5 to 120 ppm, and ROS production determined. The oxidative response mediated by TiO₂ within microglia had two components, firstly, a rapid increase in ROS production was observed at 1-5 minutes, and termed an oxidative burst. This was followed by a greater, sustained ROS release from 60-120 minutes. It is also relevant, that despite having a primary particle size of 30 nm, the particles were observed to aggregate within the cell culture medium, which increased with increasing concentration, and this appeared to promote ROS production, perhaps due to the greater uptake of larger structures by phagocytosis. The particles were internalised by cells, and found within the cytoplasm, and mitochondria located in the vicinity of the aggregates were found to be swollen and disrupted, which was postulated by the authors to promote an apoptotic or necrotic response.

Similarly, Long *et al.* (2007) investigated the neurotoxicity of nanoparticulate TiO₂ (up to 120ppm). It was illustrated that particle aggregates were phagocytosed by BV2 microglial cells, and contained within membrane bound vesicles. ROS production by cells was associated with particle exposure, and stimulated the upregulation of genes involved with inflammation, apoptosis, and cell cycling, and a down-regulation in energy metabolism. It was therefore evident that increased ROS production as a consequence of TiO₂ exposure also triggered cytotoxicity via apoptosis. However, TiO₂ exposure was non-toxic to N27 neuronal cells, following a 72 hour exposure, despite being internalised. In contrast there was evidence of neurone loss within primary cultures of rat striatum within 6 hours of exposure, which was suggested to occur via microglia mediated ROS production.

Similarly, Pisanic *et al.* (2007) found that Fe₂O₃ nanoparticles were able to induce a dose dependent cytotoxic effect, and decrease neurite generation within PC12 cells.

Information regarding the neurotoxicity of metal oxides is severely lacking. However, it is evident that the response is likely to be dictated by the cell type under investigation. Again, an oxidant driven response appears to be integral to particle toxicity. At present, there is insufficient evidence to make generalisations regarding metal oxide mediated neurotoxicity.

6.5.3.7 Kidney models

Only one study was found that assessed the toxicity of metal oxide nanoparticles to the kidneys, *in vitro*. L'azou *et al.* (2008) investigated the effect of TiO₂ (15 nm or 50 nm) on IP15 mesangial, and LLC-PK₁ proximal tubular epithelial renal cells (at concentrations up to 512 µg ml⁻¹, for 24 hours). The different cell types exhibited different sensitivities to the toxicity of TiO₂. No cytotoxicity was observed within IP15 cells on exposure to TiO₂. However, TiO₂ elicited a cytotoxic response within LLC-PK₁ cells, suggesting that this cell type was more sensitive to TiO₂ toxicity, which was postulated to derive from their high endocytic activity, and therefore greater internalisation of particles. The cytotoxic response was also observed to be size dependent, with a greater response exhibited by smaller particles. TiO₂ (15 nm) was also able to induce morphological changes within both cell types (namely cell shrinkage, and detachment), and was internalised into cytoplasmic vacuoles. TiO₂ was unable to induce an increase in ROS production. However, 13 nm carbon black nanoparticles were consistently recognised as being more toxic, in terms of cytotoxicity, and ROS production, which was evident in both cell types. Toxicity to renal cells was therefore observed to be particle size, particle composition, and cell type specific.

The findings from the only study identified as studying the toxicity of TiO₂ particles to the kidneys, suggest that the response of the individual cell populations vary. However, further investigations would be required to identify if the finding was applicable to other particle types, and if the same response is evident in vivo. Therefore it is difficult to draw definitive conclusions about particle toxicity to the kidney, due to insufficient data being available.

6.5.3.8 Immune system models

It is acknowledged that macrophages are primarily responsible for the clearance of particles at sites of exposure, and accountable for the accumulation of particles within different target sites. The consequences of uptake is therefore of interest, particularly the initiation of an oxidative driven or inflammatory response, and impact on the phagocytic function of macrophages. In addition, the ability of particles to affect other immune cell populations, such as lymphocytes, that they would be expected to encounter within the blood, will be addressed.

Renwick *et al.* (2001) determined the impact of particle exposure on the phagocytic activity of macrophages. It was observed that TiO₂ (or carbon black) particles were able to impair J774.2 macrophage cell phagocytic activity (indicated by the uptake of 2 µm latex beads), in a concentration dependent manner. TiO₂ nanoparticles (29 nm) were more effective at inhibiting macrophage phagocytosis than their microparticulate (250 nm) counterparts, and the same response was exhibited by carbon black particles. It is likely, that the impairment of phagocytosis occurs as a consequence of the fact that cells are overwhelmed by the TiO₂ particle burden, and are therefore unable to further internalise the latex particles. This was suggested to be the case, as an increase in particle-laden macrophages correlated with a decrease in phagocytic cells. The impairment in phagocytic function of macrophages would be anticipated to impact on the clearance of particles, thus increasing their retention, and thereby prolonging their interaction with cells, and thus increasing their propensity for damage.

Afaq *et al.* (1998) demonstrated that alveolar macrophages were increased in rats on intratracheal exposure (2 mg per rat, with observations made for a period of up to 16 days) to TiO₂ nanoparticles (<30 nm), which was maximal at 8 days post exposure. The exposure of alveolar macrophages to TiO₂ was associated with lipid peroxidation, glutathione depletion and enhanced H₂O₂ production, illustrating the oxidative response that developed. Exposure was also associated with a cytotoxic response.

Kang *et al.* (2008b) determined the mechanisms underlying TiO₂ (25 nm) nanoparticle toxicity within lymphocytes, at concentrations ranging from 20 to 100 µg ml⁻¹, for up to 48 hours. A particular focus of the study was on the oxidative potential of TiO₂. A dose and time dependent decrease in cell viability was observed. TiO₂ nanoparticles also exhibited a genotoxic effect, whereby the frequency of micronuclei formation increased, which insinuated chromosomal damage occurred, on exposure of cells to TiO₂. DNA damage was also observed, using the Comet assay. Importantly, the pre-treatment of cells with the antioxidant N-acetyl cysteine

(NAC), decreased TiO₂ mediated DNA damage, thereby inferring that the response was ROS mediated. ROS production was subsequently confirmed to be increased by TiO₂ exposure. The DNA damage inflicted by TiO₂ resulted in the activation of a protective response in the form of increased levels of p53 protein, highlighting an attempt at repair. TiO₂ mediated increases in ROS production, and therefore DNA damage, was thought to be central to its toxicity within lymphocytes.

The impact of particle exposure on immune cell function is lacking. Alveolar macrophages are recognised as being integral to the removal of particles from sites of deposition, and are, in the main, responsible for the accumulation of particles within different target sites. It is therefore of concern that particles appear to impair macrophage phagocytosis, and this should be considered further in future experiments. It is also suggested that particles initiate an oxidative driven response within immune cells that may have inflammatory and/or cytotoxic consequences.

6.5.4 The biological mechanisms driving metal oxide nanoparticle toxicity

6.5.4.1 Metal oxide mediated inflammatory responses

Inflammatory responses have been illustrated as being a prominent feature of a number of studies that investigated the toxicity of nanoparticles such as metal oxides. *In vivo*, the infiltration of neutrophils has been regularly demonstrated by different investigators in response to TiO₂ nanoparticles (Ferin *et al.* 1992, Renwick *et al.* 2004), in addition to elevated, particle laden macrophage numbers (Grassian *et al.* 2007, Chen *et al.* 2006). This is likely to be mediated by increases in inflammatory mediators, such as IL-8, and TNF α that have been observed *in vivo* (Chen *et al.* 2006) and *in vitro* (Singh *et al.* 2007, Wang *et al.* 2008b, Park *et al.* 2008a). In addition, the inflammogenic nature of metal oxides has been observed within a number of target sites including the lung, brain, and endothelial cells.

However different metal oxide types are also anticipated to elicit inflammatory responses that vary, with regards to their potency. This is exemplified by the finding that iron oxide nanoparticles (45 nm) failed to elicit an inflammatory response in HAEC endothelial cells, whereas ZnO particles (47 nm) induced considerable cell death and increased the expression of ICAM, IL-8 and MCP-1 in a study by Gojova *et al.* 2007. This is likely to derive from their physico-chemical characteristics that alter the cell sensitivity to the particles.

Inflammation has been demonstrated to be recurrently associated with nanoparticle exposure, with its manifestation evident on a number of occasions, within different models and for a number of particle types, including a variety of metal oxides. Inflammatory responses are often thought to derive from an increase in ROS which stimulates transcription factor activation, and therefore activation of pro-inflammatory genes.

6.5.4.2 Metal oxide mediated oxidative responses

A focus of a number of investigations has been the contribution of oxidative stress to inflammatory and cytotoxic responses elicited by metal oxides. Studies previously discussed found that oxidative stress was a prominent feature of the response to metal oxide nanoparticles. These include evidence of increased ROS production, depletion of cellular antioxidants or increase in oxidative products (such as lipid peroxidation), in a variety of cell types, including for example; lung epithelial cells (Park *et al.* 2008a and 2008b), fibroblasts (Jin *et al.* 2008), and microglia (Long *et al.* 2006).

It is acknowledged that the development of a moderate level of oxidative stress is connected to the initiation of inflammatory responses through the activation of ROS sensitive signalling cascades (Nel *et al.* 2006). Consequently Kang *et al.* (2008a) compared the ability of nano (21 nm) and microparticulate (1 μ m) forms of TiO₂ (0.5-200 μ g ml⁻¹) to initiate an oxidative response within RAW 264.7 macrophages, following exposure for up to 24 hours. No alterations in cell viability were observed on exposure of cells to both forms of TiO₂. This finding is of importance, as when assessing the processes that underlie particle toxicity, sub-lethal concentrations are

required. ROS production was increased by TiO₂, and was greatest in magnitude for the nanoparticles. The ROS sensitive mitogen-activated protein kinase (MAPK) signalling pathway was activated by TiO₂ exposure (indicated by ERK1/2 phosphorylation), which was suggested to initiate the increase in pro-inflammatory mediator production (TNF α , MIP-2). Within the endpoints measured, the response exhibited by nanoparticles was consistently greater than that of their larger counterparts, when administered at an equivalent mass basis. The oxidative stress paradigm therefore held true within this study; specifically that particle mediated ROS production (at a moderate level) stimulates signalling cascades that promote the activation of transcription factors that, in turn, initiate an inflammatory response. Accordingly, the ability of particles to promote ROS production is thought to be central to their ability to regulate inflammatory responses.

In line with these findings, Xia *et al.* (2008) investigated whether ZnO (13 nm), TiO₂ (11 nm) and CeO₂ (8 nm) oxidative stress development or particle dissolution contributed to their toxicity. RAW 264.7 macrophages and BEAS-2B epithelial cells were exposed to the nanoparticle panel for up to 15 hours, at concentrations up to 60 $\mu\text{g ml}^{-1}$. Only ZnO particles were capable of inducing cytotoxicity, within both cell types. In addition, ZnO stimulated increased ROS production, which in turn, increased HO-1 expression, and activated the JNK signalling pathway, which paralleled the release of IL-8 and TNF α . Increased calcium release was also mediated by ZnO, and was associated with mitochondrial damage. These findings therefore imply that ZnO elicits an oxidant driven response that was responsible for the initiation of an inflammatory and cytotoxic response. On the contrary, CeO₂ was able to stimulate a cytoprotective response, whereby pre-treatment of cells with CeO₂ protected against diesel exhaust particle mediated cell damage (which is known to be oxidant driven). All particle types were internalised by an endocytic mechanism, however, only ZnO accumulated within lysosomes, which was suggested to drive their ability to inflict oxidative injury, and promote particle dissolution. ZnO also induced ultrastructural alterations, including nuclear fragmentation, apoptotic body formation and mitochondrial disappearance. ZnO was therefore recognised as being the most toxic particle within the panel, however this was thought to be accounted for, in part, by their dissolution and therefore release of Zn²⁺ ions. The ability of ZnO particles to generate ROS, oxidant injury, inflammation and cell death was demonstrated within this study, highlighting how these processes are inherently linked. The toxicity of the metal oxide particles was therefore suggested to be driven by their oxidant properties, which was dependent on their composition, dissolution and intracellular fate. In contrast CeO₂ may exhibit cytoprotective response, suggesting that composition plays an important role in determining cellular responses to nanoparticles.

Lu *et al.* (2008) had a slightly different focus and determined the impact of TiO₂ (0.2-3 mg ml⁻¹) on protein tyrosine nitration (using bovine serum albumin (BSA) as a model protein). Protein nitration was associated with TiO₂ exposure, in the presence of UV light, with anatase TiO₂ forms being more photoactive than rutile forms. Antioxidants, such as GSH and vitamin E were able to prevent against the protein nitration observed. To determine if proteins contained within mouse skin were targets for TiO₂ mediated nitrosation, similar tests were conducted using mouse skin homogenate. Again, anatase forms of TiO₂ were observed to exhibit a greater propensity to nitrosate proteins. This study therefore highlighted the photocatalytic activity of TiO₂, and the superior toxicity of anatase forms, and demonstrated the ability of TiO₂ to induce nitrate stress.

There is debate surrounding the oxidative properties of cerium dioxide, as some claim it is a free radical scavenger, despite evidence of its ability to inflict oxidant injury (Park *et al.* 2008a). This has therefore been a focus of a number of investigations.

Neurones are particularly prone to oxidative stress (Das *et al.* 2007). As a result, Das *et al.* (2007) investigated the ability of CeO₂ particles (3-5 nm) to exhibit neuroprotective effects, during the culture of rat spinal cord neurones. The survival of neurones was promoted by the inclusion of CeO₂ (10 nM) within the cell culture medium, and this was thought to derive from the ability of CeO₂ to scavenge free radicals, and thereby prevent against oxidative stress. The antioxidant behaviour of CeO₂ was also suggested by the findings that CeO₂ treatment improved neurone viability following H₂O₂ exposure. However, further studies would be required to confirm this hypothesis.

In line with these findings, Schubert *et al.* (2006) investigated the neuroprotective behaviour of CeO₂ from oxidative stress mediated cell death. The HT22 neuronal cell line was exposed to CeO₂ nanoparticles (6 and 12 nm) or microparticles (1 µm) for 20 hours, at concentrations up to 100 µg ml⁻¹, and no detrimental impact on cell viability was observed. Pre-treatment with CeO₂ particles were able to protect against glutamate mediated cell death, which is known to be driven by oxidative stress. As a consequence, it was suggested that CeO₂ particles acted as antioxidants, and that this property was not size dependent. This was confirmed by the finding that glutamate mediated increases in ROS production were diminished by CeO₂ pre-treatment. The findings were also replicated using yttrium oxide particles. Therefore contrary to the findings that demonstrate CeO₂ is toxic, this study illustrated that CeO₂ may also exhibit antioxidant, cytoprotective properties.

Similarly, Niu *et al.* (2007) investigated the cardioprotective effects of CeO₂ nanoparticles (7 nm) *in vivo*. CeO₂ nanoparticles (0.15 mM, administered twice a week for 2 weeks) inhibited the progression of cardiac dysfunction (namely left ventricular dysfunction and dilatation) within a murine model of heart failure (MCP-1 transgenic mice). The inflammatory response evident within the myocardium of MCP mice was lessened by CeO₂ treatment, as witnessed by the decrease in macrophage infiltration, and reduction in cytokine production (TNFα, IL-1β, IL-6 and MCP-1). In addition, cardiac myocyte degeneration and apoptotic cell death were also reduced by CeO₂. It was also demonstrated that CeO₂ protected against myocardial oxidative stress, which was suggested due to its ability to reduce peroxynitrite production within the myocardium. CeO₂ was therefore observed to have a protective effect against oxidative and inflammatory processes, which are ordinarily associated with the progression of heart failure within the MCP mouse model. This protective effect was thought to be mediated by its antioxidant behaviour, although the mechanism is difficult to verify in the study conducted. Therefore, the applicability of the protective effects against oxidant injury exhibited by CeO₂, to other particle types requires assessment.

Oxidative responses exhibited by metal oxide particles have been a focus of a number of studies, especially for TiO₂, and its recurrence has prompted the suggestion that oxidative stress drives the inflammatory and cytotoxic responses evident. In contrast, a number of investigations have suggested that other metal oxides, particularly cerium dioxide exhibit anti-oxidant, cytoprotective properties, which is worthy of further consideration in the future.

6.5.4.3 Uptake of metal oxides into cells

The clearance of particles by phagocytic cells has been a particular focus of studies. However, a number of non-phagocytic cell populations also have the propensity to internalise particles. This is of relevance as the uptake of particles may enhance their toxicity, due to their interference with normal cellular physiology and function.

Stearns *et al.* (2001) demonstrated that 50 nm TiO₂ (40 µg ml⁻¹) was internalised by alveolar A549 epithelial cells into membrane bound vesicles after 3, 6 and 24 hour exposures. However, it was observed that the uptake of particles by these cells was limited to their aggregated form. TiO₂ internalisation was suggested to be mediated via phagocytosis, due to the fact that the particles were surrounded and engulfed prior to uptake.

A focus of a number studies has been the contribution of macrophage populations to the clearance of particles, particularly within the lung. However, Geiser *et al.* (2008) argued that alveolar macrophages were not primarily responsible for the uptake and clearance of nanoparticles within the lung *in vivo*. Rats were exposed to TiO₂, via inhalation (0.1 mg m⁻³), and alveolar macrophages were isolated, and the internalisation of particles assessed, 1 or 24 hours post exposure. Large TiO₂ particles (3-6 µm) were more effectively cleared by alveolar macrophages, than their smaller (20 nm) counterparts. Therefore TiO₂ nanoparticles were able to bypass the important alveolar macrophage mediated clearance mechanism within the lung, due to their small size. These findings could be expected due to the known size limitations of uptake processes such as phagocytosis, which is thought to be restricted to substances that are 1 to 5 µm (Patel *et al.* 2007). The ability of TiO₂ nanoparticles to evade phagocytosis was confirmed by the findings that they were not enclosed by a vesicular membrane equivalent that surrounding the larger TiO₂ particles. Therefore it was suggested that phagocytosis was not

responsible for nanoparticle uptake, and instead it was suggested that a sporadic, non-specific mechanism of uptake enabled nanoparticle uptake by macrophages. Alternatively it was suggested that nanoparticles were internalised unintentionally when macrophages phagocytosed other material. The findings of Geiser *et al.* (2008) are also able to provide an explanation for the finding that only TiO₂ agglomerates, and not individual nanoparticles, were internalised by A549 cells *in vitro* (Stearns *et al.* 2001). However, Stearns *et al.* (2001) utilised epithelial cells, and Geiser *et al.* (2008) used macrophages which are professional phagocytes, and therefore the processes that drive particle uptake may be cell specific, and reliant on their function.

Rothen-Rutishauser *et al.* (2007) also utilised an *in vitro* airway wall model (triple cell co-culture), containing, macrophages, epithelial, and dendritic cells to determine the translocation of particles between the different cell types, and their intracellular fate. Membrane-bound aggregates (>0.2µm) of TiO₂ were evident within all cell types, however smaller aggregates (<0.2µm) were apparent within the cell cytoplasm, but were not membrane bound, suggesting that the entry mechanism of particles was size dependent. However, it is relevant that the behaviour of gold, and polystyrene particles was also assessed, and it was found that their intracellular location differed, which implied that different nanoparticles are internalised by different mechanisms or follow different intracellular trafficking processes.

Pisanic *et al.* (2007) evaluated the uptake of iron oxide (Fe₂O₃) nanoparticles (2-12 nm) by the PC12 neuronal cell line. To promote the internalisation of Fe₂O₃ particles by endocytosis, they were coated with dimercaptosuccinic acid (DMSA). Particles were internalised by cells, and were contained within endosomes, accumulated in the perinuclear region and were found free in the cytoplasm. Fe₂O₃ induced a dose, and time dependent decrease in cell viability, induced changes to cell morphology via alterations to the cytoskeleton, and also decreased neurite generation and differentiation in response to nerve growth factor (NGF). Therefore cell function, morphology and viability were affected by Fe₂O₃ nanoparticle uptake.

The physico-chemical properties of particles have been illustrated to influence CeO₂ uptake and adsorption onto proteins by Patil *et al.* (2007). The surface charge of particles was observed to influence the adsorption of proteins onto the particle surface. Specifically, BSA adsorbed onto the surface of positively charged CeO₂ to the greater extent, when compared to negatively charged CeO₂. Negatively charged nanoparticles were internalised by A549 cells to a greater extent. Electrostatic interactions were suggested to be the main driving force for the protein adsorption and cellular uptake of CeO₂ nanoparticles. Surface charge therefore appears to be a determining factor to particle uptake and protein adsorption.

The uptake of metal oxides by a variety of cell types has been demonstrated on numerous occasions. The consequences of particle internalisation are anticipated to be oxidative and cytotoxic in nature. It is anticipated that particle physico-chemical properties may influence their internalisation by cells; accordingly the importance of size and surface charge has the ability to influence particle uptake. In addition, the cell type under investigation has the potential to determine the mechanism of uptake and intracellular fate of particles.

6.5.4.4 Genotoxicity of metal oxides

Evidence of genotoxicity has been previously encountered within a number of studies; micronuclei development is associated with metal oxide exposure, which is indicative of chromosomal damage (Kang *et al.* 2008b), DNA damage has also been observed in response to metal oxide particulate exposure (Sharma *et al.* 2009).

Rahman *et al.* (2002) investigated the potential for TiO₂ to elicit DNA damage within SHE fibroblasts. Micronuclei were evident within cells exposed to nano (<20 nm), but not micro (>200 nm) TiO₂ particles (up to 10 µg cm⁻², for up to 72 hours), which insinuates that chromosomal damage has occurred. The nanoparticles also triggered the induction of apoptosis within cells, which is recognised as a common response to DNA damage.

Dufour *et al.* (2006) investigated the genotoxicity of ZnO (up to 500 µg ml⁻¹), within CHO cells. The contribution of a phototoxic component to the response was evaluated, by determining the

impact of UV irradiation prior to, or during ZnO exposure. In dark conditions, ZnO elicited a dose dependent decrease in cell viability, with irradiation observed to enhance ZnO mediated cytotoxicity. ZnO caused an increase in chromosome structure aberrations, which was most pronounced within irradiated cells. The cells were therefore deemed to be more susceptible to the genotoxic effects of ZnO in the presence of UV light, as the genotoxic effects of ZnO were more potent under these conditions.

Karlsson *et al.* (2008) evaluated the ability of a variety of metal oxide nanoparticles (copper oxide (CuO), ZnO, TiO₂, Fe₃O₄) to induce oxidative stress and DNA damage within A549 lung epithelial cells, at concentrations of up to 80 µg ml⁻¹ for a period of up to 18 hours. CuO and ZnO were able to decrease cell viability, but TiO₂ and Fe₃O₄ were not capable of eliciting such an effect. In addition, all metal oxide particles, except Fe₃O₄ were able to elicit DNA damage, as determined by the Comet assay. The CuO nanoparticles were consistently demonstrated to be the most toxic particle within the panel, which was thought to be driven by their ability to induce oxidative stress, and in turn DNA damage, which prompted cell death. It was suggested that the release of Cu²⁺ ions may contribute to the response, but are not solely accountable for the toxicity of CuO nanoparticles.

Dunford *et al.* (1997) investigated the DNA damage inflicted by a panel of TiO₂ containing sunscreens on fibroblasts, when exposed for up to 60 minutes. It was demonstrated that, on sunlight illumination, the ability of TiO₂ to cause DNA damage was enhanced, in plasmid DNA and cells. Anatase forms of TiO₂ were more effective at inducing damage than rutile forms. Mannitol (an antioxidant) was able to protect against DNA damage, therefore implying that TiO₂ mediated ROS production is central to its genotoxic potential. Consequently, absorption of UV light by TiO₂ is thought to stimulate ROS generation, which, in turn, leads to DNA damage.

Nakagawa *et al.* (1997) investigated the genotoxicity of TiO₂ nanoparticles (in rutile and anatase forms), at concentrations up to 800 µg ml⁻¹, following a 24 hour treatment, in the absence or presence of UV light. Without UV light, TiO₂ nanoparticles induced no, or very limited genotoxicity. However in the presence of UV light TiO₂ elicited DNA damage and chromosome aberrations (but no gene mutations) that was greatest for anatase forms.

Theogaraj *et al.* (2007) investigated the UV dependence of the genotoxic potential of TiO₂ nanoparticles (in rutile and anatase forms) within CHO cells, at concentrations up to 5000 µg ml⁻¹ for a period of 3 hours. In contrast to previously discussed investigations, no chromosomal alterations were observed within exposed cells, in the presence or absence of UV light.

Driscoll *et al.* (1997) determined if there was a relationship between the inflammatory and genotoxic potential of several particles; namely ultrafine carbon black (14 nm), TiO₂ (0.18 µm) and α-quartz (0.9 µm). Rats were exposed to particles via intratracheal instillation, at a dose of 10 or 100 mg kg⁻¹, and analysis conducted 15 months post exposure. All particle types induced the infiltration of neutrophils into the lungs, indicating that particles could induce an inflammatory response. Hypoxanthine–guanine phosphoribosyl transferase gene mutation frequency was increased within alveolar type 2 cells, on exposure to particles. This response was replicated *in vitro*, following the exposure of RLE-6TN alveolar epithelial cells to BAL cells isolated from particle treated animals. It was thus suggested that BAL cell derived ROS were responsible for the mutagenic effects that transpired, and they were suggested to derive from neutrophil activity. Therefore the particle mediated neutrophilic driven inflammatory response within the rat lung, was postulated to increase the frequency of mutations within alveolar epithelial cells, which was a dose and material dependent finding. Accordingly, quartz was consistently found to elicit the greatest genotoxic response, followed by carbon black and then titanium dioxide, and this was related to their inflammatory potential. Therefore, the particles themselves are not thought to be inherently genotoxic, but the inflammatory response, and in particular neutrophil presence (and therefore increased oxidant burden) instigated is anticipated to mediate this effect.

Concern regarding the genotoxic potential of TiO₂, has also emanated from findings that have illustrated that tumours develop *in vivo* following a chronic exposure regime (Heinrich *et al.* 1995).

The ability of metal oxide nanoparticles to inflict DNA damage has been observed on numerous occasions, and is thought to be driven by particle mediated ROS production, with cell death often stimulated as a protective response. In addition, the manifestation of genotoxic events, as a secondary consequence of an inflammatory response is also evident, and requires further investigation. The appearance of TiO₂ mediated genotoxicity appears to be influenced by the crystal form of the sample, particle dissolution and light conditions. The finding that chronic exposure to TiO₂ can induce tumours, also implies that the genotoxicity is not limited to in vitro investigations.

6.5.4.5 Reproductive toxicology

Evaluation of metal oxide nanoparticle effects on the reproductive system is limited to a small number of *in vitro* studies.

A study conducted by Makhlu^f *et al.* (2008) assessed whether magnetite-PVA nanoparticles were able to penetrate sperm cells without affecting motility or the sperm's ability to undergo acrosome reaction; two functions which are crucial for successful fertilisation. The group used bovine sperm and iron oxide nanoparticles coated with PVA (poly-vinyl alcohol), which had a diameter of 11 nm. Nanoparticles (0.5 ml) were added to sperm cells (0.5 ml, 10⁸ cells ml⁻¹) for 1 hour at 37°C. The sperm cells were then treated with digitonin, which permeabilises the plasma membrane, or with SDS, which solubilises the cell membrane and allows for the release of bound and free particles. TEM was used to assess and validate the findings. The acrosome reaction was used to determine the ability of sperm to fertilise the egg. Particles were found bound to the acrosome and a cross-section of the tail revealed high particle binding to the sperm mitochondria. The group's data indicates that the particles crossed the sperm plasma membrane in order to reach the acrosome and mitochondria. They concluded that particle treated cells were not damaged and that sperm function was normal after treatment. The authors suggested that it may be possible to move and target these magnetic sperm cells in an animal body, thereby allowing the targeting of these magnetic sperm cells for biomedical and diagnostic applications.

Komatsu *et al.* (2008) determined the potential for TiO₂ nanoparticles, diesel exhaust particles and ultrafine carbon black to impair the male mouse reproductive system. This study evaluated the direct effect of nanoparticles on testis-constituent cells, and examined the effect of TiO₂ on mouse Leydig TM3 cells, the testosterone-producing cells of the testis. TiO₂ nanoparticles (25–70 nm) at concentrations 1 to 1000 µg ml⁻¹ were examined and uptake into Leydig cells was detected using transmission electron microscopy (TEM) or field emission type scanning electron microscopy/energy-dispersive X-ray spectroscopy (FE-SEM/EDS). TiO₂ was more cytotoxic to Leydig cells than the other carbon based particles used in the study. The proliferation of Leydig cells was suppressed transiently by treatment with TiO₂. TiO₂ nanoparticles were taken up by Leydig cells, and in turn affected cell viability, proliferation and gene expression.

There are a limited number of available studies examined the effects of metal oxide nanoparticles, namely titanium dioxide, and iron oxide on male reproductive physiology. The literature highlights toxicity of metal oxide nanoparticles to male Leydig cells and an interaction with sperm cells that did not lead to any adverse altered function; however studies are limited in number and in sample size, and therefore further studies would be required to confirm such a finding. No literature examining nanoparticle effects on organs or cell types in the female reproductive system were found.

6.5.5 Linking the physico-chemical attributes of metal oxides to their pathogenicity or toxicity

As metal oxide particles are a diverse group of materials which vary with regards to their size (and therefore surface area), composition, crystal form, and dissolution propensity, it is necessary to outline what properties are most influential to the toxicity of metal oxides. However it is also relevant to highlight that the experimental set up is also able to influence the findings, including the choice of species or cell type, method of exposure, and particle dispersal.

6.5.5.1 Size dependency

Particle dimensions are recognised as being fundamental to their toxicity. This derives from the fact that nanoparticles have been consistently capable of eliciting more pronounced toxicity than their larger (microparticulate) counterparts. The size dependency of metal oxide toxicity has been frequently demonstrated (Ferin *et al.* 1992, Renwick *et al.* 2001, Renwick *et al.* 2004, Gurr *et al.* 2005, Chen *et al.* 2006, Ogami *et al.* 2009, Wang *et al.* 2007, Kang *et al.* 2008b), and appears to be applicable to a variety of metal oxides, regardless of their composition and model used. However it is relevant that a high degree of particle aggregation and agglomeration is associated with metal oxide administration, and so exposure to particles is unlikely to occur to particles in a 'nano' form. However the nanoparticles that make up the agglomerates are within the nano size range, and this appears to be fundamental to driving their toxicity. Some authors have suggested that nanoparticle aggregation and agglomeration are distinct phenomena with agglomerates formed by clusters of particles that are held together by electrostatic interactions, whereas aggregates are formed from covalently fused or sintered particles that are not easily separated (Oberdoerster *et al.* 2007).

In particular, the superior toxicity of nanoparticulate forms of TiO₂ has not been repeatedly documented. However, a study conducted by Dick *et al.* (2003) illustrated that compared to other nanoparticles such as nickel, cobalt or carbon black, TiO₂ nanoparticles were less potent at inducing an inflammatory response within the lung, despite their similarity in surface area. This was assumed to be related to their lower capacity to generate free radicals, and therefore perhaps particle composition and surface chemistry are also important contributors to toxic responses (Dick *et al.* 2003).

Bermudez *et al.* (2004) exposed rats to TiO₂ via inhalation, to investigate their pulmonary toxicity. Despite the fact that the nanoparticles had a primary particle size of 21 nm, the aerosol generated contained particle aggregates (1.37 µm), which is a common experience in the exposure of animals or cell culture models to nanomaterials, and is likely to be encountered within the exposure of humans. However, despite this, nanoparticles exerted greater toxicity. Similarly, aggregates of TiO₂ nanoparticles (1.44 µm) have been demonstrated to be more toxic than similarly sized aggregates of their larger counterparts (Ferin *et al.* 1992). However, Grassian, *et al.* (2007) demonstrated the properties of agglomerates of particles that formed during aerosol generation were dependent on the primary particle size. Specifically, 21 nm particle based agglomerates were less dense than their 5 nm counterparts, which contained particles that were more tightly packed. It was found that 21 nm TiO₂ was more toxic than 5 nm particles in inhalation and instillation exposure scenarios. The agglomeration state was suggested to dictate their toxicity potential. Therefore although 21 nm particles were larger, it is anticipated that they would form agglomerates that would more easily deagglomerate, due to the weaker interactions that held the particles together. As a result, 21 nm particles would be available as smaller structures to stimulate an enhanced toxic response. Furthermore the aggregates formed were larger within inhalation preparations than intratracheal suspensions, and so this explains why intratracheal exposures produced a greater toxic response. However, Lin *et al.* (2006), expected 14 nm SiO₂ to exhibit greater toxicity than their 46 nm counterparts, but this did not transpire, and it was suggested that their aggregation accounted for this, as their hydrodynamic diameters were similar.

The surface area of particles has been previously suggested to dictate the toxic potential of particles (Duffin *et al.* 2002, Stoeger *et al.* 2006, Brown *et al.* 2001). Sager *et al.* (2008) addressed what dose metric was most appropriate for influencing the toxicity of TiO₂, specifically the surface area or mass of particles administered. Nanoparticles, suspended in BALF, were observed to agglomerate, so that particles were 200-300 nm in diameter (despite having a primary particle size of 21 nm), emphasising that particle exposure does not occur in an individual form. Microparticle and nanoparticle types were able to induce an inflammatory response (neutrophil infiltration, LDH activity, TNFα, MIP and IL-1β release), subsequent to intratracheal administration of rats. It was of interest that a higher mass of microparticulate TiO₂ was required to obtain the same inflammogenic response as nanoparticle TiO₂. However, when the dose administered was equivalent, in terms of surface area delivered, both particle types behaved similarly. Therefore, using a mass dose metric, nanoparticulate TiO₂ was considerably

more toxic than its larger counterpart, however when the exposure dose was normalised to equivalent surface area particles delivered, no differences in the potency of the particles were observed. Similarly, Sager *et al.* (2009) determined whether the surface area of ultrafine carbon black and TiO₂ nanoparticles drives their inflammatory potential. Particles were administered to rats via intratracheal instillation, and toxicological observations made up to 42 days post exposure. At equivalent surface area doses, both particle types induced an inflammatory response that was characterised by the infiltration of neutrophils, at day 1, and similar in magnitude. However the response decreased with time for ultrafine carbon black, but for TiO₂ the response was sustained in nature. This pattern of response was similar for BALF protein levels. This observation remains debatable as others have disputed this finding (Warheit *et al.* 2007).

*The size (and surface area) of metal oxide nanoparticles is known to be fundamental to their toxicity. The aggregation and agglomeration state of nanoparticles is also likely to be influential to their toxicity. Consequently, it is often the case that although the primary particle size is stipulated by investigators; cells and animals are not exposed to the particles in this form, which is also driven by the exposure scenario (i.e. air or suspension) (Pflucker *et al.* 1999; Sayes *et al.* 2006; Singh *et al.* 2007; Stearns *et al.* 2001; Gamer *et al.* 2006; Long *et al.* 2006 and 2007; Jin *et al.* 2009; Pan *et al.* 2009; Rehn *et al.* 2003). However it is often observed that agglomerates/aggregates of nanoparticles are more toxic than similarly sized agglomerates/aggregates of their larger counterparts. Therefore, the propensity of particles to aggregate has prompted investigators to prevent against its occurrence through the use of sonication, or inclusion of dispersants such as within particle suspensions, but this also needs to take into account what is relevant to human exposure.*

6.5.5.2 Influence of metal oxide particulate surface chemistry /modification/coatings to their toxicity

As for other nanoparticles, the surface of metal oxides can be altered through the attachment of surface moieties. In addition, the surface of TiO₂ particles can be modified through coating with aluminium oxide, or silica in order to improve an aspect of their behaviour, and has generally been encountered within sunscreens to enhance protection from UV radiation (Warheit *et al.* 2005).

Warheit *et al.* (2005) investigated the impact of surface treatments (alumina or silica) on TiO₂ toxicity, within lungs (see earlier). It was demonstrated that those exhibiting the greatest toxicity, were those that contained the highest aluminium oxide and/or silica content on the particle surface. However, overall it was observed that TiO₂ particles induced low pulmonary toxicity, despite the ability of surface coatings to influence this.

Oberdorster (2001) demonstrated that the surface properties of TiO₂ were able to influence their toxicity. This was achieved through the intratracheal exposure of rats (500 µg per animal, for 24 hours) to TiO₂ (20 nm) that remained uncoated (hydrophilic) or received a silane coating (hydrophobic). The samples varied, with regards to their inflammatory potency within the lungs, so that the uncoated particles induced a lower response, than their coated counterparts.

Hohr *et al.* (2002) investigated the impact of surface properties on TiO₂ toxicity. Microparticulate, uncoated nanoparticles, and coated nanoparticles (via methylation) of TiO₂ were exposed to rats, via intratracheal instillation (1 or 6 mg per rat), and analysis conducted 16 hours post exposure. Administration of all particle types stimulated the infiltration of neutrophils, with the effect most pronounced with administration of nanoparticles. However, it is of interest that the coated (hydrophobic) nanoparticles tended to stimulate neutrophil recruitment to a lesser extent than the uncoated (hydrophilic) particles. Protein, LDH, TNFα and MIP-2 levels in BAL also exhibited a similar pattern, with regards to the potency of the different samples, but overall the impact of surface methylation on TiO₂ toxicity was negligible. However, it was concluded that the toxicity of TiO₂ was driven by the surface area of particles, and not their surface coating, so that a stronger inflammatory response was associated with nanoparticles, compared with their larger counterparts and so the effect of methylation was negligible.

Singh *et al.* (2007) determined the impact of TiO₂ size (microparticles: 40-300 nm, nanoparticles: 20-80 nm), surface modification (using methylation, to make particles more hydrophobic) and radical generating potential on their uptake and toxicity within A549 lung epithelial cells. TiO₂ particles, regardless of their size or methylation, were phagocytosed as clusters of particles, and on some occasions it was evident that nanoparticles were internalised by clathrin mediated endocytosis, but this was only apparent for small particle clusters that were less than 30 nm. ROS generation, and IL-8 production exhibited by cells exposed to nanoparticles was greater than that of microparticles, and the findings were unaffected by the methylation of the particle surface. An important observation was that both particle types were present in an aggregated form, and that the nanoparticles were in a smaller size range (<500 nm) than their larger counterparts (2000-5000 nm). Therefore the superior toxicity of nanoparticles held true, despite their tendency to aggregate, and so, even when aggregated the toxicity of TiO₂ particles was assumed to be primarily driven by their surface area.

Thevenot *et al.* (2008) determined the impact of TiO₂ nanoparticle with various surface modifications (-COOH, -OH or -NH₂ functional groups) on the survival of a variety of cancer cell lines. Exposure concentrations were high, up to 10 mg ml⁻¹, for 3 to 24 hours. The cancer cell lines used were B16F10 and BF16F1 melanoma, Lewis lung carcinoma, JHU prostate cancer, and 3T3 fibroblast cell lines. The different cancer cell lines showed different sensitivities to the cytotoxicity of untreated TiO₂ nanoparticles. 3T3, B16F10 and BF16F1 cells were unresponsive to all types of TiO₂. However, TiO₂ decreased the viability of JHU and LLC cells in a dose dependent manner. Therefore TiO₂ appears to exhibit cell-specific effects, with regards to their cytotoxic potential. In addition, by altering the surface chemistry of TiO₂ particles, the toxicity of TiO₂ could be modified. In general, NH₂, and OH surface modified TiO₂ exhibited greater toxicity than COOH modified TiO₂. This study therefore illustrated that TiO₂ toxicity very much dependent on the cell type in question, the surface chemistry of TiO₂ nanoparticles, and concentration of particles used.

Rehn *et al.* (2003) illustrated that unmodified, or silane coated forms of TiO₂ were non toxic to the lungs. Rats were exposed to the different forms of TiO₂ (20 nm) via intratracheal instillation (up to 1.2 mg per rat) and the inflammatory and genotoxic effects within the lung were determined, at 3, 21 and 90 days post exposure. All forms of TiO₂ were able to stimulate a modest increase in neutrophils and macrophages within the lungs (at day 3), but this was not persistent in nature, and was not associated with TNF α production. In addition, no genotoxicity was evident with TiO₂ exposure. In contrast, quartz induced a persistent and progressive inflammatory response, with a genotoxic element to the response also observed. However, the only genotoxic endpoint that was assessed was the detection of 8 oxy-guanine, so perhaps a more comprehensive genotoxic study should be considered for TiO₂.

The modification of the surface of TiO₂ particles is able to influence its toxicity, however, this is likely to be dependent on the modification, and cell type in question.

6.5.5.3 Crystallinity

TiO₂ exists in two main crystal phases, termed rutile and anatase. These TiO₂ forms vary with regards to their crystalline structure and surface properties, which is responsible for differences within their toxicity (Shi *et al.* 2007). Anatase has been demonstrated to be the most toxic form of TiO₂, with a number of previously mentioned studies supporting this conclusion (see for example Wang *et al.* 2008a.). The photoactivity of TiO₂ is also dictated by the crystal phase, and therefore surface characteristics of particles (such as oxygen vacancies on the particle surface), with anatase forms having a greater capacity to generate ROS on exposure to light (see later).

Sayes *et al.* (2006) assessed the *in vitro* toxicity of anatase, rutile or anatase/rutile TiO₂ samples to A549 epithelial cells and HDF fibroblasts, at concentrations ranging from 3 μ g ml⁻¹ to 30 mg ml⁻¹, for up to 48 hours. The level of cytotoxicity exhibited by the different particle types varied. Accordingly, anatase particles were the most cytotoxic, while the rutile TiO₂ particles were the least toxic, and this response was paralleled within the release of IL-8 from cells. TiO₂ particle mediated ROS generation, and photoactivity was also greatest for pure anatase samples. Overall, the results implied that the anatase samples had the greatest toxic potency, and this is

likely to derive from their larger surface area ($153 \text{ m}^2 \text{ g}^{-1}$). However, the authors suggested that it was the phase of TiO_2 that was most integral to its toxicity. Therefore, despite the relatively large surface area of rutile samples ($112 \text{ m}^2 \text{ g}^{-1}$), their surface chemistry is thought to be less reactive than that of anatase samples, and they are therefore less toxic. The findings therefore suggested that the ability of TiO_2 particles to generate ROS, governs their cytotoxic and inflammatory potential, which is dictated by the crystal structure of the phase in question, and hence toxicity is not solely driven by surface area.

Pan *et al.* (2009) investigated the toxicity of a panel of TiO_2 particles to primary human dermal fibroblasts. Uncoated rutile TiO_2 (15 nm), polymer coated rutile TiO_2 or anatase TiO_2 (200 nm) particles were exposed to cells for up to 11 days, at concentrations up to 0.8 mg ml^{-1} . Rutile TiO_2 caused alterations in cell morphology, so that cells had a smaller cell area, became elongated, and detached from the culture surface. Anatase TiO_2 caused a greater magnitude of damage to cells, with severe morphological changes observed including breakage of actin filaments, and plasma membrane rupture within exposed cells. Rutile TiO_2 did not impact on cell viability, but did slow cell proliferation rate. Rutile particles were internalised by cells, and were contained within cytoplasmic vesicles. Anatase TiO_2 was internalised by cells, and could access the nucleus. H_2O_2 production by cells was greatest with anatase particles, implying that their ability to generate ROS was greatest within the TiO_2 particle panel. Anatase particles were therefore more potent than rutile particles at inducing cell damage. The coating of rutile particles with a polymer abolished their toxicity, which was suggested to derive from the lack of particle adherence to the cell surface, and thus limited internalisation, so that they were unable to impact on normal cell function.

The influence of the crystal phase of particles is not restricted to TiO_2 particles, and has also been exemplified with silica (SiO_2). Crystalline respirable silica (known as α -quartz) is classified by IARC as a class I carcinogen (IARC 1997). There is extensive evidence that crystalline silica induces lung cancer and silicosis (fibrosis) of the lung (Donaldson *et al.* 2001). Until recently, amorphous silica was considered to be relatively benign, although much of the evidence used to draw this conclusion is based upon μm sized particles, with little knowledge for silica nanoparticles. Nanoparticle silica is often assumed or reported to be amorphous rather than crystalline, and therefore it is necessary to outline the potential toxicity of nanoparticulate forms of SiO_2 , which is assumed to be less toxic than its crystalline counterpart (Kaewamatawong *et al.* 2006).

Kaewamatawong *et al.* (2006) investigated the pulmonary effects of colloidal nanoparticle silica (14 nm) in mice, up to 30 days post exposure. Particles were intratracheally instilled into the lungs, at doses ranging from 0.3 to $100 \mu\text{g}$ per mouse. Colloidal, amorphous silica nanoparticles induced a dose and time dependent inflammatory response and lung injury. In addition, there was evidence of increased apoptosis as well as oxidative DNA damage. Lin *et al.* (2006) compared the toxicity of SiO_2 nanoparticles (15 and 46 nm diameter) a crystalline form of silica (Min-U-Sil, 5 μm diameter) within A549 lung epithelial cells, *in vitro*, at concentrations ranging from 10 to $100 \mu\text{g ml}^{-1}$ for up to 72 hours. Both nanoparticulate silica samples induced a greater (dose and time dependent) cytotoxic response within cells, than the crystalline sample. The nanoparticles were found to generate ROS, with a concomitant depletion in GSH and increase in lipid peroxidation. Consequently, these studies suggest that silica nanoparticles have the potential to induce acute effects both *in vitro* and *in vivo*, but further work is needed before their true risk can be determined.

The phase composition of TiO_2 has been proposed to be influential in dictating the toxic potency of particles. This is likely to derive from their substantially different surface chemistry, with anatase forms exhibiting the greatest photocatalytic and biological activity. The importance of crystallinity, to the toxicity of particles has also been demonstrated for SiO_2 .

6.5.5.4 Contribution of photocatalytic behaviour of TiO_2 to its toxicity

The ability of UVA or visible light to increase the toxic potency of metal oxide particles, through increased ROS production, has been a focus of a number of previously discussed studies (Dunford *et al.* 1997; Zhang and Sun 2004; Dufour *et al.* 2006; Lu *et al.* 2006), but does not always transpire (Linnainmaa *et al.* 1997; Theogaraj *et al.* 2007). This phenomenon is

anticipated to be exploited, particularly within the treatment of cancer (Zhang and Sun 2004). The crystal form of TiO₂ has been suggested to be responsible for driving the photoactivity of particles, due to the importance of surface properties (Shi *et al.* 2007).

6.5.6 Quality of experiments conducted

The described studies vary greatly with regards to their experimental set up. It has been highlighted that the size of particles, their crystallinity, route of exposure, particle concentration, experiment duration and species used can all impact on metal oxide particle toxicity. Therefore these factors are able to influence the findings obtained by different investigators, and therefore justification of the relevancy of the experimental approach used by investigators would be useful.

There is a paucity of data relating to the systemic transfer of particles following exposure via the lungs, skin and gut, and this should be a focus of future experiments. Studies have focused on dermal and pulmonary toxicity of particles, but there is an absence of data on the consequences of exposure to the gastrointestinal tract, and within damaged/diseased skin. Other relevant target organs include the liver, kidney, cardiovascular system and brain which is necessary due to the fact that nanoparticles are likely to become systemically or neuronally available. The liver could be highlighted as a priority due to the propensity of particles to accumulate in this organ.

6.5.7 Summary

Due to historical reasons, a focus on the size (and surface area) dependence of metal oxide (particularly TiO₂) toxicity has been persistently illustrated, and confirmation that particle toxicity increases as particle size decreases has been consistent within wide ranging investigations. However, it has become evident that other physico-chemical factors are able to contribute to metal oxide toxicity; including particle aggregation, crystal phase, surface modification, and particle dissolution. The exposure method, dose administered, species used, cell type under investigations and light conditions also have the potential to impact on the toxicity of metal oxide particles, indicating that the experimental set up is also very influential. However, much of the current work has concentrated on revealing the toxicity of TiO₂, and therefore the applicability of the findings to other metal oxide types is unsure at this time, and should be approached with caution.

The toxicity of metal oxides (with most studies relating to TiO₂) has been demonstrated to be inflammogenic, oxidative, and genotoxic in nature; with all endpoints considered to be inherently linked. It is also of interest that the biological mechanisms identified as being responsible for driving the toxicity of metal oxides is replicated within *in vivo* and *in vitro* settings. Cytotoxicity is also a common end point that is evaluated within studies, although the relevance of this is questionable (in terms of human exposure levels), except when establishing sub-lethal concentrations for subsequent studies that allow the identification of mechanistic processes that are responsible for toxicity. Many of the studies identified have concentrated on TiO₂ and ZnO toxicity, due to their extensive exploitation in nanomaterial containing products. The ability of particles to exert toxicity at a variety of target sites is reliant on their transfer into blood, and this should therefore be a focus of future experimentation, as at this time, the systemic availability of metal oxide particles following exposure is uncertain. Accordingly, investigations into the toxicity of metal oxides via specific routes of delivery, or at particular cell and organ targets, are often insufficient in number to make definite conclusions about particle behaviour. In addition, the quality (including the concentrations used, experimental model), of conducted experiments is an important consideration, which is of vital importance when considering the risk associated with metal oxide exposure.

6.6 REFERENCES

- Afaq, F., Abidi, P., Matin, R. and Rahman, Q. 1998, "Cytotoxicity, pro-oxidant effects and antioxidant depletion in rat lung alveolar macrophages exposed to ultrafine titanium dioxide", *Toxicol Appl Pharmacol*, vol. 18, no. 5, pp. 307-312.
- Ahamed, M., Karns, M., Goodson, M., Rowe, J., Hussain, S.M., Schlager, J.J. and Hong, Y. 2008, "DNA damage response to different surface chemistry of silver nanoparticles in mammalian cells", *Toxicol Appl Pharmacol*, vol. 233, no. 3, pp. 404-410.
- Ahn, M. H., Kang, C. M., Park, C. S., Park, S. J., Rhim, T., Yoon, P. O., Chang, H. S., Kim, S. H., Kyono, H. and Kim, K. C. 2005, "Titanium dioxide particle-induced goblet cell hyperplasia: association with mast cells and IL-13", *Respir Res*, vol. 6, pp. 34-43.
- Arora, S., Jain, J., Rajwade, J. M., and Paknikar, K. M. 2008, "Cellular responses induced by silver nanoparticles: In vitro studies", *Toxicol Lett*, vol. 179, no. 2, pp. 93-100.
- Arora, S., Jain, J., Rajwade, J. M., and Paknikar, K. M. 2009, "Interactions of silver nanoparticles with primary mouse fibroblasts and liver cells", *Toxicol Appl Pharmacol*, vol. 236, no. 3, pp. 310-318.
- AshaRani, P. V., Low Kah, M. G., Hande, M. P., and Valiyaveetil, S. 2009, "Cytotoxicity and genotoxicity of silver nanoparticles in human cells", *ACS Nano*, vol. 3, no. 2, pp. 279-290.
- Baker, G.L., Gupta, A., Clark, M.L., Valenzuela, B.R., Staska, L.M., Harbo, S.J., Pierce, J.T. and Dill, J.A. 2008, "Inhalation toxicity and lung toxicokinetics of C₆₀ fullerene nanoparticles and microparticles", *Toxicol Sci*, vol. 101, no. 1, pp.122-131.
- Bar-Ilan, O., Albrecht, R.M., Fako, V.E. and Furgeson, D.Y. 2009, "Toxicity assessments of multisized gold and silver nanoparticles in zebrafish embryos", *Small*. Epub before print.
- Barlow, P. G., Clouter-Baker, A., Donaldson, K., Maccallum, J. and Stone, V. 2005, "Carbon black nanoparticles induce type II epithelial cells to release chemotaxins for alveolar macrophages", *Part Fibre Toxicol*, vol. 2, pp. 11-25.
- Bedrov, D., Smith, G.D., Davande, H. and Li, L. 2008, "Passive transport of C₆₀ fullerenes through a lipid membrane: a molecular dynamics simulation study", *J Phys Chem B*, vol. 112, no.7, pp. 2078-2084.
- Belyanskaya, L., Manser, P., Spohn, P., Bruinink, A. and Wick, P. 2007, "The reliability and limits of the MTT reduction assay for carbon nanotubes-cell interaction", *Carbon*, vol. 45, no. 13, pp. 2643-2648.
- Bermudez, E., Mangum, J. B., Wong, B. A., Asgharian, B., Hext, P. M., Warheit, D. B. and Everitt, J. I. 2004, "Pulmonary responses of mice, rats, and hamsters to subchronic inhalation of ultrafine titanium dioxide particles", *Toxicol Sci*, vol. 77, no. 2, pp. 347-357.
- Bogdanovic, V., Stankov, K., Icevic, I., Zikic, D., Nikolic, A., Solajic, S., Djordjevic, A. and Bogdanovic, G. 2008, "Fullerenol C₆₀(OH)₂₄ effects on antioxidative enzymes activity in irradiated human erythroleukemia cell line", *J Radiat Res*, vol. 49, no. 3, pp. 321-327.
- Borm, P. J., Robbins, D., Haubold, S., Kuhlbusch, T., Fissan, H., Donaldson, K., Schins, R., Stone, V., Kreyling, W., Lademann, J., Krutmann, J., Warheit, D. and Oberdorster, E. 2006, "The potential risks of nanomaterials: a review carried out for ECETOC", *Part Fibre Toxicol*, vol. 3, 11-46.
- Bottini, M., Bruckner, S., Nika, K., Bottini, N., Bellucci, S., Magrini, A., Bergamaschi, A. and Mustelin, T. 2006, "Multi-walled carbon nanotubes induce T lymphocyte apoptosis", *Toxicol Lett*, vol. 160, no. 2, pp. 121-126.

Braydich-Stolle, L., Hussain, S., Schlager, J.J. and Hofmann, M.C. 2005, "In Vitro cytotoxicity of nanoparticles in mammalian germline stem cells", *Toxicol Sci*, vol. 88, no.2, pp. 412–419

Brown, D.M., Beswick, P.H. and Donaldson, K. 1999, "Induction of nuclear translocation of NF-kappaB in epithelial cells by respirable mineral fibres", *The Journal of Pathology*, vol 189, no. 2, pp. 258-264.

Brown, D.M., Wilson, M.R., MacNee, W., Stone, V. and Donaldson, K. 2001, and "Size-dependent proinflammatory effects of ultrafine polystyrene particles: a role for surface area and oxidative stress in the enhanced activity of ultrafines", *Toxicology and Applied Pharmacology*, vol. 175, no. 3, pp. 191-199.

Brown, D., Donaldson, K. and Stone, V.. 2004, "Effects of PM10 in human peripheral blood monocytes and J774 macrophages", *Respiratory Research*, vol. 5, no. 1, pp. 29-41.

Brown, D.M., Kinloch, I.A., Bangert, U., Windle, A.H., Walter, D.M., Walker, G.S., Scotchford, C.A., Donaldson, K. and Stone, V. 2007, "An *in vitro* study of the potential of carbon nanotubes and nanofibres to induce inflammation mediators and frustrated phagocytosis", *Carbon*, vol. 45, no. 9, pp. 1743-1756.

Bullard-Dillard, R., Creek, K.E., Scrivens, W.A. and Tour, J.M. 1996, "Tissue sites of uptake of ¹⁴C labelled C₆₀", *Bioorg Chem*, vol. 24, no. 4, pp. 376-385.

Carrero-Sanchez, J.C., Elias, A.L., Mancilla, R., Arrellin, G., Terrones, H., Laclette, J.P. and Terrones, M. 2006, "Biocompatibility and toxicological studies of carbon nanotubes doped with nitrogen", *Nano Letters*, vol. 6, no. 8, pp. 1609-1616.

Casey, A., Herzog, E., Davoren, M., Lyng, F.M., Byrne, H.J. and Chambers G. 2007a, "Spectroscopic analysis confirms the interactions between single-walled carbon nanotubes and various dyes commonly used to assess cytotoxicity", *Carbon*, vol.45, no.7, pp. 1425-1432.

Casey, A., Davoren, M., Herzog, E., Lyng, F.M., Byrne, H.J. and Chambers, G. 2007b, "Probing the interaction of single-walled carbon nanotubes within cell culture medium as a precursor to toxicity testing", *Carbon*, vol. 45, no.1, pp. 34-40.

Cha, K., Hong, H. W., Choi, Y. G., Lee, M. J., Park, J. H., Chae, H. K., Ryu, G., and Myung, H. 2008b, "Comparison of acute responses of mice livers to short-term exposure to nano-sized or micro-sized silver particles", *Biotechnol Lett*, vol. 30, no. 11, pp. 1893-1899.

Cha, K., Hong, H. W., Choi, Y. G., Lee, M. J., Park, J. H., Chae, H. K., Ryu, G., and Myung, H. 2008a, "Comparison of acute responses of mice livers to short-term exposure to nano-sized or micro-sized silver particles", *Biotechnol Lett*, vol. 30, no. 11, pp. 1893-1899.

Chang, A.L.S., Khosravi, V. and Egbert, B. 2006, "A case of argyria development after colloidal silver digestion", *J Cut Pathol*, vol. 33, no. 12, pp. 809-811.

Chen, B.X., Wilson, S.R., Das, M., Coughlin, D.J. and Erlanger, B.F. 1998a, " Antigenicity of fullerenes: antibodies specific for fullerenes and their characteristics ", *PNAS USA*, vol. 95, no.18, pp.10809-10813.

Chen, C., Xing, G., Wang, J., Zhao, Y., Li, B., Tang, J., Jia, G., Wang, T., Sun, J., Xing, L., Yuan, H., Gao, Y., Meng, H., Chen, Z., Zhao, F, Chai, Z. and Fang, X. 2005, "Multihydroxylated [Gd@C₈₂(OH)₂₂]_n nanoparticles: antineoplastic activity of high efficiency and low toxicity", *Nano Letters*, vol. 5, no.10, pp. 2050-2057.

Chen, H.H., Yu, C., Ueng, T.H., Chen, S., Chen, B.J., Huang, K.J. and Chiang, L.Y. 1998b, "Acute and subacute toxicity study of water-soluble polyalkylsulfonated C₆₀ in rats", *Toxicol Pathol*, vol. 26, no. 1, pp.143-151.

Engineered Nanoparticles: Review of Health and Environmental Safety

Chen, H. W., Su, S. F., Chien, C. T., Lin, W. H., Yu, S. L., Chou, C. C., Chen, J. J. and Yang, P. C. 2006, "Titanium dioxide nanoparticles induce emphysema-like lung injury in mice", *FASEB Journal*, vol. 20, no. 13, pp. 2393-2395.

Chen, J., Dong, X., Zhao, J. and Tang, G. 2008, "In vivo acute toxicity of titanium dioxide nanoparticles to mice after intraperitoneal injection", *J Appl Toxicol*, vol. 29, no. 4, pp. 330-337.

Cheng, J., Chan, C.M., Veca, L.M., Poon, W.L., Chan, P.K., Qu, L., Sun, Y.P. and Cheng, S.H. 2009, "Acute and long-term effects after single loading of functionalised multi-walled carbon nanotubes into zebrafish (*Danio rerio*)", *Toxicology and Applied Pharmacology*, vol. 235, no. 2, pp. 216-225.

Cheng, J., Flahaut, E. and Cheng, S.H. 2007, "Effect of carbon nanotubes on developing zebrafish (*Danio rerio*) embryos", *Environ Toxicol Chem*, vol. 26, no. 4, pp. 708-716.

Cherukuri, P., Bachilo, S.M., Litovsky, S.H. and Weisman, R.B. 2004, "Near-infrared fluorescence microscopy of single-walled carbon nanotubes in phagocytic cells", *J American Chem Soc*, vol. 126, no. 48, pp.15638-15639.

Cherukuri, P., Gannon, C.J., Leeuw, T.K., Schmidt, H.K., Smalley, R.E., Curley, S.A. and Weisman, R.B. 2006, "Mammalian pharmacokinetics of carbon nanotubes using intrinsic near-infrared fluorescence", *PNAS USA*, vol. 103, no. 50, pp. 18882-18886.

Chi, Z., Liu, R., Zhao, L., Qin, P., Pan, X., Sun, F., and Hao, X. 2009, "A new strategy to probe the genotoxicity of silver nanoparticles combined with cetylpyridine bromide", *Spectrochim Acta A Mol Biomol Spectrosc*, vol. 72, no. 3, pp. 577-581.

Chithrani, B.D., Ghazani, A.A. and Chan, W.C.W. 2006, "Determining the size and shape dependence of gold nanoparticle uptake by mammalian cells", *Nano Letters*, vol. 6, no. 4, pp. 662-668.

Chithrani, B.D. and Chan, W.C.W. 2007, "Elucidating the mechanism of cellular uptake and removal of protein-coated gold nanoparticles of different sizes and shapes", *Nano Letters*, vol. 7, no. 6, pp. 1542-1550.

Cho, W-S., Cho, M., Jeong, J., Choi, M., Cho, H-Y., Han, B.S., Kim, S.H., Kim, H.O., Lim, Y.T., Chung, B.H. and Jeong, J. 2009, "Acute toxicity and pharmacokinetics of 13 nm-sized PEG-coated gold nanoparticles", *Toxicol Appl Pharmacol*, vol. 236, no.1, pp. 16-24.

Chou, C.C., Hsiao, H.Y., Hong, Q.S., Chen, C.H., Peng, Y.W., Chen, H.W. and Yang, P.C. 2008, "Single-walled carbon nanotubes can induce pulmonary injury in mouse model", *Nano Lett*, vol. 8, no. 2, pp. 437-445.

Churg, A., Gilks, B. and Dai, J. 1999. "Induction of fibrogenic mediators by fine and ultrafine titanium dioxide in rat tracheal explants". *Am J Physiol*, vol. 277, no.5, pp. L975-L982.

Connor E.E., Mwamuka, J, Gole, A., Murphy, C.J. and Wyatt, M.D. 2005, "Gold nanoparticles are taken up by human cells but do not cause acute cytotoxicity", *Small*, vol.1, no. 3, pp. 325-327.

Courtois, A., Andujar, P., Ladeira, Y., Baudrimont, I., Delannoy, E., Leblais, V., Begueret, H., Galland, M. A., Brochard, P., Marano, F., Marthan, R. and Muller, B. 2008. "Impairment of NO-dependent relaxation in intralobar pulmonary arteries: comparison of urban particulate matter and manufactured nanoparticles", *Environ Health Perspect*, vol. 116, no.10, pp. 1294-1299.

Cross, S. E., Innes, B., Roberts, M. S., Tsuzuki, T., Robertson, T. A. and McCormick, P. 2007, "Human skin penetration of sunscreen nanoparticles: *in-vitro* assessment of a novel micronized zinc oxide formulation", *Skin Pharmacol Physiol*, vol. 20, no. 3, pp. 148-154.

Das, M., Patil, S., Bhargava, N., Kang, J. F., Riedel, L. M., Seal, S. and Hickman, J. J. 2007, "Auto-catalytic ceria nanoparticles offer neuroprotection to adult rat spinal cord neurons", *Biomaterials*, vol. 28, no. 10, pp. 1918-1925.

Engineered Nanoparticles: Review of Health and Environmental Safety

Davoren, M., Herzog, E., Casey, A., Cottineau, B., Chambers, G., Byrne, H.J. and Lyng, F.M. 2007, "In vitro toxicity evaluation of single-walled carbon nanotubes on human A549 lung cells", *Toxicol In Vitro*, vol. 21, no. 3, pp. 438-448.

De Jong, W. H., Hagens, W.I, Krystek, P., Burger, M.C., Sips, A. J.A.M., Geertsma, R.E., 2008, "Particle size-dependent organ distribution of gold nanoparticles after intravenous administration", *Biomaterials*, vol. 29, no. 12, pp. 1912-1919.

Deng, X., Jia, G., Wang, H., Sun, H., Wang, X., Yang, S., Wang, T. and Liu, Y. 2007, "Translocation and fate of multi-walled carbon nanotubes *in vivo*", *Carbon*, vol. 45, no. 7, pp. 1419-1424.

Dhawan, A., Taurozzi, J.S., Pandey, A.K., Shan, W., Miller, S.M., Hashsham, S.A. and Tarabara, V.V. 2006, "Stable colloidal dispersions of C₆₀ fullerenes in water: evidence for genotoxicity", *Environ Sci Technol*, vol. 40, no. 23, pp. 7394-7401.

Di, S.A., Chiaretti, M., Carru, G.A., Bellucci, S. and Mazzanti, G. 2009, "Multi-walled carbon nanotubes: Lack of mutagenic activity in the bacterial reverse mutation assay", *Toxicology Letters*, vol, 184, no. 3, pp. 192-197.

Dick, C. A., Brown, D. M., Donaldson, K. and Stone, V. 2003, "The role of free radicals in the toxic and inflammatory effects of four different ultrafine particle types", *Inhal Toxicol*, vol. 15, no.1, pp. 39-52.

Ding, L., Stilwell, J., Zhang, T., Elboudwarej, O., Jiang, H., Selegue, J.P., Cooke, P.A., Gray, J.W. and Chen, F.F. 2005, "Molecular characterization of the cytotoxic mechanism of multiwall carbon nanotubes and nano-onions on human skin fibroblast", *Nano Letters*, vol. 5, no.12, pp. 2448-2464.

Donaldson, K., Stone, V., Duffin, R., Clouter, A., Schins, R. and Borm, P. 2001, "The quartz hazard: effects of surface and matrix on inflammatory activity", *J Environ Pathol Toxicol Oncol*, vol. 20, no. 1, pp. 109-118.

Donaldson, K. and Stone, V. 2003, "Current hypotheses on the mechanisms of toxicity of ultrafine particles", *Annali dell 'Istituto Superiore di Sanita*, vol. 39, no. 3, pp. 405-410.

Donaldson, K., Tran, C.L., Jimenez, L.A., Duffin, R., Newby, D., Mills, N., MacNee, W. and Stone, V. 2005, "Combustion-derived nanoparticles: A critical review of their toxicology following inhalation exposure", *Part Fibre Toxicol*, vol. 2, no.10, pp.1-14.

Driscoll, K. E., Deyo, L. C., Carter, J. M., Howard, B. W., Hassenbein, D. G. and Bertram, T. A. 1997, "Effects of particle exposure and particle-elicited inflammatory cells on mutation in rat alveolar epithelial cells", *Carcinogenesis*, vol. 18, no.2, pp. 423-430.

Duffin, R., Tran, C. L., Clouter, A., Brown, D. M., MacNee, W., Stone, V. and Donaldson, K. 2002, "The importance of surface area and specific reactivity in the acute pulmonary inflammatory response to particles", *Ann Occup Hyg*, vol. 46, no. 1, pp. 242-245.

Duffin, R., Tran, L., Brown, D., Stone V. and Donaldson, K. 2007, "Proinflammatory effects of low-toxicity and metal nanoparticles *in vivo* and *in vitro*: highlighting the role of particle surface area and surface reactivity", *Inhal Toxicol*, vol. 19, no.10, pp. 849-856.

Dufour, E. K., Kumaravel, T., Nohynek, G. J., Kirkland, D. and Toutain, H. 2006, "Clastogenicity, photo-clastogenicity or pseudo-photo-clastogenicity: Genotoxic effects of zinc oxide in the dark, in pre-irradiated or simultaneously irradiated Chinese hamster ovary cells", *Mut Res*, vol. 607, no.2, pp. 215-224.

Dugan, L.L., Gabrielsen, J.K., Yu, S.P., Lin, T.S. and Choi, D.W. 1996, "Buckminsterfullerene free radical scavengers reduce excitotoxic and apoptotic death of cultured cortical neurons", *Neurobiol Dis*, vol. 3, no.2, pp. 129-135.

Engineered Nanoparticles: Review of Health and Environmental Safety

- Dumortier, H., Lacotte, S., Pastorin, G., Marega, R., Wu, W., Bonifazi, D., Briand, J.P., Prato, M., Muller, S. and Bianco, A. 2006, "Functionalised carbon nanotubes are non-cytotoxic and preserve the functionality of primary immune cells", *Nano Letters*, vol. 6, no. 7, pp. 1522-1528.
- Dunford, R., Salinaro, A., Cai, L., Serpone, N., Horikoshi, S., Hidaka, H. and Knowland, J. 1997, "Chemical oxidation and DNA damage catalysed by inorganic sunscreen ingredients", *FEBS Letters*, vol. 418, no. 1-2, pp. 87-90.
- Dussert, A. S., Gooris, E. and Hemmerle, J. 1997, "Characterization of the mineral content of a physical sunscreen emulsion and its distribution onto human stratum corneum", *Int J Cos Sci*, vol. 19, no. 3, pp. 119-129.
- Elder, A., Gelein, R., Silva, V., Feikert, T., Opanashuk, L., Carter, J., Potter, R., Maynard, A., Ito, Y., Finkelstein, J. and Oberdorster, G. 2006, "Translocation of inhaled ultrafine manganese oxide particles to the central nervous system" *Environ Health Perspect*, vol. 114, no.8, pp. 1172-1178.
- Erlanger, B.F., Chen, B.X., Zhu, M. and Brus, L. 2001, "Binding of an anti-fullerene IgG monoclonal antibody to single wall carbon nanotubes", *Nano Letters*, vol. 1, no.9, pp. 465-467.
- Fabian, E., Landsiedel, R., Ma-Hock, L., Wiench, K., Wohlleben, W., and Van, R. B. 2008, "Tissue distribution and toxicity of intravenously administered titanium dioxide nanoparticles in rats", *Arch Toxicol*, vol. 82, no. 3, pp. 151-157.
- Fall, M., Guerbet, M., Park, B., Gouriou, F., Dionnet, F. and Morin, J-P. 2007, "Evaluation of cerium oxide and cerium oxide based fuel additive safety on organotypic cultures of lung slices", *Nanotoxicol*, vol. 1, no. 3, pp. 226-233.
- Ferin, J., Oberdorster, G. and Penney, D. P. 1992, "Pulmonary retention of ultrafine and fine particles in rats", *Am J Respir Cell Mol Biol*, vol. 6, no.5, pp. 535-542.
- Ferin, J., Oberdorster, G., Penney, D. P., Soderholm, S. C., Gelein, R. and Piper, H. C. 1990, "Increased pulmonary toxicity of ultrafine particles? I. Particle clearance, translocation, morphology", *J Aerosol Sc*, vol. 21, no.3, pp. 381-384.
- Fortner, J.D., Lyon, D.Y., Sayes, C.M., Boyd, A.M., Falkner, J.C., Hotze, E.M., Alemany, L.B., Tao, Y.J., Guo, W., Ausman, K.D., Colvin, V.L. and Hughes, J.B. 2005, "C₆₀ in water: nanocrystal formation and microbial response", *Environ Sci Technol*, vol. 39, no. 11, pp. 4307-4316.
- Fujita, K., Morimoto, Y., Ogami, A., Myojo, T., Tanaka, I., Shimada, M., Wang, W.N., Endoh, S., Uchida, K., Nakazato, T., Yamamoto, K., Fukui, H., Horie, M., Yoshida, Y., Iwahashi, H. and Nakanishi, J. 2009, "Gene expression profiles in rat lung after inhalation exposure to C(60) fullerene particles" *Toxicology*, vol. 258, no. 1, pp. 47-55.
- Gamer, A. O., Leibold, E. and Van, R. B. 2006, "The in vitro absorption of microfine zinc oxide and titanium dioxide through porcine skin", *Toxicol In Vitro*, vol. 20, no. 3, pp. 301-307.
- Geiser, M., Casaulta, M., Kupferschmid, B., Schulz, H., Semmler-Behnke, M. and Kreyling, W. 2008, "The role of macrophages in the clearance of inhaled ultrafine titanium dioxide particles", *Am J of Respir Cell Mol Biol*, vol. 38, no. 3, pp. 371-376.
- Gojova, A., Guo, B., Kota, R. S., Rutledge, J. C., Kennedy, I. M. and Barakat, A. I. 2007, "Induction of inflammation in vascular endothelial cells by metal oxide nanoparticles: effect of particle composition", *Environ Health Perspect*, vol. 115, no. 3, pp. 403-409.
- Gharbi, N., Pressac, M., Hadchouel, M., Szwarc, H., Wilson, S.R. and Moussa, F. 2005. "[60]Fullerene is a powerful antioxidant *in vivo* with no acute or subacute toxicity" *Nano Letters*, vol. 5, no. 12, pp. 2578-2585.

Goodman, C.M., Chari, N.S., Han, G., Hong, R., Ghosh, P. and Rotello, V.M. 2006, "DNA-binding by functionalised gold nanoparticles: mechanism and structural requirements", *Chem Biol Drug Des*, vol. 67, no.4, pp. 297-304.

Grassian, V. H., Adamcakova-Dodd, A., Pettibone, J. M., O'shaughnessy, P. T. and Thorne, P. S. 2007, "Inflammatory response of mice to manufactured titanium dioxide nanoparticles: comparison of size effects through different exposure routes", *Nanotoxicol*, vol. 1, no. 3, pp. 211-226

Grubek-Jaworska, H., Nejman, P., Czuminiska, K., Przybylowski, T., Huczko, A., Lange, H., Bystrejewski, M., Baranowski, P. and Chazan, R. 2006, "Preliminary results on the pathogenic effects of intratracheal exposure to one-dimensional nanocarbons", *Carbon*, vol. 44, no. 6, pp. 1057-1063.

Guo, L., Morris, D.G., Liu, X., Vaslet, C., Kane, A.B. and Hurt, R.H. 2007, "Iron bioavailability and redox activity in diverse carbon nanotube samples", *Chem Mater*, vol. 19, no. 4, pp. 3472-3478.

Gurr, J. R., Wang, A. S., Chen, C. H. and Jan, K. Y. 2005, "Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells", *Toxicol*, vol. 213, no.1-2, pp.66-73.

Han, S.G., Andrews, R., Gairola, C.G. and Bhalla, D.K. 2008, "Acute pulmonary effects of combined exposure to carbon nanotubes and ozone in mice", *Inhal Toxicol*, vol. 20, no. 4, pp. 391-398.

Han, B. and Karim, M.N. 2009, Cytotoxicity of aggregated fullerene C₆₀ particles on CHO and MDCK cells", *Scanning*, vol. 30, no.2, pp. 213-220.

Harhaji, L., Isakovic, A., Vucicevic, L., Janjetovic, K., Misirkic, M., Markovic, Z., Todorovic-Markovic, B., Nikolic, N., Vranjes-Djuric, S., Nikolic, Z. and Trajkovic, V. 2008, "Modulation of tumor necrosis factor-mediated cell death by fullerenes", *Pharmaceut Res*, vol. 25, no. 6, pp. 1365-1376.

Heinrich, U., Fuhst, R., Rittinghausen, S., Creutzenberg, O., Bellmann, B., Koch, W., and Levsen, K. 1995, "Chronic inhalation exposure of wistar rats and two different strains of mice to diesel engine exhaust, carbon black, and titanium dioxide", *Inhal Toxicol*, vol. 7, no. 4, pp. 533-556.

Helfenstein, M., Miragoli, M., Rohr, S., Muller, L., Wick, P., Mohr, M., Gehr, P. and Rothen-Rutishauser, B. 2008, "Effects of combustion-derived ultrafine particles and manufactured nanoparticles on heart cells *in vitro*", *Toxicol*, vol. 253, no. 1-3, pp. 70-78.

Hirano, S., Kanno, S. and Furuyama, A. 2008, "Multi-walled carbon nanotubes injure the plasma membrane of macrophages", *Toxicol Appl Pharmacol*, vol. 232, no. 2, pp. 244-251.

Hohr, D., Steinfartz, Y., Schins, R. P., Knaapen, A. M., Martra, G., Fubini, B. and Borm, P. J. 2002, "The surface area rather than the surface coating determines the acute inflammatory response after instillation of fine and ultrafine TiO₂ in the rat", *Int J Hyg Environ Health* vol. 205, no.3, pp. 239-244.

Hsin, Y.H., Chen, C.F., Huang, S., Shih, T.S., Lai, P.S. and Chueh, P.J. 2008, "The apoptotic effect of nanosilver is mediated by a ROS- and JNK dependent mechanism involving the mitochondrial pathway in NIH3T3 cells", *Toxicol Lett*, vol. 179, no. 3, pp. 130-139.

Huang, S.T., Liao, J.S., Fang, H.W. and Lin, C.M. 2008, "Synthesis and anti-inflammation evaluation of new C₆₀ fulleropyrrolidines bearing biologically active xanthine", *Bioorg Med Chem Lett*, vol. 18, no. 1, pp. 99-103.

Huczko, A., Lange, H. and Calko, E. 1999, "Fullerenes: experimental evidence for a null risk of skin irritation and allergy", *Fullerene Sci Technol*, vol. 7, no. 5, pp. 935-939.

- Hussain, S. M., Hess, K. L., Gearhart, J. M., Geiss, K. T. and Schlager, J. J. 2005, "In vitro toxicity of nanoparticles in BRL 3A rat liver cells", *Toxicol In Vitro*, vol. 19, no. 7, pp. 975-983.
- Hutchison, G.R., Brown, D.M., Hibbs, L.R., Heal, M.R., Donaldson, K., Maynard, R.L., Monaghan, M., Nichol, I. A. and Stone, V. 2005, "The effect of refurbishing a UK steel plant on PM10 metal composition and ability to induce inflammation", *Respir Res*, vol. 18, no. 6, pp. 43-59.
- Hyun, J. S., Lee, B. S., Ryu, H. Y., Sung, J. H., Chung, K. H., and Yu, I. J. 2008, "Effects of repeated silver nanoparticles exposure on the histological structure and mucins of nasal respiratory mucosa in rats", *Toxicol Lett*, vol. 182, no. 1-3, pp. 24-28.
- Injac, R., Perse, M., Cerne, M., Potocnik, N., Radic, N., Govedarica, B., Djordjevic, A., Cerar, A. and Strukelj, B. 2009, "Protective effects of fullereneol C₆₀(OH)₂₄ against doxorubicin-induced cardiotoxicity and hepatotoxicity in rats with colorectal cancer", *Biomaterials*, vol. 30, no. 6, pp. 1184-1196.
- Inoue, K., Takano, H., Ohnuki, M., Yanagisawa, R., Sakurai, M., Shimada, A., Mizushima, K. and Yoshikawa, T. 2008, "Size effects of nanomaterials on lung inflammation and coagulatory disturbance", *Int J Immunopathol Pharmacol*, vol. 21, no. 1, pp. 197-206.
- Inoue, K., Takano, H., Koike, E., Yanagisawa, R., Sakurai, M., Tasaka, S., Ishizaka, A. and Shimada, A. 2008, "Effects of pulmonary exposure to carbon nanotubes on lung and systemic inflammation with coagulatory disturbance induced by lipopolysaccharide in mice", *Experiment Biol Med*, vol. 233, no. 12, pp. 1583-1590.
- Isakovic, A., Markovic, Z., Nikolic, N., Todorovic-Markovic, B., Vranjes-Djuric, S., Harhaji, L., Raicevic, N., Romcevic, N., Vasiljevic-Radovic, D., Dramicanin, M. and Trajkovic, V. 2006, "Inactivation of nanocrystalline C₆₀ cytotoxicity by gamma-irradiation", *Biomaterials*, vol. 27, no. 29, pp. 5049-5058.
- Jacobsen, N.R., Pojana, G., White, P., Moller, P., Cohn, C.A., Korsholm, K.S., Vogel, U., Marcomini, A., Loft, S. and Wallin, H. 2008, "Genotoxicity, cytotoxicity, and reactive oxygen species induced by single-walled carbon nanotubes and C(60) fullerenes in the FE1-Mutatrade mark Mouse lung epithelial cells", *Environ Mol Mutagen*, vol. 49, no. 6, pp. 476-487.
- Janssen, Y.M., Barchowsky, A., Treadwell, M., Driscoll, K.E. and Mossman, B.T. 1995, "Asbestos induces nuclear factor kappa B (NF-kappa B) DNA-binding activity and NF-kappa B-dependent gene expression in tracheal epithelial cells", *PNAS USA*, vol. 92, no. 18, 8458-8462.
- Ji, J.H., Jung, J.H., Kim, S.S., Yoon, J.U., Park, J.D., Choi, B.S., Chung, Y.H., Kwon, I.H., Jeong, J., Han, B.S., Shin, J.H., Sung, J.H., Song, K.S. and Yu, I.J. 2007, "Twenty-eight-day inhalation toxicity study of silver nanoparticles in sprague-dawley rats", *Inhal Toxicol*, vol. 19, no. 10, pp. 857-871.
- Jia, G., Wang, H., Yan, L., Wang, X., Pei, R., Yan, T., Zhao, Y. and Guo X. 2005, "Cytotoxicity of carbon nanomaterials: single-wall nanotube, multi-wall nanotube, and fullerene", *Environ Sci Technol*, vol. 39, no. 5, pp. 1378-1383.
- Jimenez, L.A., Thompson, J., Brown, D.A., Rahman, I., Antonicelli, F., Duffin, R., Drost, E.M., Hay, R.T., Donaldson, K. and MacNee, W. 2000, "Activation of NF-kappaB by PM(10) occurs via an iron-mediated mechanism in the absence of IkappaB degradation", *Toxicol Appl Pharmacol*, vol. 166, no. 2, pp. 101-110.
- Jin, C. Y., Zhu, B. S., Wang, X. F. and Lu, Q. H. 2008, "Cytotoxicity of titanium dioxide nanoparticles in mouse fibroblast cells", *Chem Res Toxicol*, vol. 21, no. 9, pp. 1871-1877.
- Kaewamatawong, T., Shimada, A., Okajima, M., Inoue, H., Morita, T., Inoue, K. and Takano, H. 2006, "Acute and subacute pulmonary toxicity of low dose of ultrafine colloidal silica particles in mice after intratracheal instillation", *Toxicol Pathol*, vol. 34, no. 7, pp. 958-965.

- Kagan, V.E., Tyurina, Y.Y., Tyurin, V.A., Konduru, N.V., Potapovich, A.I., Osipov, A.N., Kisin, E.R., Schwegler-Berry, D., Mercer, R., Castranova, V. and Shvedova, A.A. 2006, "Direct and indirect effects of single-walled carbon nanotubes on RAW 264.7 macrophages: Role of iron", *Toxicol Lett*, vol. 165, no. 1, pp. 88-100.
- Kagan, V.E., Bayir, H. and Shvedova, A.A. 2005, "Nanomedicine and nanotoxicology: two sides of the same coin", *Nanomed*, vol 1, no. 4, pp. 313-316.
- Kam, N.W., Jessop, T.C., Wender, P.A. and Dai, H. 2004, "Nanotube molecular transporters: internalisation of carbon nanotube-protein conjugates into mammalian cells", *J Am Soc* vol. 126, no. 22, pp. 6850-6851.
- Kamat, J.P., Devasagayam, T.P.A., Priyadarsini, K.I. and Mohan, H. 2000, "Reactive oxygen species mediated membrane damage induced by fullerene derivatives and its possible biological implications", *Toxicol*, vol. 155, no. 1-3, pp. 55-61.
- Kang, J. L., Moon, C., Lee, H. S., Lee, H. W., Park, E. M., Kim, H. S. and Castranova, V. 2008a, "Comparison of the biological activity between ultrafine and fine titanium dioxide particles in RAW 264.7 cells associated with oxidative stress", *J Toxicol Environ Health A*, vol. 71, no. 8, pp. 478-485.
- Kang, S. J., Kim, B. M., Lee, Y. J. and Chung, H. W. 2008b, "Titanium dioxide nanoparticles trigger p53-mediated damage response in peripheral blood lymphocytes", *Environ Mol Mutagen*, vol. 49, no. 5, pp. 399-405.
- Karajanagi, S.S., Vertegel, A.A., Kane, R.S. and Dordick, J.S. 2004, "Structure and function of enzymes adsorbed onto single-walled carbon nanotubes", *Langmuir*, vol. 20, no. 26, pp. 11594-11599.
- Karlsson, H. L., Cronholm, P., Gustafsson, J. and Moller, L. 2008, "Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes", *Chem Res Toxicol*, vol. 21, no. 9, pp. 1726-1732.
- Kim, J. K., Lee, W. K., Lee, E. J., Cho, Y. J., Lee, K. H., Kim, H. S., Chung, Y., Kim, K. A. and Lim, Y. 1999, "Mechanism of silica- and titanium dioxide-induced cytotoxicity in alveolar macrophages", *J Toxicol Environ Health A*, vol. 58, no. 7, pp. 437-450.
- Kim, Y., Suh, H.S., Cha, H.J, Kim, S.H., Jeong, K.S., Kim, D.H. 2009, "A case of generalised argyria after ingestion of colloidal silver solution", *Am J Ind Med*, vol. 52, no. 3, pp. 246-250.
- Kisin, E.R., Murray, A.R., Keane, M.J., Shi, X.C., Schwegler-Berry, D., Gorelik, O., Arepalli, S., Castranova, V., Wallace, W.E., Kagan, V.E. and Shvedova, A.A. 2007, "Single-walled carbon nanotubes: geno- and cytotoxic effects in lung fibroblast V79 cells", *J Toxicol Environ Health A*, vol. 70, no. 24, pp. 2071-2079.
- Kiss, B., Biro, T., Czifra, G., Toth, B. I., Kertesz, Z., Szikszai, Z., Kiss, A. Z., Juhasz, I., Zouboulis, C. C. and Hunyadi, J. 2008, "Investigation of micronized titanium dioxide penetration in human skin xenografts and its effect on cellular functions of human skin-derived cells", *Exp Dermatol*, vol. 17, no. 8, pp. 659-667.
- Knaapen, A.M., Seiler, F., Schilderman, P.A., Nehls, P., Bruch, J., Schins, R.P. and Borm, P.J. 1999, "Neutrophils cause oxidative DNA damage in alveolar epithelial cells", *Free Rad Biol Med*, vol. 27, no. 1-2, pp. 234-240.
- Komatsu, T., Tabata, M., Kubo-Irie, M., Shimizu, T., Suzuki, K.I., Nihei, Y. and Takeda K. 2008, "The effects of nanoparticles on mouse testis Leydig cells *in vitro*", *Toxicol in Vitro*, vol. 22, no. 8, pp. 1825-1831.
- Kostarelos, K., Lacerda, L., Pastorin, G., Wu, W., Wieckowski, S., Luangsivilay, J., Godefroy, S., Pantarotto, D., Briand, J.P., Muller, S., Prato, M. and Bianco, A. 2007, "Cellular uptake of

functionalised carbon nanotubes is independent of functional group and cell type", *Nature Nanotechnol*, vol. 2, no. 2, pp108-113.

Koyama, S., Endo, M., Kim, Y-A., Hayashi, T., Yanagisawa, T., Osaka, K., Koyama, H., Hania, H. and Kuroiwa, N. 2006, "Role of systemic T-cells and histopathological aspects after subcutaneous implantation of various carbon nanotubes in mice", *Carbon*, vol. 44, no. 6, pp. 1079-1092.

Krajcik, R., Jung, A., Hirsch, A., Neuhuber, W. and Zolk, O. 2008, "Functionalisation of carbon nanotubes enables non-covalent binding and intracellular delivery of small interfering RNA for efficient knock-down of genes", *Biochem Biophys Res Comm*, vol. 369, no. 2, pp. 595-602.

L'azou, B., Jorly, J., On, D., Sellier, E., Moisan, F., Fleury-Feith, J., Cambar, J., Brochard, P. and Ohayon-Court, C. 2008, "In vitro effects of nanoparticles on renal cells", *Part Fibre Toxicol*, vol. 5, pp. 22-36.

Lacerda, L., li-Boucetta, H., Herrero, M.A., Pastorin, G., Bianco, A., Prato, M. and Kostarelos, K. 2008, "Tissue histology and physiology following intravenous administration of different types of functionalised multiwalled carbon nanotubes", *Nanomed*, vol. 3, no. 2, pp.149-161.

Lam, C.W., James, J.T., McCluskey, R., Arepalli, S. and Hunter, R.L. 2006, "A review of carbon nanotube toxicity and assessment of potential occupational and environmental health risks", *Crit Rev Toxicol*, vol. 36, no. 3, pp.189-217.

Lam, C.W., James, J.T., McCluskey, R. and Hunter, R.L.. 2004, "Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation" *Toxicol Sci*, vol. 77, no. 1, pp. 126-134.

Larese, F.F., D'Agostin, F., Crosera, M., Adami, G., Renzi, N., Bovenzi, M. and Maina, G. 2009, "Human skin penetration of silver nanoparticles through intact and damaged skin", *Toxicol*, vol. 225, no. 1-2, pp.33-37.

Lee, K.J., Nallathamby, P.D., Browning, L.M., Osgood, C.L. and Xu XHN. 2007, "In vivo imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. *ACS Nano*, vol. 1, no. 2, pp. 133-143.

Li, J.G., Li, W.X., Xu, J.Y., Cai, X.Q., Liu, R.L., Li, Y.J., Zhao, Q.F. and Li, Q.N. 2007a, "Comparative study of pathological lesions induced by multiwalled carbon nanotubes in lungs of mice by intratracheal instillation and inhalation", *Environ Toxicol*, vol. 22, no. 4, pp. 415-421.

Li, Z. Hulderman, T., Salmen, R., Chapman, R., Leonard, S.S., Young, S.H., Shvedova, A.A., Luster, M.I. and Simeonova, P.P. 2007b, "Cardiovascular effects of pulmonary exposure to single-wall carbon nanotubes", *Environ Health Perspect*, vol. 115, no. 3, pp. 377-382.

Lin, A.M., Fang, S.F., Lin, S.Z., Chou, C.K., Luh, T.Y. and Ho, L.T. 2002, "Local carboxyfullerene protects cortical infarction in rat brain" *Neurosci Rese*, vol. 43, no. 4, pp. 317-321.

Lin, W., Huang, Y. W., Zhou, X. D. and Ma, Y. 2006, "Toxicity of cerium oxide nanoparticles in human lung cancer cells", *Int J Toxicol*, vol. 25, no.6, pp. 451-457.

Linnainmaa, K., Kivipensas, P. and Vainio, H. 1997 "Toxicity and cytogenetic studies of ultrafine titanium dioxide in cultured rat liver epithelial cells", *Toxicol In Vitro*, vol. 11, no.4, pp. 329-335.

Liu, H.F., Liu, Z.L., Xie, C.S., Yu, J. and Zhu, C.H. 2007, "The antifertility effectiveness of copper/low-density polyethylene nanocomposite and its influence on the endometrial environment in rats", *Contraception* vol. 75, no. 2, pp.157- 161

Engineered Nanoparticles: Review of Health and Environmental Safety

Liu, R., Sun, F., Zhang, L., Zong, W., Zhao, X., Wang, L., Wu, R., and Hao, X. 2009, "Evaluation on the toxicity of nanoAg to bovine serum albumin", *Sci Total Environ*, vol. 407, no. 13, pp. 4184-4188.

Long, T. C., Saleh, N., Tilton, R. D., Lowry, G. V. and Veronesi, B. 2006, "Titanium dioxide (P25) produces reactive oxygen species in immortalized brain microglia (BV2): implications for nanoparticle neurotoxicity", *Environ Sci Technol*, vol. 40, no. 14, pp. 4346-4352.

Long, T. C., Tajuba, J., Sama, P., Saleh, N., Swartz, C., Parker, J., Hester, S., Lowry, G. V. and Veronesi, B. 2007, "Nanosize titanium dioxide stimulates reactive oxygen species in brain microglia and damages neurons *in vitro*", *EnvironHealth Perspect*, vol. 115, no. 11, pp. 1631-1637.

Lu, N., Zhu, Z., Zhao, X., Tao, R., Yang, X. and Gao, Z. 2008, "Nano titanium dioxide photocatalytic protein tyrosine nitration: a potential hazard of TiO₂ on skin", *Biochem Biophys Res Communications*, vol. 370, no. 4, pp. 675-680.

Lubick N. 2008, "Nanosilver toxicity: ions, nanoparticles--or both?", *Environ Sci Technol*, vol, 42, no.23, pp. 8617.

Luoma, S. N. 2008, *Silver Nanotechnologies and The Environment: Old Problems or New Challenges*, The Pew Charitable Trusts and the Woodrow Wilson International Center for Scholars.

Lyon, D.Y., Adams, L.K., Falkner, J.C. and Alvarez, P.J. 2006, "Antibacterial activity of fullerene water suspensions: effects of preparation method and particle size", *Environ Sci Technol*, vol. 40, no. 14, pp. 4360-4366.

MacNee, W. and Donaldson, K. 2003, "Mechanism of lung injury caused by PM10 and ultrafine particles with special reference to COPD. *Eur Respir J*, vol. 21, no. 40, pp. 47s-51s.

Makhluf, S.B.D., Abu-Mukh, R., Rubinstein, S., Breitbart, H. and Gedanken, A. 2008, "Modified PVA-Fe₃O₄ nanoparticles as protein carriers into sperm cells", *Small* vol. 4, no. 9, pp. 1453-1458

Magrez, A., Kasas, S., Salicio, V., Pasquier, N., Seo, J.W., Celio, M., Catsicas, S., Schwaller, B. and Forro, L. 2006, "Cellular toxicity of carbon-based nanomaterials", *Nano Letters*, vol. 6, no. 6, pp. 1121-1125.

Manna, S.K., Sarkar, S., Barr, J., Wise, K., Barrera, E.V., Jejelowo, O., Rice-Ficht, A.C. and Ramesh, G.T. 2005, "Single-walled carbon nanotube induces oxidative stress and activates nuclear transcription factor-kappaB in human keratinocytes", *Nano Letters*, vol. 5, no. 9, pp.1676-1684.

Manninger, S. P., Muldoon, L. L., Nesbit, G., Murillo, T., Jacobs, P. M. and Neuwelt, E. A. 2005, "An exploratory study of ferumoxtran-10 nanoparticles as a blood-brain barrier imaging agent targeting phagocytic cells in CNS inflammatory lesions", *AJNR Am J Neuroradiol*, vol. 26, no. 9, pp. 2290-2300.

Markovic, Z., Todorovic-Markovic, B., Kleut, D., Nikolic, N., Vranjes-Djuric, S., Misirkic, M., Vucicevic, L., Janjetovic, K., Isakovic, A., Harhaji, L., Babic-Stojic, B., Dramicanin, M. and Trajkovic, V. 2007, "The mechanism of cell-damaging reactive oxygen generation by colloidal fullerenes", *Biomaterials*, vol. 28, no. 36, pp. 5437-5448.

Mavon, A., Miquel, C., Lejeune, O., Payre, B. and Moretto, P. 2007, "*In vitro* percutaneous absorption and *in vivo* stratum corneum distribution of an organic and a mineral sunscreen", *Skin Pharmacol Physiol*, vol. 20, no.1, pp. 10-20.

Maynard, A.D., Baron, P.A., Foley, M., Shvedova, A.A., Kisin, E.R. and Castranova V. 2004, "Exposure to carbon nanotube material: aerosol release during the handling of unrefined single-

walled carbon nanotube material", *Journal of Toxicol Environ Health Part A*, vol. 67. no. 1, pp. 87-107.

Maynard, A.D., Aitken, R.J., Butz, T., Colvin, V., Donaldson, K., Oberdörster, G., Philbert, M.A., Ryan, J., Seaton, A., Stone, V., Tinkle, S.S., Tran, L., Walker, N.J. and Warheit, D.B. 2006, "Safe Handling in nanotechnology", *Nature*, vol. 444, no. 7117, pp. 267-269.

Mercer, R.R., Scabilloni, J., Wang, L., Kisin, E., Murray, A.R., Schwegler-Berry, D., Shvedova, A.A. and Castranova, V. 2008, "Alteration of deposition pattern and pulmonary response as a result of improved dispersion of aspirated single-walled carbon nanotubes in a mouse model", *Am J Physiol- Lung Cell Mol Physiol*, vol. 294, no. 1, pp. L87-L97.

Mitchell, L.A., Gao, J., Wal, R.V., Gigliotti, A., Burchiel, S.W. and McDonald, J.D. 2007, "Pulmonary and systemic immune response to inhaled multiwalled carbon nanotubes", *Toxicol Sci*, vol. 100, no. 1, pp. 203-214.

Monteiro-Riviere, N.A., Inman, A.O., Wang, Y.Y. and Nemanich, R.J. 2005, "Surfactant effects on carbon nanotube interactions with human keratinocytes", *Nanomed*, vol. 1, no. 4, pp.293-299.

Monteiro-Riviere, N.A., Nemanich, R.J., Inman, A.O., Wang, Y.Y., Riviere, J.E.. 2005, "Multi-walled carbon nanotube interactions with human epidermal keratinocytes", *Toxicol Lett*, vol. 155, no. 3, pp. 377-384.

Monteiro-Riviere, N. and Inman, A.O. 2006, "Challenges for assessing carbon nanomaterial toxicity to the skin", *Carbon*, vol. 44, no. 6, pp/ 1070-1078.

Mori, T., Takada, H., Ito, S., Matsubayashi, K., Miwa, N. and Sawaguchi, T. 2006, "Preclinical studies on safety of fullerene upon acute oral administration and evaluation for no mutagenesis", *Toxicol*, vol. 225, no. 1, pp. 48-54.

Mori, T., Ito, S., Matsubayashi, K. and Sawaguchi, T. 2007, "Comparison of nitric oxide synthase inhibitors, phospholipase A2 inhibitor and free radical scavengers as attenuators of opioid withdrawal syndrome", *Behav Pharmacol*, vol. 18, no. 8, pp. 725-729.

Mossman, B.T., Kamp, D.W. and Weitzman, S.A. 1996, "Mechanisms of carcinogenesis and clinical features of asbestos-associated cancers", *Cancer Invest*, vol. 14, no. 5, pp. 466-480.

Muller, J., Decordier, I., Hoet, P.H., Lombaert, N., Thomassen, L., Huaux, F., Lison, D. and Kirsch-Volders, M. 2008, "Clastogenic and aneugenic effects of multi-wall carbon nanotubes in epithelial cells", *Carcinogenesis*, vol. 29, no. 2, pp. 427-433.

Muller, J., Huaux, F., Moreau, N., Misson, P., Heilier, J.F., Delos, M., Arras, M., Fonseca, A., Nagy, J.B. and Lison, D. 2005, "Respiratory toxicity of multi-wall carbon nanotubes", *Toxicol Appl Pharmacol*, vol. 207, no. 3, pp. 221-231.

Muldoon, L. L., Sandor, M., Pinkston, K. E. and Neuwelt, E. A. 2005, "Imaging, distribution, and toxicity of superparamagnetic iron oxide magnetic resonance nanoparticles in the rat brain and intracerebral tumor", *Neurosurgery*, vol. 57, no.4, pp. 785-796.

Murr, L.E., Garza, K.M., Soto, K.F., Carrasco, A., Powell, T.G., Ramirez, D.A., Guerrero, P.A., Lopez, D.A. and Venzor, J. III. 2005, "Cytotoxicity assessment of some carbon nanotubes and related carbon nanoparticle aggregates and the implications for anthropogenic carbon nanotube aggregates in the environment", *Int J Environ Res Public Health*, vol. 2, no. 1, pp. 31-42.

Murray, A.R., Kisin, E., Leonard, S.S., Young, S.H., Kommineni, C., Kagan, V.E., Castranova, V. and Shvedova, A.A. 2009, "Oxidative stress and inflammatory response in dermal toxicity of single-walled carbon nanotubes", *Toxicol*, vol. 257. no. 3, pp. 161-171.

Engineered Nanoparticles: Review of Health and Environmental Safety

Nakagawa, Y., Wakuri, S., Sakamoto, K. and Tanaka, N. 1997, "The photogenotoxicity of titanium dioxide particles", *Mut Res*, vol. 394, no.1-3, pp. 125-132.

Nel, A., Xia, T., Madler, L. and Li, N. 2006, "Toxic potential of materials at the nanolevel", *Science*, vol. 311, no. 5761, pp. 622-627.

Nielsen, G.D., Roursgaard, M., Jensen, K.A., Poulsen, S.S. and Larsen, S.T. 2008, " *In vivo* biology and toxicology of fullerenes and their derivatives", *Bas Clin Pharmacol Toxicol*, vol. 103, no. 3, pp. 197-208.

Nimmagadda, A., Thurston, K., Nollert, M.U. and McFetridge, P.S. 2006, "Chemical modification of SWNT alters in vitro cell-SWNT interactions" *J Biomed Mat Res A*, vol. 76, no. 3, pp. 614-625.

Niu, J., Azfer, A., Rogers, L. M., Wang, X. and Kolattukudy, P. E. 2007, "Cardioprotective effects of cerium oxide nanoparticles in a transgenic murine model of cardiomyopathy", *Cardiovas Res*, vol. 73, no. 3, pp. 549-559.

Nurkiewicz, T. R., Porter, D. W., Barger, M., Millecchia, L., Rao, K. M., Marvar, P. J., Hubbs, A. F., Castranova, V. and Boegehold, M. A. 2006, "Systemic microvascular dysfunction and inflammation after pulmonary particulate matter exposure", *Environ Health Perspect*, vol. 114, no.3, pp. 412-419.

Oberdorster, G. 2001, "Pulmonary effects of inhaled ultrafine particles. *Int Arch Occup Environ Health*, vol. 74, no.1, pp. 1-8.

Oberdorster, G., Ferin, J., Finkelstein, J., Wade, P. and Corson, N. 1990, "Increased pulmonary toxicity of ultrafine particles? II. Lung lavage studies", *J Aerosol Sci*, vol. 21, no.3, pp. 384-387.
Oberdorster, G., Stone, V. and Donaldson, K. 2007, "Toxicology of nanoparticles: A historical perspective", *Nanotoxicol*, vol. 1, no.1, pp. 2-25.

Oesterling, E., Chopra, N., Gavalas, V., Arzuaga, X., Lim, E. J., Sultana, R., Butterfield, D. A., Bachas, L. and Hennig, B. 2008, "Alumina nanoparticles induce expression of endothelial cell adhesion molecules", *Toxicol Lett*, vol. 178, no. 3, pp. 160-166.

Ogami, A., Morimoto, Y., Myojo, T., Oyabu, T., Murakami, M., Todoroki, M., Nishi, K., Kadoya, C., Yamamoto, M. and Tanaka, I. 2009, "Pathological features of different sizes of nickel oxide following intratracheal instillation in rats", *Inhal Toxicol*, [Epub ahead of print]

Pacurari, M., Yin, X.J., Zhao, J., Ding, M., Leonard, S.S., Schwegler-Berry, D., Ducatman, B.S., Sbarra, D., Hoover, M.D., Castranova, V. and Vallyathan V. 2008, "Raw single-wall carbon nanotubes induce oxidative stress and activate MAPKs, AP-1, NF-kappaB, and Akt in normal and malignant human mesothelial cells", *Environ Health Perspect*, vol. 116. no. 9, pp. 1211-1217.

Pan, Z., Lee, W., Slutsky, L., Clark, R. A., Pernodet, N. and Rafailovich, M. H. 2009, "Adverse effects of titanium dioxide nanoparticles on human dermal fibroblasts and how to protect cells", *Small*, vol. 5, no.4, pp. 511-520.

Pantarotto, D., Briand, J.P., Prato, M. and Bianco, A. 2004, "Translocation of bioactive peptides across cell membranes by carbon nanotubes", *Chem Commun (Cambridge)*, vol. 7, no.1, pp. 16-17.

Park, B., Martin, P., Harris, C., Guest, R., Whittingham, A., Jenkinson, P. and Handley, J. 2007, "Initial *in vitro* screening approach to investigate the potential health and environmental hazards of Enviroxtrade mark - a nanoparticulate cerium oxide diesel fuel additive", *Part Fibre Toxicol*, vol. 4, pp. 12-22.

Park, E. J., Choi, J., Park, Y. K., and Park, K. 2008a, "Oxidative stress induced by cerium oxide nanoparticles in cultured BEAS-2B cells", *Toxicol*, vol. 245, no. 1-2, pp. 90-100.

Park, E. J., Yi, J., Chung, K. H., Ryu, D. Y., Choi, J., and Park, K. 2008b, "Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells", *Toxicol Lett*, vol. 180, no. 3, pp. 222-229.

Patel, L.N., Zaro, J.L. and Shen, W.-C. 2007, "Cell Penetrating peptides: intracellular pathways. *Pharma Res* **24**, no.11, pp. 1977-1992.

Patil, S., Sandberg, A., Heckert, E., Self, W. and Seal, S. 2007, "Protein adsorption and cellular uptake of cerium oxide nanoparticles as a function of zeta potential", *Biomaterials*, vol. 28, no. 31, pp. 4600-4607.

Peters, K., Unger, R. E., Kirkpatrick, C. J., Gatti, A. M. and Monari, E. 2004, "Effects of nano-scaled particles on endothelial cell function *in vitro*: studies on viability, proliferation and inflammation", *J Mater Sci. Mater Med*, vol. 15, no. 4, pp. 321-325.

Pflucker, F., Hohenberg, H., Holzle, E., Will, T., Pfeiffer, S., Wepf, R., Diembeck, W., Wenck, H. and Gers-Barlag, H. 1999, "The outermost stratum corneum layer is an effective barrier against dermal uptake of topically applied micronized titanium dioxide", *Int J Cos Sci*, vol. 21, no.6, pp. 399-411.

Pisanic, T. R., Blackwell, J. D., Shubayev, V. I., Finones, R. R. and Jin, S. 2007, "Nanotoxicity of iron oxide nanoparticle internalization in growing neurons" *Biomaterials*, vol. 28, no. 16, pp. 2572-2581.

Poland, C.A., Duffin, R., Kinloch, I.A., Maynard, A., Wallace, W.A.H., Seaton, A., Stone, V., Brown, S., MacNee, W. and Donaldson, K. 2008, "Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study", *Nature Nanotechnol*, vol. 3, no.7, pp. 423-428.

Pope, C.A. and Dockery, D.W. 1999, "Epidemiology of particle effects". In Holgate ST, Samet JM, Koren HS eds, *Air Pollution and Health*; San Diego, Academic Press, 673-705.

Porter, A.E., Muller, K., Skepper, J., Midgley, P. and Welland, M. 2006, "Uptake of C₆₀ by human monocyte macrophages, its localization and implications for toxicity: studied by high resolution electron microscopy and electron tomography", *Acta Biomaterialia*, vol. 2, no. 4, pp. 409-419.

Porter, A.E., Gass, M., Muller, K., Skepper, J.N., Midgley, P.A. and Welland, M. 2007, "Direct imaging of single-walled carbon nanotubes in cells", *Nature Nanotechnol*, vol. 2, no. 11, pp. 713-717.

Pulskamp, K., Diabate, S. and Krug, H.F. 2007, "Carbon nanotubes show no sign of acute toxicity but induce intracellular reactive oxygen species in dependence on contaminants", *Toxicol Lett*, vol. 168, no. 1, pp. 58-74.

Qiao, R., Roberts, A.P., Mount, A.S., Klaine, S.J. and Ke, P.C. 2007, "Translocation of C₆₀ and its derivatives across a lipid bilayer", *Nano Letters*, vol. 7, no. 3, pp. 614-619.

Radomski, A., Jurasz, P., Alonso-Escolano, D., Drews, M., Morandi, M., Malinski, T. and Radomski, M.W. 2005, "Nanoparticle-induced platelet aggregation and vascular thrombosis", *Brit J Pharmacol*, vol. 146, no. 6, pp. 882-893.

Rahman, Q., Lohani, M., Dopp, E., Pemsel, H., Jonas, L., Weiss, D. G. and Schiffmann, D. 2002, "Evidence that ultrafine titanium dioxide induces micronuclei and apoptosis in Syrian hamster embryo fibroblasts", *Environ Health Perspect*, vol. 110, no. 8, pp. 797-800.

Rahman, M. F., Wang, J., Patterson, T. A., Saini, U. T., Robinson, B. L., Newport, G. D., Murdock, R. C., Schlager, J. J., Hussain, S. M., and Ali, S. F. 2009, "Expression of genes related to oxidative stress in the mouse brain after exposure to silver-25 nanoparticles", *Toxicol Lett*, vol. 187, no. 1, pp. 15-21.

Rehn, B., Seiler, F., Rehn, S., Bruch, J. and Maier, M. 2003, "Investigations on the inflammatory and genotoxic lung effects of two types of titanium dioxide: untreated and surface treated", *Toxicol Appl Pharmacol*, vol. 189, no. 2, pp. 84-95.

Raja, P.M., Connolley, J., Ganesan, G.P., Ci, L., Ajayan, P.M., Nalamasu, O. and Thompson, D.M. 2007, "Impact of carbon nanotube exposure, dosage and aggregation on smooth muscle cells", *Toxicol Lett*, vol. 169, no. 1, pp. 51-63.

Renwick, L. C., Brown, D., Clouter, A. and Donaldson, K. 2004, "Increased inflammation and altered macrophage chemotactic responses caused by two ultrafine particle types", *Occupational and Environ Med*, vol. 61, no.5, pp. 442-447.

Renwick, L. C., Donaldson, K. and Clouter, A. 2001, "Impairment of alveolar macrophage phagocytosis by ultrafine particles", *Toxicol Appl Pharmacol*, vol. 172, no. 2, pp. 119-127.

Reynolds, P. R., Larkman, D. J., Haskard, D. O., Hajnal, J. V., Kennea, N. L., George, A. J. and Edwards, A. D. 2006, "Detection of vascular expression of E-selectin in vivo with MR imaging", *Radiol*, vol. 241, no. 2, pp. 469-476.

Roberts, J.E., Wielgus, A.R., Boyes, W.K., Andley, U. and Chignell, C.F. 2008, "Phototoxicity and cytotoxicity of fullerol in human lens epithelial cells", *Toxicol Appl Pharmacol*, vol. 228, no 1, pp. 49-58.

Rothen-Rutishauser, B., Muhlfield, C., Blank, F., Musso, C. and Gehr, P. 2007, "Translocation of particles and inflammatory responses after exposure to fine particles and nanoparticles in an epithelial airway model", *Part Fibre Toxicol*, vol. 4, pp. 9-18.

Rotoli, B.M., Bussolatim, O., Bianchim, M.G., Barillim, A., Balasubramanianm, C., Bellucci, S. and Bergamaschi, E. 2008, "Non-functionalised multi-walled carbon nanotubes alter the paracellular permeability of human airway epithelial cells", *Toxicol Lett*, vol. 178, no. 2, pp. 95-102.

Roursgaard, M., Poulsen, S.S., Kepley, C.L., Hammer, M., Nielsen, G.D. and Larsen, S.T. 2008, "Polyhydroxylated C₆₀ fullerene (fullerenol) attenuates neutrophilic lung inflammation in mice", *Bas Clin Pharmacol Toxicol*, vol. 103, no. 4, pp. 386-388.

Rouse, J.G., Yang, J., Barron, A.R. and Monteiro-Riviere, N.A. 2006, "Fullerene-based amino acid nanoparticle interactions with human epidermal keratinocytes", *Toxicol In Vitro*, vol. 20, no. 8, pp. 1313-1320.

Rouse, J.G., Yang, J., Ryman-Rasmussen, J.P., Barron, A.R. and Monteiro-Riviere, N.A. 2007, "Effects of mechanical flexion on the penetration of fullerene amino acid-derivatized peptide nanoparticles through skin", *Nano Letters*, vol. 7, no. 1, pp. 155-160.

Sager, T. M. and Castranova, V. 2009, "Surface area of particle administered versus mass in determining the pulmonary toxicity of ultrafine and fine carbon black: comparison to ultrafine titanium dioxide", *Part Fibre Toxicol*. vol. 6, pp. 15-46.

Sager, T. M., Kommineni, C. and Castranova, V. 2008, "Pulmonary response to intratracheal instillation of ultrafine versus fine titanium dioxide: role of particle surface area", *PartiFibre Toxicol*, vol. 5, pp. 17-32.

Sato, Y., Yokoyama, A., Shibata, K., Akimoto, Y., Ogino, S., Nodasaka, Y., Kohgo, T., Tamura, K., Akasaka, T., Uo, M., Motomiya, K., Jeyadevan, B., Ishiguro, M., Hatakeyama, R., Watari, F. and Tohji, K. 2005, "Influence of length on cytotoxicity of multi-walled carbon nanotubes against human acute monocytic leukemia cell line THP-1 *in vitro* and subcutaneous tissue of rats *in vivo*", *Mol Biosys*, vol. 1, no. 2, pp.176-182.

Engineered Nanoparticles: Review of Health and Environmental Safety

- Sayes, C.M., Fortner, J.D., Guo, W., Lyon, D.Y., Boyd, A.M., Ausman, K., Tao, Y.J., Sitharaman, B., Wilson, L.J., Hughes, J.B., West, J.L. and Colvin, V.L. 2004, "The differential cytotoxicity of water-soluble fullerenes", *Nano Letters*, vol. 4, no. 10, pp. 1881-1887.
- Sayes, C. M., Wahi, R., Kurian, P. A., Liu, Y., West, J. L., Ausman, K. D., Warheit, D. B. and Colvin, V. L. 2006, "Correlating nanoscale titania structure with toxicity: a cytotoxicity and inflammatory response study with human dermal fibroblasts and human lung epithelial cells", *Toxicol Sci*, vol. 92, no. 1, pp. 174-185.
- Sayes, C.M., Gobin, A.M., Ausman, K.D., Mendez, J, West, J,L. and Colvin, V.L. 2005, "Nano-C₆₀ cytotoxicity is due to lipid peroxidation", *Biomaterials*, vol. 26, no. 36, pp. 7587-7595.
- Sayes, C.M., Liang, F., Hudson, J.L., Mendez, J., Guo, W., Beach, J.M., Moore, V.C., Doyle, C.D., West, J.L., Billups, W.E., Ausman, K.D. and Colvin, V.L. 2006, "Functionalisation density dependence of single-walled carbon nanotubes cytotoxicity *in vitro*", *Toxicol Lett*, vol, 161, no. 2, pp. 135-142.
- Sayes, C. M., Reed, K. L. and Warheit, D. B. 2007, "Assessing toxicity of fine and nanoparticles: comparing *in vitro* measurements to *in vivo* pulmonary toxicity profiles", *Toxicol Sci*, vol. 97, no.1, pp. 163-180.
- Sayes, C.M., Marchione, A.A., Reed, K.L. and Warheit, D.B. 2007, "Comparative pulmonary toxicity assessments of C₆₀ water suspensions in rats: few differences in fullerene toxicity *in vivo* in contrast to *in vitro* profiles", *Nano Letters*, vol. 7, no. 8, pp. 2399-2406.
- Schins, R.P. 2002, "Mechanisms of genotoxicity of particles and fibers", *Inhal Toxicol*, vol. 14, no. 1, pp. 57-78.
- Schubert, D., Dargusch, R., Raitano, J. and Chan, S. W. 2006, "Cerium and yttrium oxide nanoparticles are neuroprotective", *Biochem Biophys Res Comm*, vol. 342, no. 1, pp. 86-91.
- Schulz, J., Hohenberg, H., Pflucker, F., Gartner, E., Will, T., Pfeiffer, S., Wepf, R., Wendel, V., Gers-Barlag, H. and Wittern, K. P. 2002, "Distribution of sunscreens on skin", *Adv Drug Del Rev*, vol. 54, no. 1, pp. S157-S163.
- Schwartz, J. 1994, "Air pollution and daily mortality: a review and meta analysis", *Environ Res*, vol. 64, no. 1, pp. 36-52.
- Scrivens, W.A., Tour, J.M., Creek, K.E. and Pirisi, L. 1994, "Synthesis of ¹⁴C-labelled C₆₀, its suspension in water, and its uptake by human keratinocytes", *J Am Chem Soc*, vol. 116, no.10, pp. 4517-4518.
- Sera, N., Tokiwa, H. and Miyata, N. 1996, "Mutagenicity of the fullerene C₆₀-generated singlet oxygen dependent formation of lipid peroxides", *Carcinogenesis*, vol. 17, no. 10, pp. 2163-2169.
- Selikoff, I.J. 1990, "Historical developments and perspectives in inorganic fiber toxicity in man", *Environ Health Perspect*, vol. 88, pp. 269-76.
- Semmler-Behnke, M., Kreyling, W.G., Lipka, J., Fertsch, S., Wenk, A., Takenaka, S., Schmid, G. and Brandau, W. 2008, "Biodistribution of 1.4- and 18-nm Gold Particles in Rats", *Small*, vol. 4, no. 12, pp. 2108-2111.
- Sharma, V., Shukla, R. K., Saxena, N., Parmar, D., Das, M. and Dhawan, A. 2009, "DNA damaging potential of zinc oxide nanoparticles in human epidermal cells", *Toxicol Lett*, vol. 185, no.3, pp. 211-218.
- Shvedova, A.A., Castranova, V., Kisin, E.R., Schwegler-Berry, D., Murray, A.R., Gandelsman, V.Z., Maynard, A., and Baron, P. 2003, "Exposure to carbon nanotube material: assessment of nanotube cytotoxicity using human keratinocyte cells", *J Toxicol Environ Health A*, vol. 66, no. 20, pp. 1909-1926.

Shvedova, A.A., Kisin, E.R., Mercer, R., Murray, A.R., Johnson, V.J., Potapovich, A.I., Tyurina, Y.Y., Gorelik, O., Arepalli, S., Schwegler-Berry, D., Hubbs, A.F., Antonini, J., Evans, D.E., Ku, B.K., Ramsey, D., Maynard, A., Kagan, V.E., Castranova, V. and Baron, P.. 2005, "Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice", *Am J Physiol- Lung Cell Mol Physiol*, vol. 289, no. 5, pp. L698-L708.

Shvedova, A.A., Kisin, E.R., Murray, A.R., Gorelik, O., Arepalli, S., Castranova, V., Young, S.H., Gao, F., Tyurina, Y.Y., Oury, T.D. and Kagan, V.E.. 2007, "Vitamin E deficiency enhances pulmonary inflammatory response and oxidative stress induced by single-walled carbon nanotubes in C57BL/6 mice", *Toxicol Appl Pharmacol*, vol. 221, no. 3, pp. 339-348.

Shvedova, A.A., Kisin, E., Murray, A.R., Johnson, V.J., Gorelik, O., Arepalli, S., Hubbs, A.F., Mercer, R.R., Keohavong, P., Sussman, N., Jin, J., Yin, J., Stone, S., Chen, B.T., Deye, G., Maynard, A., Castranova, V., Baron, P.A. and Kagan, V.E. 2008a, "Inhalation vs. aspiration of single-walled carbon nanotubes in C57BL/6 mice: inflammation, fibrosis, oxidative stress, and mutagenesis", *Am J Physiol- Lung Cell Mol Physiol*, vol. 295, no. 4, pp. L552-L565.

Shvedova, A.A., Kisin, E.R., Murray, A.R., Kommineni, C., Castranova, V., Fadeel, B. and Kagan, V.E. 2008b, "Increased accumulation of neutrophils and decreased fibrosis in the lung of NADPH oxidase-deficient C57BL/6 mice exposed to carbon nanotubes", *Toxicol Appl Pharmacol*, vol. 231, no. 2, pp. 235-240.

Shi, L., Chen, J., Feng, Z., Chen, T., Lian, Y., Wang, X. and Li, C. 2007, "Photoluminescence characteristics of TiO₂ and their relationship to the photoassisted reaction of water/methanol mixture", *J Phys Chem*, vol. 111, no.2, pp. 693-699.

Simon-Deckers, A., Gouget, B., Mayne-L'hermite, M., Herlin-Boime, N., Reynaud, C. and Carriere, M. 2008, "*In vitro* investigation of oxide nanoparticle and carbon nanotube toxicity and intracellular accumulation in A549 human pneumocytes", *Toxicol*, vol. 253, no. 1-3 pp. 137-146.

Singh, R., Pantarotto, D., Lacerda, L., Pastorin, G., Klumpp, C., Prato, M., Bianco, A. and Kostarelos, K. 2006, "Tissue biodistribution and blood clearance rates of intravenously administered carbon nanotube radiotracers", *PNAS USA*, vol. 103, no. 9, pp. 3357-3362.

Singh, S., Shi, T., Duffin, R., Albrecht, C., van, B. D., Hohr, D., Fubini, B., Martra, G., Fenoglio, I., Borm, P. J. and Schins, R. P. 2007, "Endocytosis, oxidative stress and IL-8 expression in human lung epithelial cells upon treatment with fine and ultrafine TiO₂: role of the specific surface area and of surface methylation of the particles", *Toxicol Appl Pharmacol*, vol. 222, no. 2, pp. 141-151.

Sonavane, G., Tomoda, K., Sano, A., Ohshima, H., Terada, H. and Makino, K. 2008a, "*In vitro* permeation of gold nanoparticles through rat skin and rat intestine: effect of particle size", *Coll Surf B Biointerfaces*, vol. 65, no. 1, pp. 1-10.

Sonavane, G., Tomoda, K. and Makino, K. 2008b, "Biodistribution of colloidal gold nanoparticles after intravenous administration: Effect of particle size", *Coll Surf B Biointerfaces*, vol. 66, no. 2, pp. 274-280 .

Sosnovik, D. E., Nahrendorf, M. and Weissleder, R. 2008, "Magnetic nanoparticles for MR imaging: agents, techniques and cardiovascular applications", *Bas Res Cardiol*, vol. 103, no. 2, pp. 122-130.

Soto, K., Garza, K.M. and Murr, L.E. 2007, "Cytotoxic effects of aggregated nanomaterials *Acta Biomaterialia*. vol. 3, no. 3, pp. 351-358.

Stearns, R. C., Paulauskis, J. D. and Godleski, J. J. 2001, "Endocytosis of ultrafine particles by A549 cells", *Am J Respir Cell Mol Biol*, vol. 24, no.2, pp. 108-115.

Stoeger, T., Reinhard, C., Takenaka, S., Schroepfel, A., Karg, E., Ritter, B., Heyder, J. and Schulz, H. 2006, "Instillation of six different ultrafine carbon particles indicates a surface area

threshold dose for acute lung inflammation in mice", *Environ Health Perspect.* vol. 114, no.3, pp. 328-333.

Stone, V., Shaw, J., Brown, D.M., MacNee, W., Faux, S.P. and Donaldson, K. 1998, "The role of oxidative stress in the prolonged inhibitory effect of ultrafine carbon black on epithelial cell function", *Toxicol In Vitro*, vol. 12, no 6, pp. 649-659.

Sung, J.H., Ji, J.H., Park, J.D., Yoon, J.U., Kim, D.S., Jeon, K.S., Song, M.Y., Jeong, J., Han, B.S., Han, J.H., Chung, Y.H., Chang, H.K., Lee, J.H., Cho, M.H., Kelman, B.J. and Yu, I.J. 2009, "Subchronic inhalation toxicity of silver nanoparticles", *Toxicol Sciences*, vol. 108, no.2, 452-461.

Tabata, Y., Murakami, Y. and Ikada, Y. 1997, "Photodynamic effect of polyethylene glycol-modified fullerene on tumour", *Jap J Cancer Res*, vol. 88, no. 11, pp. 1108-1116.

Takagi, A., Hirose, A., Nishimura, T., Fukumori, N., Ogata, A., Ohashi, N., Kitajima, S. and Kanno, J. 2008, "Induction of mesothelioma in p53[±] mouse by intraperitoneal application of multi-wall carbon nanotube", *J Toxicol Sci*, vol. 33, no. 1, pp.105-116.

Takenaka, S., Karg, E., Roth, C., Schulz, H., Ziesenis, A., Heinzmann, U., Schramel, P., and Heyder, J. 2001, "Pulmonary and systemic distribution of inhaled ultrafine silver particles in rats", *Environ.Health Perspect.*, vol. 109 Suppl 4, pp. 547-551.

Theogaraj, E., Riley, S., Hughes, L., Maier, M. and Kirkland, D. 2007, "An investigation of the photo-clastogenic potential of ultrafine titanium dioxide particles", *Mut Res*, vol. 634, no. 1-2, pp. 205-219.

Thevenot, P., Cho, J., Wavhal, D., Timmons, R. B. and Tang, L. 2008, "Surface chemistry influences cancer killing effect of TiO₂ nanoparticles", *Nanomed*, vol. 4, no. 3, pp. 226-236.

Tian, F., Cui, D., Schwarz, H., Estrada, G.G. and Kobayashi, H. 2006, "Cytotoxicity of single-wall carbon nanotubes on human fibroblasts", *Toxicol In Vitro*, vol. 20, no. 7, pp. 1202-1212.

Tian, J., Wong, K.K., Ho, C.M., Lok, C.N., Yu, W.Y., Che, C.M., Chiu, J.F. and Tam, P.K. 2007 Topical Delivery of Silver Nanoparticles Promotes Wound Healing, *ChemMedChem*, vol. 2, no.1, pp. 129-136.

Trajkovic, S., Dobric, S., Jacevic, V., Dragojevic-Simic, V., Milovanovic, Z. and Dordevic, A. 2007, "Tissue-protective effects of fullereneol C₆₀(OH)₂₄ and amifostine in irradiated rats", *Coll Surf B: Biointerfaces*, vol. 58, no. 1, pp. 39-43.

Trop, M., Novak, M., Rodl, S., Hellbom, B., Kroell, W., Goessler, W., 2006, "Silver coated dressing Acticoat caused raised liver enzymes and argyria-like symptoms in burn patient", *J Trauma*, vol. 60, no. 3, pp. 648-652.

Tsao, N., Kanakamma, P.P., Luh, T.Y., Chou, C.K. and Lei, H.Y. 1999, "Inhibition of Escherichia coli-induced meningitis by carboxyfullerene", *Antimicrob Agents Chemo*, vol. 43, no. 9, pp. 2273-2277.

Tsoli, M., Kuhn, H., Brandau, W., Esche, H. and Schmid, G. 2005, "Cellular uptake and toxicity of Au₅₅ clusters", *Small*, vol. 1, no. 8-9, pp. 841-844.

Tsuchiya, T., Oguri, I., Yamakoshi, Y.N. and Miyata, N. 1996, "Novel harmful effects of [60]fullerene on mouse embryos *in vitro* and *in vivo*", *FEBS Letters*, vol. 393, no. 1, pp. 139-145.

Tykhomyrov, A.A., Nedzvetsky, V.S., Klochkov, V.K. and Andrievsky, G.V. 2008, "Nanostructures of hydrated C₆₀ fullerene (C₆₀HyFn) protect rat brain against alcohol impact and attenuate behavioural impairments of alcoholized animals", *Toxicol*, vol. 246, no. 2-3, pp.158-165.

- Ueng, T.H., Kang, J.J., Wang, H.W., Cheng, Y.W. and Chiang, L.Y. 1997, "Suppression of microsomal cytochrome P450-dependent monooxygenases and mitochondrial oxidative phosphorylation by fullerene, a polyhydroxylated fullerene C₆₀", *Toxicol Lett*, vol. 93, no 1, pp. 29-37.
- Usenko, C.Y., Harper, S.L. and Tanguay, R.L. 2008, "Fullerene C₆₀ exposure elicits an oxidative stress response in embryonic zebrafish", *Toxicol Appl Pharmacol*, vol. 229, no. 1, pp. 44-55.
- Vlachou, E., Chipp, E., Shale, E., Wilson, Y.T., Papini, R. and Moiemmen, N.S. 2007, "The safety of nanocrystalline silver dressings on burns: A study of systemic silver absorption", *Burns*, vol. 33, no. 8, pp. 979-985.
- Wadhwa, A., and Fung, M. (2005). Systemic argyria associated with ingestion of colloidal silver. *Dermatol Online J*, 11(1): 12. Available at <http://dermatology.cdlib.org>
- Wang, I.C., Tai, L.A., Lee, D.D., Kanakamma, P.P., Shen, C.K., Luh, T.Y., Cheng, C.H. and Hwang, K.C. 1999, "C(60) and water-soluble fullerene derivatives as antioxidants against radical-initiated lipid peroxidation", *J Med Chem*, vol. 42, no. 22, pp. 4614-4620.
- Wang, H., Wang, J., Deng, X., Sun, H., Shi, Z., Gu, Z., Liu, Y. and Zhao, Y. 2004, "Biodistribution of carbon single-walled nanotubes in mice", *J Nanosci Nanotechnol*, vol. 4, no.8, pp. 1019-1024.
- Wang, J., Chen, C., Liu, Y., Jiao, F., Li, W., Lao, F., Li, Y., Li, B., Ge, C., Zhou, G., Gao, Y., Zhao, Y. and Chai, Z. 2008a, "Potential neurological lesion after nasal instillation of TiO₂ nanoparticles in the anatase and rutile crystal phases", *Toxicology Letters*, vol. 183, no. 1-3, pp. 72-80.
- Wang, J., Liu, Y., Jiao, F., Lao, F., Li, W., Gu, Y., Li, Y., Ge, C., Zhou, G., Li, B., Zhao, Y., Chai, Z. and Chen, C. 2008b, "Time-dependent translocation and potential impairment on central nervous system by intranasally instilled TiO₂ nanoparticles", *Toxicol*, vol. 254, no. 1-2, pp. 82-90.
- Wang, J., Zhou, G., Chen, C., Yu, H., Wang, T., Ma, Y., Jia, G., Gao, Y., Li, B., Sun, J., Li, Y., Jiao, F., Zhao, Y. and Chai, Z. 2007, "Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration", *Toxicol Lett*, vol. 168, no. 2, pp. 176-185.
- Warheit, D.B., Laurence, B.R., Reed, K.L., Roach, D.H., Reynolds, G.A. and Webb, T.R. 2004, "Comparative pulmonary toxicity assessment of single-wall carbon nanotubes in rats", *Toxicol Sci*, vol. 77, no. 1, pp. 117-125.
- Warheit, D. B., Brock, W. J., Lee, K. P., Webb, T. R. and Reed, K. L. 2005, "Comparative pulmonary toxicity inhalation and instillation studies with different TiO₂ particle formulations: impact of surface treatments on particle toxicity" *Toxicol Sci*, vol. 88, no.2, pp. 514-524.
- Warheit, D. B., Webb, T. R., Reed, K. L., Frerichs, S. and Sayes, C. M. 2007, "Pulmonary toxicity study in rats with three forms of ultrafine-TiO₂ particles: differential responses related to surface properties. *Toxicol*, vol. 230, no.1, pp. 90-104.
- Wick, P., Manser, P., Limbach, L.K., Dettlaff-Weglikowska, U., Krumeich, F., Roth, S., Stark, W.J. and Bruinink, A. 2007, "The degree and kind of agglomeration affect carbon nanotube cytotoxicity", *Toxicol Lett*, vol. 168, no. 2, pp. 121-131.
- Wilson, M.R., Lightbody, J.H., Donaldson, K., Sales, J. and Stone, V. 2002, "Interactions between ultrafine particles and transition metals *in vivo* and *in vitro*", *Toxicol Appl Pharmacol*, vol. 184, no. 3, pp. 172-179.
- Witzmann, F.A. and Monteiro-Riviere, N.A. 2006, "Multi-walled carbon nanotube exposure alters protein expression in human keratinocytes", *Nanomed*, vol. 2, no. 3, pp.158-168.

- Wiwanitkit, V., Sereemasun A. and Rojanathanes R. 2009, "Effect of gold nanoparticles on spermatozoa", *FertilSteril*, vol. 91, no.1, pp.7-8
- Wong-Ekkabut, J., Baoukina, S., Triampo, W., Tang, I.M., Tieleman, D.P. and Monticelli, L. 2008, "Computer simulation study of fullerene translocation through lipid membranes", *Nature Nanotechnol*, vol. 3, no. 6, pp.363-368.
- Wu, W., Wieckowski, S., Pastorin, G., Benincasa, M., Klumpp, C., Briand, J.P., Gennaro, R., Prato, M. and Bianco, A. 2005, "Targeted delivery of amphotericin B to cells by using functionalised carbon nanotubes", *Angewandte Chemie (International Edition)*, vol. 44, no. 39, pp. 6358-6362.
- Xia, T., Kovochich, M., Brant, J., Hotze, M., Sempf, J., Oberley, T., Sioutas, C., Yeh, J.I., Wiesner, M.R. and Nel, A.E. 2006, "Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm", *Nano Lett*, vol. 6, no. 8, pp. 1794-1807.
- Xia, T., Kovochich, M., Liang, M., Madler, L., Gilbert, B., Shi, H., Yeh, J. I., Zink, J. I. and Nel, A. E. 2008, "Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties", *ACS Nano*, vol. 2, no. 10, pp. 2121-2134.
- Xiao, L., Takada, H., Gan, X., Miwa, N. 2006, "The water-soluble fullerene derivative "Radical Sponge" exerts cytoprotective action against UVA irradiation but not visible-light-catalyzed cytotoxicity in human skin keratinocytes", *Bioorg Med Chem Lett*, vol. 16, no. 6, pp.1590-1595.
- Yamago, S., Tokuyama, H., Nakamura, E., Kikuchi, K., Kananishi, S., Sueki, K., Nakahara, H., Enomoto, S. and Ambe F. 1995, "In vivo biological behaviour of a water-miscible fullerene: ¹⁴C labelling, absorption, distribution, excretion and acute toxicity", *Chem Biol*, vol. 2, no.6, pp. 385-389.
- Yamawaki, H. and Iwai, N. 2006, "Cytotoxicity of water-soluble fullerene in vascular endothelial cells" *Am J Physiol-Cell Physiol*, vol. 290, no. 6, pp. C1495-C1502.
- Yang, S.T., Guo, W., Lin, Y., Deng, X-Y., Wang, H-F., Sun, H-F., Liu, X-F., Wang, X., Chen, M., Huang, Y-P. and Sun, Y-P. 2007, "Biodistribution of pristine single-walled carbon nanotubes in vivo", *J Phys Chem C*, vol. 111, no. 48, pp. 17761-17764.
- Yang, S.T., Wang, X., Jia, G., Gu, Y., Wang, T., Nie, H., Ge, C., Wang, H. and Liu Y. 2008, "Long-term accumulation and low toxicity of single-walled carbon nanotubes in intravenously exposed mice", *Toxicol Lett*, vol. 181, no. 3, pp. 182-189.
- Ye, S.F., Wu, Y.H., Hou, Z.Q. and Zhang, Q.Q. 2009, "ROS and NF-kappaB are involved in upregulation of IL-8 in A549 cells exposed to multi-walled carbon nanotubes", *Biochem Biophys Res Comm*, vol. 379, no. 2, pp. 643-648.
- Yin, J-J., Lao, F., Fu, P.P., Wamer, W.G., Zhao, Y., Wang, P.C., Qiu, Y., Sun, B., Xing, G., Dong, J., Liang, X-J. and Chen, C. 2009, "The scavenging of reactive oxygen species and the potential for cell protection by functionalised fullerene materials", *Biomaterials*, vol. 30, no.4, pp. 611-612.
- Yin, J.J., Lao, F., Meng, J., Fu, P.P., Zhao, Y., Xing, G., Gao, X., Sun, B., Wang, P.C., Chen, C. and Liang, X.J. 2008, "Inhibition of tumor growth by endohedral metallofullerenol nanoparticles optimized as reactive oxygen species scavenger", *Mol Pharmacol*, vol. 74, no. 4, pp. 1132-1140.
- Yokoyama, A., Sato, Y., Nodasaka, Y., Yamamoto, S., Kawasaki, T., Shindoh, M., Kohgo, T., Akasaka, T., Uo, M., Watari, F., and Tohji, K. 2005, "Biological behavior of hat-stacked carbon nanofibers in the subcutaneous tissue in rats", *Nano Lett*, vol. 5, no. 1, pp. 157-161.

Engineered Nanoparticles: Review of Health and Environmental Safety

Zhang, A. P., and Sun, Y. P. 2004, "Photocatalytic killing effect of TiO₂ nanoparticles on Ls-174-t human colon carcinoma cells", *World J Gastroenterol*, vol. 10, no. 21, pp. 3191-3193.

Zhang, B., Cho, M., Fortner, J.D., Lee, J., Huang, C.H., Hughes, J.B. and Kim, J.H. 2009, "Delineating oxidative processes of aqueous C₆₀ preparations: role of THF peroxide", *Environ Sci Technol*, vol. 43, no. 1, pp. 108-113.

Zhang, L.W., Zeng, L., Barron, A.R. and Monteiro-Riviere, N.A. 2007, "Biological interactions of functionalised single-wall carbon nanotubes in human epidermal keratinocytes", *Int J Toxicol*, vol. 26, no. 2, pp. 103-113.

Zhu, L., Chang, D.W., Dai, L. and Hong, Y. 2007, "DNA damage induced by multiwalled carbon nanotubes in mouse embryonic stem cells", *Nano Lett*, vol. 7, no. 12, pp. 3592-3597.

Zhu, J.T., He, J., Chen, J.Y., Lu, D.R. and Zhou, L.W. 2008, "Fast differential interference contrast imaging combined with autocorrelation treatments to measure the heart rate of embryonic fish" *J Biomed Optics*, vol. 13, no. 2, pp. 020503.

7 EPIDEMIOLOGY AND HUMAN EXPOSURE STUDIES

7.1 EPIDEMIOLOGY

7.1.1 Introduction

An extensive body of knowledge has been gained from epidemiology studies concerning the health effects arising from exposure to particles in the workplace, as well as in outdoor air and indoor air and was reviewed as part of a scoping study to identify hazard data needs for addressing the risks presented by nanoparticles and nanotubes (Tran *et al.* 2005). The extent of the resulting health effects are dependent upon numerous factors including: i) the intensity and duration of exposure; ii) the composition and size distribution of the particles; iii) the susceptibility (including pre-existing health condition) of those exposed; and iv) interactions with other risk factors, such as smoking habits and socio-economic considerations.

In the context of the ENRHES review, we have undertaken a review of the relevant epidemiological studies relating to engineered nanoparticles (i.e. those manufactured during the development of new products and materials), with a view to potentially informing a risk assessment. Particular attention has been paid to i) published results on the epidemiology studies on carbon black and titanium dioxide industries, focusing on case-reports of the exposure-health effect relationship; ii) epidemiological studies on particles with a known nano-size range such as welding fumes; iii) published data on particle number studies of exposure to particles in ambient air.

The number of directly relevant epidemiological studies identified was limited as there is, to date, no epidemiological study on engineered nanoparticles such as the fullerenes and carbon nanotubes focused upon in this report. Thus, included in this chapter is a discussion strongly focused on identifying the methodological issues that arise in any in the context of future epidemiology studies in the nanotechnology industries.

7.1.2 Epidemiology studies on the carbon black industry

7.1.2.1 Mortality and carcinogenicity

Hodgson and Jones (1985) studied mortality in 1422 male carbon black workers at five UK factories. The size characteristics of the carbon black products were: 9-29 nm channel black, 13-100 nm furnace; 30-200 nm lampblack; and 150-500 nm thermal black. The original aim was to study every person who had worked at any of the five factories from 1947 (when production started) to 1974. However, due to incomplete personnel data, the effective start of follow-up at two of the plants was 1967-68. The authors report the following findings:

- A statistically significant *deficit* of deaths from non-malignant respiratory disease (ICD8 460-519), including the three factories with complete data. Results, standardised for region, showed 5 observed (Obs), 11.1 expected (Exp) deaths, giving a standardised mortality ratio (SMR: 100 times the ratio of observed to expected deaths) of 45. This means that the death rate associated with malignant respiratory disease in this occupational group was lower than expected compared to the general population. The deficit occurred almost entirely in men with a mortality follow-up of less than 15 years;
- A *deficit* of deaths from circulatory disease (ICD8 390-458), with results of 45 Obs, 52.6 Exp, SMR 86. The reduced incidence of deaths due to cardiovascular disease occurred entirely in the first 10 years of mortality follow-up, consistent with “healthy worker” selection and survival effects (i.e. in order to maintain employment, workers are healthier than the general population of the same age). Hodgson and Jones (1985) suggest this effect may be strengthened in this case, as the visibility dusty nature of the factories may have deterred potential new employees with pre-existing respiratory problems;

Engineered Nanoparticles: Review of Health and Environmental Safety

- A non-statistically significant excess of lung cancer overall (25 Obs, 16.5 Exp, SMR 152), occurred primarily in the two factories with incomplete data. Allowing for a latency of at least 10 years since first exposure, an excess remained at 21 Obs, 13 Exp, SMR 162, of which 11 Obs, 7.9 Exp, SMR 139 occurred at the other three factories. The excess occurred principally in the factories with lowest exposures, with cases and controls having similar employment periods. Following a detailed investigation (including a case-control study matched for date of birth and factory), Hodgson and Jones (1985) conclude that the evidence of an excess of lung cancer due to carbon black exposure is almost entirely non-existent, which seems well justified;
- A small excess of bladder cancer (3 Obs, 1.2 Exp, SMR 250) was also reported, but could not be attributed to exposure due to being based on too few cases.

As noted by Hodgson and Jones (1985), carbon black contains traces of polycyclic aromatic hydrocarbons (PAHs), an established carcinogenic substance. It is unclear as to whether these PAHs were biologically available, but there is the potential that PAH exposure may have contributed to the recorded cancer occurrences.

Sorahan *et al.* (2001) followed up the cohort of Hodgson and Jones (1985) until the end of 1996, providing a further 16 years of follow-up data, with the following updates:

- Some updating of work histories, where possible, at two of the five factories;
- Detailed dust measurements at these same two factories, undertaken as part of the European carbon black respiratory study;
- Development of a job-exposure matrix (JEM);
- Estimates of cumulative exposure derived for all subjects, based on the assumption that dust conditions at the three closed factories were similar to those at one of the two open factories where measurements had been taken.

Reported results focus on those for 1147 male manual workers employed for a minimum of 12 months. In assessing lung cancer mortality, results indicated a statistically significant excess of lung cancer compared to deaths expected (61 Obs, 35.3 Exp, SMR 173, 95% CI 132-222), based on death rates for England and Wales. Using local death rates, the resulting excess was marginally higher (SMR 185). A statistically significant difference in SMRs between factories was determined, with a statistically significant excess at two factories (SMRs 278 and 315). The authors assume this to be due to differences in the factories but, upon investigation, could not determine the nature of the difference due to lack of smoking and prior work history data. No evidence of a gradient with time since first employment was reported, but there was some (non-statistically significant) evidence of increasing risks with duration of employment, with relative risks (RRs) of 1.58, 1.72 and 1.21 for durations of 5-9 years, 10-19 years and 20+ years respectively (as compared to a baseline of 1-4 years duration). Weak evidence ($P = 0.16$) of a trend with estimated cumulative carbon black exposure was not sustained after adjustment for factory and other factors, and there was no evidence of a positive trend with estimated cumulative exposure lagged 20 years.

Sorahan *et al.* (2001) conclude that the results do not associate the excess lung cancer mortality with carbon black exposure, but highlight that the exposure data is somewhat limited due to a lack of sufficient work history data, and a potentially inappropriate exposure metric (surface area would be desirable). Such limitations may cause the study to underestimate the extent of the occupational hazards. Thus, the possibility of an occupational effect as a result of carbon black exposure cannot be entirely excluded.

When assessing non-malignant respiratory disease (ICD8 460-519), observed mortality was unexceptional with a resultant SMR of 107 (35 deaths), lower than the all-cause SMR of 113. No marked differences between factories were determined, indicating that the cause of the factory-specific lung cancer excess had no bearing on the incidence of non-malignant respiratory diseases. A statistically significant trend with time since first employment was reported, with a deficit in 1-19 years and an excess at 30+ years. No evidence of a relationship with estimated cumulative carbon black exposure unlagged was determined. Results for

relative risk with duration of employment are not reported and are thus assumed to be not statistically significant.

Sorahan *et al.* (2001) conclude that the study does not show an effect of carbon black exposure on non-malignant respiratory mortality. However, as in the lung cancer mortality assessment, there are limitations to the study size and exposure data. The possibility of a respiratory effect of carbon black exposure therefore cannot be excluded, but it seems reasonable to conclude that there is no risk of strong or severe effects.

In terms of circulatory disease (ICD8 390-458), observed mortality was unexceptional with a reported SMR of 100 (157 deaths), again lower than the all-cause SMR of 113.

In 1995, and more recently in February 2006, an IARC panel of experts conducted a comprehensive review of carbon black (IARC 1996). They considered the afore-mentioned study to be the most informative, but deemed the evidence to be weak. As such, IARC concluded there to be “inadequate evidence” of carcinogenicity in humans, placing a Group 2B “possible human carcinogen” classification on carbon black based evidence from animal studies.

7.1.2.2 Morbidity

Long-running study of the respiratory health of European carbon black industry workers have been carried out. The health effects studied are those based on chest radiographs (Gardiner *et al.* 1993; van Tongeren *et al.* 2002) and lung function and respiratory symptoms (Gardiner *et al.* 1993; Gardiner *et al.* 2001).

Gardiner *et al.* (1993) reported results from a cross-sectional survey of 1742 employees in 15 plants for respiratory symptoms and spirometry, and 1096 chest radiographs from 10 plants for radiology. Lung function and respiratory symptoms were associated with current exposure, rather than cumulative. Small category 2 opacities were found in only 3% of those studied, yet small opacities (of varying degrees of profusion) were clearly associated with indices of cumulative exposure. Gardiner *et al.* (1993) concluded that these findings indicate a non-irritant effect of carbon black dust on the airways combined with dust retention in the lungs. However, in a review of respiratory health effects of occupational exposure to carbon black, Gardiner (1995) concluded that, despite methodological limitations, exposure-related effects are evident in the studied populations in terms of small opacities, symptoms of chronic bronchitis and reduction in forced expiratory volume in one second (FEV₁) and forced mid-expiratory flow (FEF_{25%-75%}). More recent updates have reinforced this conclusion.

For example, Gardiner *et al.* (2001) reported results from two cross-sectional surveys of lung function and respiratory symptoms: i) Phase 2 (1991-2), with 2324 workers from 19 plants and; ii) Phase 3 (1994-95), with 1994 workers from 16 plants. A substantial overlap of workers occurred across the phases. Mean current exposure to inhalable dust was determined to be 0.77 mg m⁻³ in Phase 2 (highest concentration of 7.41 mg m⁻³) and 0.57 mg m⁻³ in Phase 3 (highest concentration of 3.26 mg m⁻³). However, estimated cumulative exposures were consistent across both phases, with mean of approximately 250 mg month m⁻³ and maximum of approximately 3500 mg month m⁻³. On both occasions, the mean duration of employment was almost 15 years. A range of respiratory symptoms were studied for both phases, including: i) cough; ii) sputum production; iii) cough and sputum production together and iv) chronic bronchitis (both together, for at least 3 months of the year).

The results (adjusted for age and cumulative smoking) indicate a consistent effect of *current* exposure to carbon black across the range of respiratory symptoms. The odds ratios for current concentration of 1 mg m⁻³ were in the range 1.3-1.8, which, while not insignificant, are considerably less than that of the highest category of cumulative cigarette smoking (odds ratio of 5-12). Evidence of a relationship between *cumulative* exposure and the studied respiratory symptoms is more ambiguous. However, both current and cumulative exposure to carbon black were related to lower lung function through assessing FEV₁, FEF_{25%-75%} and ratio of FEV₁ to the force vital capacity (FVC). There was some evidence to suggest that the effect of exposure was weakest in current smokers (according to respiratory symptoms) and/or those with higher

cumulative smoking (according to lung function). An attempt was made to separate the effects of current and cumulative exposures through additional analyses, but this was largely unsuccessful.

Van Tongeren *et al.* (2002) commented that the cross-sectional results from Phases 2 and 3 of the above study confirm the radiological findings of Gardiner *et al.* (1993) of a strong relationship with cumulative exposures. Given that the subjects overlap on the three occasions and the cumulative exposures within the three surveys are also strongly related, this is not an unexpected consistency but still worth highlighting.

Within their own research, Van Tongeren *et al.* (2001) studied radiological changes longitudinally based on 675 workers, with a focus on changes between 1st and 3rd surveys. Cumulative exposure to inhalable dust was used as the main dust exposure index between surveys 1 and 3, due to the ability to measure inhalable dust more precisely than respirable dust. Small opacities were noted, which tended to be irregular rather than rounded. Progression of such opacities was marked in one of the 11 factories studied, and higher in smokers and those with poor film quality during survey 1. There was evidence of a relationship between progression, especially of new cases, with inter-survey cumulative exposure (when adjusted for smoking and film quality). Regression and reversion were also indicated, but were not related to exposure.

*In summary, the results from the discussed epidemiology studies of the carbon black industry do indicate some adverse effects of exposure to carbon black dust on respiratory health. However, the main implications are reassuring. There is some consistent evidence of effects on respiratory symptoms and lung function (FEV₁ and mid-flow rather than FVC), which appear to be associated primarily with current exposure rather than cumulative. There is clear radiological evidence of dust retention, but limited evidence of radiologically-identified disease (i.e. small opacities of category 2 or above). The mortality study in particular (Sorahan *et al.* 2001) indicated no strong and little suggestive evidence of excess non-malignant respiratory disease. Although there is evidence of excess mortality from lung cancer at two of the five factories, the study has failed to link exposure to carbon black with elevated risks of lung cancer. All of the morbidity studies seem unexceptional in that they indicate relationships between exposure and respiratory health endpoints, but do not signify an extreme response to workplace dusts.*

7.1.3 Epidemiology studies on the titanium dioxide industry

The production of titanium dioxide (TiO₂) can include the production of nanoparticles. Most of the epidemiological studies of TiO₂ industry workers focus on cancer and the findings are usually negative, i.e. show no association. For example, in a study of the mortality of 15017 workers (14331 of which were male) in 11 European TiO₂-producing factories, Boffetta *et al.* (2004) concluded that their results do not suggest a carcinogenic effect of TiO₂ dust on the human lung. In line with this, a review by Hext *et al.* (2005) concluded that similar mortality studies (such as Fryzek *et al.* 2003) do not show a relationship between past exposure to TiO₂ and lung cancer. However, Hext *et al.* did highlight two studies of respiratory morbidity which, although showing no evidence of serious lung disease, indicate suggestive evidence (not statistically significant) of dust retention (Chen and Fairweather 1988) and reduced lung function (Garabrant *et al.* 1987). Overall, these results are similar in nature to those for carbon black.

However, these epidemiology studies may not be directly relevant to assessing nanoparticulate TiO₂. This view is supported by Hext *et al.* (2005) who highlight that, although some of the dust arising from the manufacture of TiO₂ pigments will contain small amounts of ultrafine particles, such ultrafine particles are known to agglomerate before release into workplace air. Thus, it is unlikely that exposure to a true ultrafine dust explains the variations in lung cancer mortality between studies and factories. Hext *et al.* (2005) therefore conclude that previous TiO₂ industry epidemiology studies provide little information to evaluate the health risks associated with ultrafine particle manufacture.

It is worthwhile highlighting that the majority of toxicology studies employ agglomerated particles or nanoparticles, yet the aggregated nanoparticle form is always more potent at driving inflammation. This is consistent with the hypothesis that surface area is the primary driver of

toxicity, rather than diameter, since aggregated nanoparticles will possess a surface area similar to that of the individual particles due to the fact that the area of particle contact is very small compared to the total particle surface area.

In summary, epidemiology studies may not be directly relevant to assessing nanoparticulate TiO₂. Previous TiO₂ industry epidemiology studies provide little information to evaluate the health risks associated with ultrafine particle manufacture. It is unlikely that exposure to a true ultrafine dust explains the variations in lung cancer mortality between studies and factories.

7.1.4 Epidemiology studies on welding

There is an extensive body of literature on mortality and morbidity of welders, of relevance as welders are exposed to very fine particles. In a review for NIOSH, Antonini *et al.* (2003) concluded that the pulmonary effects observed in full-time welders have included: airway irritation; lung function changes; metal fume fever; susceptibility to pulmonary infection; and a potential increase of lung cancer incidence. However, the effects depend on the type of metals that are being welded. It is difficult to extrapolate from the epidemiology of welders and welding fumes to that of the nanoparticles generated within the nanotechnology industries. Some of the effects may be acute, as reported by Fishwick *et al.* (2004) for example, even where the exposures are not considered to be particularly high.

In summary, it is difficult to extrapolate from the epidemiology of welders and welding fumes to that of the nanoparticles generated within the nanotechnology industries because of the type of metals that are being welded.

7.1.5 Particle number studies on particles in ambient air

Daily variations in particles in outdoor air are associated with numerous adverse health effects, including increased cardio-respiratory mortality, exacerbations of asthma and alterations in lung function and heart rate variability. Long-term exposure to outdoor particles is also associated with reduced life expectancy in adults and increased mortality in infants. These findings are based on studies of mass concentrations of ambient particles (e.g. Particulate Matters – PM – PM₁₀ and PM_{2.5}) as these parameters are most routinely measured. However, due to a conjecture that small combustion-derived particles play a key role in driving PM-health relationships, there has been an increased interest in epidemiology, which can differentiate between the effects of ultrafine and bulk respirable particles using, for example, particle size measurements.

To date, there exists a limited number of relevant epidemiological studies. Wichmann *et al.* (2000) examined mortality, while other studies have assessed subject panels with impaired health, such as pollution-related changes in the respiratory health of asthmatics and cardiovascular impairments. Following a comprehensive review of the literature, Morawska *et al.* (2004) concluded that there are adverse health effects associated with the ultrafine fraction of respirable particles, with effects indicated on mortality in the general population and panels of individuals with asthma or other impaired health. The reviewed studies suggest an immediate, acute effect of fine particles as conventionally measured (PM_{2.5}), in contrast to the effect of ultrafine particles which appears delayed by a few days. This conclusion therefore suggests that fine particles are insufficient as a surrogate for ultrafine particles.

More recent publications have further supported the use of the number metric as a surrogate measure for nanoparticles. Forastiere (2005), in a study of out-of-hospital sudden death, showed that death was related to particle numbers in the nano-size range (particularly on the day of death) to a greater degree than either particle mass or carbon monoxide concentrations. In contrast, a study by de Hartog *et al.* (2003) on elderly individuals with coronary artery disease suggested that PM_{2.5} was the more important driver of health effects, although particle numbers did correspond to activity restriction.

To summarise, there exists a limited number of relevant epidemiological studies. The general conclusions are: (i) that there are adverse health effects associated with the ultrafine fraction of

respirable particles, with effects indicated on mortality in the general population and panels of susceptible individuals, and; (ii) death was related to particle numbers in the nano-size range.

7.1.6 Air pollution, pulmonary and cardio-vascular effects

It is now well established that there is a relationship between exposure to particulate matter (PM) and pulmonary and cardio-vascular effects. In this section we will critically review the evidence which demonstrate the relationship between the particle size and the adverse effects.

Peters *et al.* (1997) investigated the association between the numbers of ultrafine particles and respiratory effects. The association between fine and ultrafine particles and respiratory health was studied in adults with a history of asthma in Erfurt Eastern Germany. Twenty-seven non smoking asthmatics recorded their peak expiratory flow (PEF) and respiratory symptoms daily. The size distribution of ambient particles in the range of 0.01 to 2.5 μm was determined with an aerosol spectrometer during the winter season 1991-1992. Most of the particles (73%) were in the ultrafine fraction (smaller than 0.1 μm in diameter), whereas most of the mass (82%) was attributable the particles in the size range of 0.1 to 0.5 μm . Because these two fractions did not have similar time courses (correlation coefficient $r = 0.51$), a comparison of their health effects was possible. Both fractions were associated with a decrease of PEF and an increase in cough and feeling ill during the day. Health effects of the 5 day mean of the number of ultrafine particles were larger than those of the mass of the fine particles, In addition, the effects of the number of the ultrafine particles on PEF were stronger than those of particulate matter smaller than 10 μm (PM10). Therefore, the authors suggested that the size distribution of ambient particles helps to elucidate the properties of ambient aerosols responsible for respiratory effects.

Mulli *et al.* (2002) have shown epidemiological evidence on the health effects of ultrafine particles. The authors focused on particles less than 100 nm diameters. The data were from a six panel studies with patients suffering from chronic pulmonary diseases have been performed in Germany, Finland and the United Kingdom. Overall, a decrease of peak expiratory flow and an increase of daily symptoms and medication use was found for elevated daily particle concentrations. Effects were seen with both fine and ultrafine particles. One large study on daily mortality from Germany showed comparable effects of fine and ultrafine particles in all size classes considered. However, fine particles showed more immediate effects while ultrafine particles showed more delayed effects on mortality. The limited number of epidemiological studies suggests that there are health effects of fine and ultrafine particles which might be independent of each other.

Despite major improvements in air quality resulting from increasingly stringent legislation, there remains a strong association between daily mortality and current levels of air pollution. Growing epidemiological evidence suggests that many, perhaps the majority, of these deaths are caused by cardiovascular disease.

Routledge *et al.* (2006) studied the effect of inhaled sulphur dioxide and carbon particles on heart rate variability and markers of inflammation and coagulation in human subjects. The authors measured the inflammatory and autonomic responses of healthy humans and patients with coronary artery disease to controlled concentrations of two specific components of vehicle derived air pollution, carbon particles and sulphur dioxide (SO_2). They found that, in healthy volunteers, markers of cardiac vagal control did not fall in response to particle exposure but, compared with the response to air, increased transiently immediately after exposure (root mean square of successive RR interval differences (RMSSD) 15 (5) ms with carbon particles and 4 (3) ms) with air, $p < 0.05$). Sulphur dioxide exposure resulted in no immediate change but a significant reduction in HRV markers of cardiac vagal control at four hours (RMSSD 22 (3.6) ms with air, 27 (2.7) ms with SO_2 , $p < 0.05$). No such changes were seen in patients with stable angina. Neither pollutant caused any change in markers of inflammation or coagulation at zero, four, or 24 hours. The authors concluded that, in healthy volunteers, short term exposure to pure carbon particles does not cause adverse effects on HRV or a systemic inflammatory response. The adverse effects of vehicle derived particulates are likely to be caused by more reactive species found on the particle surface. Sulphur dioxide exposure does, however,

reduce cardiac vagal control, a response that would be expected to increase susceptibility to ventricular arrhythmia.

Wichmann *et al.* (2000) studied the daily mortality and fine and ultrafine particles in Erfurt, Germany, specifically the role of particle number and particle mass. Data were collected prospectively over a 3.5-year period from September 1995 to December 1998. Death certificates were obtained from the local authorities and aggregated to daily time series of total counts and counts for subgroups. In addition to standard data for particle mass with diameters less than or equal to 2.5 μm (PM_{2.5}) or less than or equal to 10 μm (PM₁₀) from impactors, a mobile aerosol spectrometer (MAS) was used to obtain size-specific number and mass concentration data in six size classes between 0.01 μm and 2.5 μm . Particles smaller than 0.1 μm were labelled ultrafine particles (three size classes), and particles between 0.1 and 2.5 μm were termed fine particles (three size classes). Concentrations of the gases SO₂, NO₂, and CO were also measured. The daily average total number concentration was 18000 particles cm⁻³ with 88% of particles below 0.1 μm and 58% below 0.03 μm in diameter. The average mass concentration (PM_{2.5}) was 26 $\mu\text{g m}^{-3}$; of this, 75% of particles were between 0.1 and 0.5 μm in diameter. Other average concentrations were 38 $\mu\text{g m}^{-3}$ for PM₁₀, 17 $\mu\text{g m}^{-3}$ for SO₂, 36 $\mu\text{g m}^{-3}$ for NO₂, and 600 $\mu\text{g m}^{-3}$ for CO. Ambient air pollution demonstrated a strong seasonality with maximum concentrations in winter. Across the study period, fine particle mass decreased, whereas ultrafine particle number was unchanged. The proportion of ultrafine particles below 0.03 μm diameter increased compared with the proportion of other particles. During the study, concentrations of SO₂ and CO also decreased, whereas the concentration of NO₂ remained unchanged. The data were analysed using Poisson regression techniques with generalised additive modeling (GAM) to allow nonparametric adjustment for the confounders. Both the best single-day lag and the overall association of multiple days fitted by a polynomial distributed lag model were used to assess the lag structure between air pollution and death. Mortality increased in association with level of ambient air pollution after adjustment for season, influenza epidemics, day of week, and weather. In the sensitivity analyses, the results proved stable against changes of the confounder model. The authors compared associations for ultrafine and fine particles in a distributed lag model where the contribution of the previous 4 to 5 days was considered. Furthermore, the data suggest a somewhat more delayed association of ultrafine particles than of fine particles if single-day lags are considered. The associations tended to be stronger in winter than in summer and at ages below 70 years compared to ages above 70 years. Analysis of the prevalent diseases mentioned on death certificates revealed that the overall association for respiratory diseases was slightly stronger than for cardiovascular diseases. In two-pollutant models, associations of ultrafine and fine particles seemed to be largely independent of each other, and the risk was enhanced if both were considered at the same time. Furthermore, when the associations were summed for the six size classes between 0.01 and 2.5 μm , the overall association was clearly stronger than the associations of the individual size classes alone. Associations were observed for SO₂, NO₂, and CO with mortality despite low concentrations of these gases. These associations disappeared in two-pollutant models for NO₂ and CO, but they remained stable for SO₂. The persistence of the SO₂ effect was interpreted as artefact, however, because the SO₂ concentration was much below levels at which effects are usually expected. Furthermore, the results for SO₂ were inconsistent with those from earlier studies conducted in Erfurt. The authors conclude that both fine particles (represented by particle mass) and ultrafine particles (represented by particle number) showed independent effects on mortality at ambient concentrations. Comparable associations for gaseous pollutants were interpreted as artefacts of collinearity with particles from the same sources.

In summary, particles from ambient air of the ultrafine range have been shown to be associated with both pulmonary and cardio-vascular effects. The cellular mechanisms for pulmonary effects have been investigated in many experimental (in vivo, in vitro) models. For the cardio-vascular effects, the people affected are those with pre-disposed cardio-vascular problems. The mechanisms here could be an indirect effect of pulmonary inflammation, cardiac arrhythmia or a direct effect due to particle translocation to the plaque area and causing plaque disruption leading to a stroke or fatal heart attack. Further studies are needed to elucidate the mechanisms behind the association between ultrafine particles exposure and cardio-vascular effects.

7.2 HUMAN EXPOSURE STUDIES

7.2.1 Introduction

Human exposure studies using chambers, masks or head domes have been utilised for decades to assess biological responses to controlled levels of specific air mixtures, whereby particles are added to the inhaled air, the proportions of inhaled gases are altered, or a combination of both. Such studies are ideal for assessing acute (i.e. immediate to 24 hours) responses of human exposure to various pollutants, offering numerous advantages including:

- Providing precise control of delivered concentrations and (where pulmonary ventilation rates are known) lung dose;
- Allowing ventilation rates to be varied by introducing structured exercise protocols which not only increased lung dose but also induced changes in cardio-pulmonary responses.

However, these human studies of this type are not without limitations or complexities:

- They are largely unsuitable for assessing the effects of long-term exposure to specific substances or mixtures;
- No studies have yet considered the effects of changes in co-exposures such as temperature or humidity in understanding responses to particle exposures;
- The range of biological responses that can be studied vary depending on the invasiveness of the procedures involved (e.g. biopsy of lower respiratory tract mucosa can only be done by bronchoscopy);
- The timing of measurements is critical in order to successfully monitor the dynamics of the responses;
- In some cases, the measured responses may simply reflect a normal physiological response to an exposure rather than an abnormal response. Thus, it is crucial that any observed changes are considered in the context of responses to exposures which would be regarded as normal events (e.g. breathing cold air), whilst not down-playing subtle patho-physiological changes. There is still a large knowledge gap with regards addressing this issue.

The majority of human challenge work to date has concentrated on assessing the effects of ambient air pollution. In the context of responses to inhaled nanomaterials, human challenge studies are limited to assessing immediate responses, which may or may not be relevant in understanding the mechanisms involved in long-term exposures. There are, as yet, no studies specifically of engineered particles. Much of the available information therefore comes from studies of particles derived from the internal combustion engine or from laboratory generated particles, whose content is part of that seen in ambient particles.

Thus, in the context of the ENRHES review, we have aimed to review the existing literature on human exposure studies in the laboratory setting which considered nano-sized particles even if only as part of an exposure which incorporated particles of larger size fraction, focussing on i) studies of diesel exhaust exposure; ii) studies of concentrated ambient particles; iii) other human studies on particles with a known nano-size range, such as zinc oxide and ultra-fine carbon.

The majority of these studies considered a source of particles for which either the exact particle size range was unknown or where the range included, but was not limited to, the nanoparticle range. This is, to some extent, inevitable, reflecting the real life setting. The only studies of particles solely in the nanoparticle range are those where the particles are specifically generated in the laboratory (usually using an electrical spark generator) and thus usually comprising a single type of particle (e.g. carbon, iron or zinc).

7.2.2 Studies of exposure to diesel exhaust particles

Diesel exhaust particles (DEPs) do contain nanoparticles but, by definition, all exposures to DEPs will also contain larger particles. As a result, any changes observed in human exposures to DEPs cannot be confidently deemed a consequence of nanoparticles alone. Numerous studies have assessed inflammatory and immunologic changes following exposure to captured diesel exhaust re-suspended and instilled in the nose, with Diaz-Sanchez undertaking leading work in this area. However, such studies have not been considered here due to the occurrence of particle aggregation during collection and reinstallation. This leads to difficulty in interpreting studies of this nature in the context of nanoparticles.

A small number of studies, investigating a range of outcomes, were identified which both assess diesel exhaust exposure and provide adequate information on particle size distribution (e.g. Rudell *et al.* 1996; Salvi *et al.* 1999; Nordenhall *et al.* 2000; Salvi *et al.* 2000; Nightingale *et al.* 2000). The majority of these studies delivered moderately high concentration in mass terms ($100\text{-}300\ \mu\text{g m}^{-3}$), and utilised normal and mild asthmatic subjects. Consistent observed changes were as follows: evidence of a neutrophilic inflammatory response, assessed by cell counts in i) broncho-alveolar lavage; ii) bronchial wash; iii) some biopsies and; iv) evidence upon biopsy of increased high IL8 mRNA. Increased IL8 mRNA was also seen in bronchial wash on a couple of occasions. Biopsies also tended to indicate increased evidence of adhesion marker expression (ICAM1, VCAM1) in the endothelial wall. These afore-mentioned studies suggest that DEPs can cause a neutrophilic inflammatory response in both normal and asthmatic subjects. This is likely mediated through IL8 mRNA, with some evidence of endothelial activation (especially in asthmatic subjects).

7.2.3 Studies of exposure to concentrated ambient particles

A study was identified (Gong *et al.* 2003) which looked at human exposure to concentrated ambient particles (CAPs) and, although the particle size incorporated particles of around 100 to 1500 nm, it is likely that some ultrafine particles would have been present. This study also indicated an increased neutrophil response. Gong *et al.* (2003) monitored healthy and mild asthmatic volunteers upon CAPs exposure and reported small, variable changes in systemic inflammatory and cardio-pulmonary responses. However, this study indicated similar ultrafine exposure in the control "air" exposure, as that determined with CAPs.

In a double-blind, randomized, cross-over study, Mills *et al.* (2005) used 30 healthy men were exposed to diluted diesel exhaust ($300\ \mu\text{g m}^{-3}$ particulate concentration) or air for 1 hour during intermittent exercise. Bilateral forearm blood flow and inflammatory factors were measured before and during unilateral intrabrachial bradykinin ($100\text{ to }1000\ \text{pmol min}^{-1}$), acetylcholine ($5\text{ to }20\ \mu\text{g min}^{-1}$), sodium nitroprusside ($2\text{ to }8\ \mu\text{g min}^{-1}$), and verapamil ($10\text{ to }100\ \mu\text{g min}^{-1}$) infusions 2 and 6 hours after exposure. There were no differences in resting forearm blood flow or inflammatory markers after exposure to diesel exhaust or air. Although there was a dose-dependent increase in blood flow with each vasodilator, this response was attenuated with bradykinin, acetylcholine, and sodium nitroprusside infusions 2 hours after exposure to diesel exhaust, which persisted at 6 hours. Bradykinin caused a dose-dependent increase in plasma tissue plasminogen activator that was suppressed 6 hours after exposure to diesel. The authors conclude that at levels encountered in an urban environment, inhalation of dilute diesel exhaust impairs 2 important and complementary aspects of vascular function in humans: the regulation of vascular tone and endogenous fibrinolysis. These important findings provide a potential mechanism that links air pollution to the pathogenesis of atherothrombosis and acute myocardial infarction.

Mills *et al.* (2007), in a double-blind, randomised crossover study, 20 men with prior myocardial infarction were exposed, in two separate sessions, to dilute diesel exhaust ($300\ \mu\text{g m}^{-3}$) or filtered air for 1 hour during periods of rest and moderate exercise in a controlled-exposure facility. During the exposure, myocardial ischemia was quantified by ST-segment analysis using continuous 12-lead electrocardiography. Six hours after exposure, vasomotor and fibrinolytic function were assessed by means of intra arterial agonist infusions. During both exposure sessions, the heart rate increased with exercise; the increase was similar during exposure to diesel exhaust and exposure to filtered air. Exercise-induced ST-segment depression was

present in all patients, but there was a greater increase in the ischemic burden during exposure to diesel exhaust. Exposure to diesel exhaust did not aggravate pre-existing vasomotor dysfunction, but it did reduce the acute release of endothelial tissue plasminogen activator ($P = 0.009$; 35% decrease in the area under the curve).

Tornqvist *et al.* (2007) used fifteen healthy men who were exposed to diesel exhaust (particulate concentration, $300 \mu\text{g m}^{-3}$) or filtered air for 1 hour in a double-blind, randomized cross over study. Twenty four hours after exposure, bilateral forearm blood flow, and inflammatory and fibrinolytic markers were measured before and during unilateral intrabrachial bradykinin ($100\text{--}1000 \text{ pmol min}^{-1}$), acetylcholine ($5\text{--}20 \mu\text{g min}^{-1}$), sodium nitroprusside ($2\text{--}8 \text{ g min}^{-1}$), and verapamil ($10\text{--}100 \text{ g min}^{-1}$) infusions. The authors found that resting forearm blood flow, blood pressure, and basal fibrinolytic markers were similar 24 hours after either exposure. Diesel exhaust increased plasma cytokine concentrations (tumor necrosis factor and interleukin-6,) but appeared to reduce acetylcholine, and bradykinin ($p=0.08$) induced forearm vasodilatation. In contrast, there were no differences in either endothelium-independent (sodium nitroprusside and verapamil) vasodilatation or bradykinin-induced acute plasma tissue plasminogen activator release. They conclude that twenty-four hours after diesel exposure, there is a selective and persistent impairment of endothelium-dependent vaso-dilatation that occurs in the presence of mild systemic inflammation. These findings suggest that combustion-derived air pollution may have important systemic and adverse vascular effects for at least 24 hours after exposure.

Lucking *et al.* (2008) used a double-blind randomized cross over study, 20 healthy volunteers were exposed to diluted diesel exhaust ($350 \mu\text{g m}^{-3}$) and filtered air. Thrombus formation, coagulation, platelet activation, and inflammatory markers were measured at 2 and 6 hours following exposure. Thrombus formation was measured using the Badimon ex vivo perfusion chamber. Platelet activation was assessed by flow cytometry. Compared with filtered air, diesel exhaust inhalation increased thrombus formation under low- and high-shear conditions by 24%. This increased thrombogenicity was seen at 2 and 6 hours, using two different diesel engines and fuels. Diesel exhaust also increased platelet–neutrophil and platelet–monocyte aggregates by 52% at 2 hours following exposure compared with filtered air. The authors concluded that Inhalation of diesel exhaust increases ex vivo thrombus formation and causes *in vivo* platelet activation in man. These findings provide a potential mechanism linking exposure to combustion-derived air pollution with the triggering of acute myocardial infarction.

7.2.4 Studies of exposure to zinc oxide

A small number of studies have been undertaken which investigate exposure to zinc oxide fume in an attempt to recreate metal fume fever (e.g. Kuschner *et al.* 1995 and 1997), but exposures were high at up to 37 mg m^{-3} . At such doses, systemic effects typical of metal fume fever (e.g. raised temperature and muscle aches) and increased plasma IL6 levels were observed. There was also a dose related increase in BAL neutrophils. In contrast, Beckett *et al.* (2005) generated pure zinc oxide particles of two particle size fractions (fine $\sim 260 \mu\text{m}$ and ultra-fine $\sim 41 \mu\text{m}$) in the laboratory using an electrical spark generator, and monitored effects of exposure at airborne concentrations of approximately $500 \mu\text{g m}^{-3}$ for both fine and ultrafine zinc oxide. Pre-exposure and follow up studies of symptoms, leukocyte surface markers, haemostasis and cardiac electrophysiology were conducted to 24 hours post-exposure. Induced sputum was also sampled 24 hours post-exposure. No effects on any inflammatory marker were observed. The authors concluded that freshly generated zinc oxide in the fine or ultrafine fractions inhaled by healthy subjects at rest at a concentration level of $500 \mu\text{g m}^{-3}$ for 2 hours have generated an internal dose below the threshold for acute systemic effects as detected by these endpoints.

7.2.5 Studies of exposure to ultra-fine carbon

Routledge *et al.* (2005) studied effects on patients with severe coronary artery disease following oral-nasal exposure to ultra-fine carbon, as compared to age-matched healthy controls. The normal subjects displayed an immediate increase in cardiac vagal control, whilst no such response was observed in those with pre-existing coronary disease. Although this may have been a therapy-related effect, it does suggest that ultra-fine carbon may induce a cardio-protective effect upon exposure in normal subjects. Frampton *et al.* (2004) studied normal and asthmatic patients following oral exposure to ultra-fine carbon in the nano-metre size range. No

change in lung function, inflammatory markers, heart rate or heart rate variability was observed. However, following exposure there was some suggestion of shortening of the Q-T interval of the ECG upon exercise.

Pietropaoli *et al.* (2004) studied effects on gas exchange in normal and asthmatic individuals following ultrafine carbon exposure. No effect on lung function or inflammatory parameters was observed in either subject group at a dose of $25 \mu\text{g m}^{-3}$. However, the normal subjects were also exposed to a higher dose of $50 \mu\text{g m}^{-3}$ and displayed a significant fall in gas transfer and maximal mid-expiratory flow but with no concomitant change in inflammatory markers. This suggests a non-inflammatory change in ventilation/perfusion balance.

7.2.6 Mathematical models of particle deposition in the respiratory tract

Mathematical deposition models of nanosized particles and fibers in anatomically-accurate lung geometries under physiologically-realistic breathing conditions have been constructed (Asgharian and Price 2007). These dosimetry models allow data extrapolation from laboratory animal inhalation studies to human exposure scenarios (Kelly *et al.* 2005). Dosimetry models can also be used to target the site and predict the deposition of inhaled pharmaceutical drugs within the human respiratory tract (Kimbell *et al.* 2007).

A complementary area of research involves validation of the dosimetry models. Experiments are conducted in nasal replicas of humans and rats to measure local and overall deposition of particles (Kelly *et al.* 2001). Inhalation exposures are also conducted to obtain the deposition fraction of inhaled particles in various regions of the rat respiratory tract.

The information generated from the model simulation is used to assess the internal deposited dose at various sites in the human respiratory tracts and helped in evaluating potential health risk to humans from exposure to airborne materials. However, without a human based model describing the retention/distribution of nanoparticles in the body and the resulting adverse effects, the role of deposition models are rather limited.

Overall, these findings indicate that ultrafine particles have the capability to induce physiological and inflammatory responses in humans. The effects are those of a neutrophilic inflammation mediated through IL8 m-RNA, with evidence of endothelial activation measured directly in the CAPs studies. Some studies, but not all, have also demonstrated changes in heart rate variability.

The majority of the subjects used in these studies have been young, healthy volunteers, although some studies have exposed subjects with mild asthma. Air pollution epidemiology shows that older subjects with pre-existing cardiopulmonary disease are most affected by everyday air pollution changes. Thus, the meaning of these results remains unclear. It is likely that availability of volunteers and ethical concerns over potential risky exposure of subjects with coronary artery or pulmonary disease has limited the subject range for these studies. However, it could be argued that the exposures used in these types of studies are typical of those of severe pollution days.

7.3 LIMITATIONS AND STRENGTHS OF EPIDEMIOLOGY

Epidemiology has its limitations, methodologically, but also its strengths and studies to date give rise to similar issues as would be expected to arise in any long-term occupational study of mortality or morbidity.

Small methodological variations may give rise to somewhat different results, although not to markedly different conclusions. Whereas some work-related influence on risks of lung cancer or non-malignant respiratory mortality cannot be excluded, particularly because of unavoidable limitations in estimating cumulative exposures, it is most highly unlikely that severe work-related risks remain unidentified. Results from the morbidity studies also seem generally consistent across different medical surveys, and across radiological classifications by different readers. The evidence from morbidity and mortality studies is consistent, in that neither points to a marked serious adverse effect. While the results from the carbon black studies are reassuring,

there are several reasons why they do not justify such a generalisation to other engineered nanoparticles.

An understanding of how different primary particle sizes underlie workers exposures would require a review of which carbon black factories produced nanomaterials, when that production started, and how different occupations were differently affected at different points in time - in effect, an elaboration of the job-exposure matrices developed by the study team. Assuming that in general particle size has reduced over time, it is worth noting that different indices of exposure, and so different analyses, may contain different information about primary particles of different sizes.

Lifetime cumulative exposures (relevant to mortality and some analyses of respiratory morbidity) will have been dominated by high occupational exposures in the past, when dusts were coarser.

Current exposures will reflect more recent conditions. The cross-sectional analyses of lung function and symptoms relative to current concentrations, and the longitudinal analyses of radiology in relation to recent inter-survey dust exposures, may therefore better reflect the effects of nanomaterial production than do the studies of cumulative exposure.

Epidemiology is not sufficiently precise to identify reliably small real changes in risk coefficients across surveys, against the background of all the other sources of variation that are necessarily in the data.

Nanoparticles in the airborne dust as sampled for the carbon black studies are found primarily in agglomerated form (Aitken *et al.* 2004). It is unclear to what extent the results of the carbon black studies translate to other situations, for example where exposure is to particles in their nanoparticle form. Similarly, it is unclear what happens to agglomerated particles following inhalation whether they remain bound, or they separate, what influence surfactant may have, and what implications this may have for toxicity. It is likely that agglomerated particles from different sources and substances may behave differently in that respect.

The literature review above suggests that there may be relatively mild adverse respiratory effects from short-term or longer-term occupational exposure to the nanoparticles considered, although the extent to which these effects may be attributable to (agglomerated) nanoparticles is unclear.

The literature review suggests also, however, that the methodological issues involved in estimating these risks epidemiologically are not intrinsically different from those involved in designing and carrying out long-term epidemiological studies in an industry.

Recommendations for further epidemiology on the effects on health of exposure to ultrafine particles in ambient air (Morawska *et al.* 2004; Schwela *et al.* 2002) highlighted the need for:

- Sufficient contrast, between high and low exposed people (or, between people exposed at high and low concentrations);
- Long enough periods of observation to enable study of latency;
- Sufficiently detailed exposure characterisation such that the effects related to particle size of interest (< 0.1 μm) could be decoupled from other characteristics of the particles or complex pollutant mixtures;
- Large enough sample sizes to enable adjustment for confounders and the study of susceptibility;
- Studies in different locations, with people exposed to different mixtures.

It is widely recognised that there is a need to look at these issues on a case-by-case base. Until we have a better understanding of the relationships between different metrics, for different nanoparticles in different scenarios, we need to consider each case and devise the most appropriate strategy and combination of measurements to arrive at the most useful metric(s) for epidemiological studies. Different aerosols in different industry scenarios will lead to different risks which may be assessed by different combinations of measurement.

The importance and relevance of past occupational history will depend hugely on who is employed in the new nanotechnology industries; and, especially, on the extent to which workers in these new industries will have had a prior history of exposure in dusty occupations. Nevertheless, a full lifetime work history will need to be taken, with trigger questions about prior occupations and/or exposures of particular interest. It may be that such a questionnaire can be administered on recruitment of the individual, rather than only at medical surveys subsequently. This especially would be useful for studies of mortality, where attendance at medical survey is not required. Mortality studies, the tracking date and cause of death, are relatively easy to put in place and monitoring of outcome is often via standard systems. The disadvantages are that time is needed to get sufficient exposures, to get sufficient deaths for study (death rates will increase anyway as the cohort ages), and to allow artefacts such as the healthy worker effect to work their way through the data, though exposure-response relationships should be robust to the healthy worker effect.

7.4 RECOMMENDATIONS FOR FUTURE EPIDEMIOLOGICAL RESEARCH IN NANOTECHNOLOGY INDUSTRIES

The studies of carbon black and titanium dioxide industries have focussed on respiratory health, including lung function, respiratory symptoms and radiologically identified dust retention or lung changes, as indeed had the large-scale studies in the UK coal industry many years previously (Ashford 1958). Issues of design would include whether new studies should be repeated cross-sections, or strictly longitudinal, studies. Given the numbers, longitudinal with new entrants on each occasion seems best.

Studies of outdoor air have however shown the importance of cardiovascular endpoints for mortality, both in studies of long-term average exposure and short-term (daily variations). Other indicators of cardiovascular health, namely pulse rate, blood pressure and heart rate variability, have been shown to be related to short-term exposure to ambient air pollution. It would be important, therefore, to include measurements of cardiovascular changes among the health endpoints to be studied. It may be useful and important to carry out some studies of effects of short-term exposure at an early stage, to give guidance on whether or not exposure-related changes are identifiable, and if so of what magnitude, even if these changes may be transient and to do in themselves constitute cardiovascular or lung 'disease'. Neurological endpoints may also be of interest.

Conducting epidemiological studies in the future will be greatly enhanced if management, unions, and others can be involved. Ideally, the co-ordination of methods across industries would limit duplication of effort and enhance the ability to compare results across industries. The proactive development and implementation of systems in new industries, as production of nanomaterials in different contexts comes on-stream would include setting up systems to track workers in the newly emerging industries as they move employers, incentives to individuals to continue to participate in such schemes, collection of baseline data on entering an industry, the recording of occupational histories in a way that is relevant to future epidemiology, and the detailed tracking of occupation and location in a way that can be linked effectively with a workplace dust measurement programme. It also would need to involve active collaboration between countries, to gain sufficient study power from including a wider range of facilities and large numbers of study subjects. The measurement of exposure and of health for future epidemiology could be linked with a planned programme of measurements for surveillance and control. Adhering to guidelines for Good Epidemiology Practice (for example, those published by the International Epidemiological Association; www.dundee.ac.uk/iea/GEP07.htm; accessed 15/10/09) is important.

Studies have shown the usefulness of standard respiratory health outcomes. However, these were designed for measuring the larger effects of heavy exposures in large cohorts, and it is unclear if they will be sufficient if exposures are lower and/or effects are more subtle. Future epidemiology should include consideration of what newer outcomes might be used, or might be developed, for the direct investigation of early effects such as inflammation, e.g. lavage, blood samples etc. Such methods might be especially useful in short-term studies, and indeed would be practicable only on a small scale. As well as standard confounding factors such as smoking habits, other characteristics (diet; possible genetic factors) need to be considered.

7.4.1 Conclusions

There is still very little published work on human responses to nano-particle exposures and none on manufactured nano-material exposure in the laboratory setting. The work to date relevant to understanding the acute effects of nano-particle exposure has come largely from the air pollution field and it is likely that this field of work will continue to provide very useful and transferable information with respect to other nano-material exposures. Consideration is starting to be given to aspects of study design(s) which would specifically deal with occupational nanoparticle exposures (for example Schulte *et al.* 2009).

While there may be concern about chronic exposure (for which an experimental approach is not best suited), biological responses to acute exposures may also be of relevance. It may be hypothesised that inflammatory responses after repeated acute exposures may result in airway remodelling, alveolar and peri-alveolar inflammation which may induce vascular responses which will contribute to atherogenesis. In this context, a better understanding of what constitutes physiological and patho-physiological responses is needed across a range of exposures and outcomes.

Allied to this is the interest in understanding whether co-exposures are interfering with the interpretation of results. This is important in terms of apportioning contribution and where a range of interventions and control measures might be required. Studies to elucidate the effects of potential co-exposures are needed for a wide range of exposures.

At present, the systems for delivering nano-materials in the laboratory setting are relatively simple. Electrical spark generators are limited in the amounts being able to be delivered and are not suitable for generating exposures of already manufactured nano-materials. The current technology for creating, characterising and quantifying 'test' aerosols needs to be enhanced as well as other potential approaches developed.

The dose received is also critically dependent on the portal of entry, target organ(s) and where in the body the materials deposit. There is very limited information on lung deposition in the context of nanoparticles, beyond the modelling based on the long established ICRP model. With the development of increasingly sophisticated imaging techniques such as PET-CT, there is now the opportunity for understanding better where nano-materials deposit once inhaled in relation to specific size/property/surface characteristics and how these may behave during the process of inhalation. These new approaches also offer the opportunity to explore the nose as a portal of entry to the brain and perhaps also the gastro-intestinal route.

7.5 REFERENCES

- Aitken, R.J., Creely, K.S. and Tran, C.L. 2004, *Nanoparticles: An occupational hygiene review*. HSE Research Report 274, HSE Books, London, UK.
- Antonini, J.M., Lewis, A.B., Roberts, J.R. and Whaley, D.A. 2003, "Pulmonary effects of welding fumes: review of worker and experimental animal studies.", *American Journal of Industrial Medicine*, vol. 43, pp. 350-360.
- Asgharian, B., and Price, O.T. 2007, "Deposition of Ultrafine (nano) Particles in the Human Lung", *Inhalation Toxicology*, vol. 19, pp. 1045-1052.
- Ashford, J.R. 1958, "The design of a long-term sampling programme to measure the hazard associated with an industrial environment", *J.R.Stat.Soc.*, A121, pp. 333-347.
- Boffetta, P., Boffetta P, Soutar A, Cherrie J.W., Granath, F., Andersen, A., Anttila, A., Blettner, M., Valerie Gaborieau, V., Klug, S.J., Langard, S., Luce, D., Merletti, F., Miller, B., Mirabelli, D., Pukkala, E., Adami, H.O., Weiderpass, E. 2004, "Mortality among workers employed in the titanium dioxide production industry in Europe", *Cancer Causes and Control*, vol. 15, pp. 697-706.
- Beckett, W.S., Chalupa, D.F., Pauly-Brown, A., Speers, D.M., Stewart, J.C., Frampton, M.W., Utell, M.J., Huang, L.S., Cox, C., Zareba, W., and Oberdorster, G. 2005, "Comparing inhaled ultrafine versus fine zinc oxide particles in healthy adults: a human inhalation study", *Am.J.Respir.Crit Care Med.*, vol. 171, pp. 1129-1135.
- CEN. 1993, *Workplace atmospheres: size fraction definitions for measurements of airborne particles in the workplace*, CEN standard EN481, CEN, Bruxelles, Belgium.
- Chen, J.L. and Fayerweather, W.E. 1988, "Epidemiologic study of workers exposed to titanium dioxide", *Journal of Occupational Medicine*, vol. 30, pp. 937-942.
- de Hartog, J.J., Hoek, G., Peters, A., Timonen, K.L., Ibald-Mulli, A., Brunekreef, A.B., Heinrich Tiittanen, J.P., van Wijnen, J.H., Kreyling, W., Kulmala, M. and Pekkanen J. 2003, "Effects of Fine and Ultrafine Particles on Cardiorespiratory Symptoms in Elderly Subjects with Coronary Heart Disease, The ULTRA Study", *Am J Epidemiol*, vol. 157, pp. 613-23.
- Fishwick, D., Bradshaw, L., Slater, T., Curran, A. and Pearce N. 2004, "Respiratory symptoms and lung function change in welders: are they associated with workplace exposures?", *New Zealand Medical Journal*, vol. 117, U872.
- Forastiere, F., Stafoggia, M., Picciotto, S., Bellander D'Ippoliti, T.D., Lanki, T., von Klot, S., Nyberg, F., Paatero, P., Peters, A., Pekkanen, J., Sunyer, J., and Perucci C.A. 2005, "A Case-crossover Analysis of Out-of-Hospital Coronary Deaths and Air pollution in Rome, Italy", *Am. J. Respir. Crit. Care Med.*, vol. 172, pp. 1549 – 1555.
- Frampton, M.W., Utell, M.J., Zareba, W., Oberdorster, G., Cox, C., Huang, L.S., Morrow, P.E., Lee, F.E., Chalupa, D., Frasier, L.M., Speers, D.M. and Stewart, J. 2004, "Effects of exposure to ultrafine carbon particles in healthy subjects and subjects with asthma", *Res.Rep.Health Eff.Inst.* vol. 126, pp. 1-47.
- Fryzek, J.P., Chadda, B., Marano, D., White, K., Schweitzer, S., McLaughlin, J.K. and Blot, W.J. 2003, "A cohort mortality study among titanium dioxide manufacturing workers in the United States", *J of Occup and Environ Med / Am Collof Occup and Environ Med*, vol. 45, pp. 400- 409.
- Garabrant, D.H., Fine, L.J., Oliver, C., Bernstein, L. and Peters, J.M. 1987, "Abnormalities of pulmonary function and pleural disease among titanium metal production workers", *Scand J of Work, Environ and Health*, vol. 13, pp. 47-51.

Engineered Nanoparticles: Review of Health and Environmental Safety

Gardiner, K., Trethowan, N.W., Harrington, J.M., Rossiter, C.E. and Calvert, I.A. 1993, "Respiratory health effects of carbon black: a survey of European carbon black workers," *British Journal of Industrial Medicine*, vol. 50, pp.1082-1096.

Gardiner, K. 1995, "Effects on respiratory morbidity of occupational exposure to carbon black: a review." *Archives of Environmental Health*, vol 50, pp. 44-60.

Gardiner, K., van Tongeren, M. and Harrington, M. 2001, "Respiratory health effects from exposure to carbon black: results of the phase 2 and 3 cross sectional studies in the European carbon black manufacturing industry", *Occup. Environ. Med.*, vol. 58, pp. 496-503.

Gong, H.Jr, Sioutas, C., Linn, W.S. 2003, *Controlled Exposures of Healthy and Asthmatic Volunteers to Concentrated Ambient Particles in Metropolitan Los Angeles*. Research Report - Effects of Health Institute, vol. 118, pp. 1-36.

Gong, H., Linn, W.S., Sioutas, C., Terrell, S.L., Clark, K.W., Anderson, K.R. and Terrell, L.,L.. 2003, "Controlled exposures of healthy and asthmatic volunteers to concentrated fine particles in Los Angeles", *Inhal Toxicol.*, vol. 15, pp. 305-25.

Groat, S., Kauffer, E., Lovett, M., Miller, B.G., Kidd, M.W., Davies, L.S.T., McIntosh, C., Vigneron, J.C., Cherrie, J.W., Johnston, A., Robertson, A. and Hurley J.F. 1999, *Epidemiological research in the European ceramic fibre industry 1994 -1998. Workplace concentrations of airborne dust and fibres*", IOM Report TM/99/01, Institute of Occupational Medicine, Edinburgh, UK.

Hext, P.M., Tomenson, J.A. and Thompson, P. 2005, "Titanium dioxide: inhalation toxicology and epidemiology", *Ann Occup Hyg.*, vol. 49, no. 6, pp. 461-72.

Hodgson, J.T. and Jones, R.D. 1985, "A mortality study of carbon black workers employed at five United Kingdom factories between 1947 and 1980", *Archives of Environmental Health*, vol. 40, pp. 261-268

IARC (1996). *Printing trades, printing jobs, carbon black and some nitro compounds*, IARC Monographs, Working Group on the Evaluation of Carcinogenic Risks to Humans.

Kelly, J.T., Asgharian, B. and Wong, B.A. 2005, "Inertial particle deposition in a monkey nasal mold compared with that in human nasal replicas", *Inhal. Toxicol.* vol. 17, pp. 823-830.

Kelly, J.T., Kimbell, J.S. and Asgharian, B. 2001, "Deposition of fine and coarse aerosols in a rat nasal mold", *Inhal. Toxicol.* vol. 13, pp. 577-588.

Kimbell, J.S., Segal, R.A., Asgharian, B., Wong, B.A., Schroeter, J.D., Southall, J.P., Dickens C.J., Brace, G. and Miller, F.J. 2007, "Characterization of deposition from nasal spray devices using a computational fluid dynamics model of the human nasal passages", *J. Aerosol Med.*, vol. 20, pp. 59-74.

Kuschner, W.G., D'Alesandro, A., Wintermeyer, S.F., Wong, G.H., Boushey, H.A. and Blank, P.D.,1995, "Pulmonary responses to purified zinc oxide fume", *Journal of Investigative Medicine*. vol 43, pp. 371-8.

Kuschner, W.G., Wong, H., D'Alessandro, A., Quinlan, P. and Blanc, P.D. 1997, "Human pulmonary responses to experimental inhalation of high concentration fine and ultrafine magnesium oxide particles", *Environ. Health Perspect.*, vol 105, pp. 1234-7.

Lucking, A.L., Lundback, M., Mills, N.L., Faratian, D., Barath, S.L., Pourazar, J., Cassee, F.R., Donaldson, K., Boon, N.A., Badimon, J.J., Sandstrom, T., Blomberg, A. and Newby, D.E. 2008, "Diesel exhaust inhalation increases thrombus formation in man", *European Heart Journal*, vol 29, no. 24, pp. 3043-51.

Engineered Nanoparticles: Review of Health and Environmental Safety

Miller, B.G., Donnan, P.T., Sinclair, A., Edwards, J.C., Soutar, C.A., Hurley, J.F. 1996, "The respiratory and cardiovascular health of iron and steel process workers. Part II: results of the field studies and of the analyses of the data" IOM Report TM/96/05, Institute of Occupational Medicine, Edinburgh, UK.

Mills, N., Tornquist, S., Robinson, S., Darnley, K., Boon, N.A., MacNee, W., Donaldson, K., Blomberg, A., Sandstrom, T., and Newby D. 2005, "Diesel exhaust inhalation causes vascular dysfunction and impaired endogenous fibrinolysis", *Circulation*, vol. 112, pp. 3930-3936.

Mills, N.L.; Törnqvist, H.; Gonzalez, M.C., Vink, E., Robinson, S.D., Söderberg, S., Boon, N.A., Donaldson, K., Sandström, T. Blomberg, A., Newby, D.E. 2007, "Ischemic and thrombotic effects of dilute diesel-exhaust inhalation in men with coronary heart disease.", *The New England journal of medicine*, vol. 357, no. 11, pp.1075-82.

Mills, N.L., Robinson, S.D., Fokkens, P.H.B., Leseman Daan, L.A.C., Miller, M.R., Anderson, D., Freney, E.J., Heal, M.R., Donovan, R.J., Blomberg, A., Sandström, T., MacNee, W., Boon, N.A., Donaldson, K., Newby, D.E., Cassee, F.R. 2008, "Exposure to concentrated ambient particles does not affect vascular function in patients with coronary heart disease." *Environmental health perspectives*, vol. 116, no. 6, pp. 709-15.

Morawska, L., Moore, M.R., Ristovski, Z.D. 2004, *Health impacts of ultrafine particles - desktop literature review and analysis*, Department of the Environment and Heritage, Commonwealth of Australia. Accessed at:
<http://www.environment.gov.au/atmosphere/airquality/publications/health-impacts/index.html>
(15/10/09)

Nightingale, J.A., Maggs, R., Cullinan, P., Donnelly, L.E., Rogers, D.F., Kinnersley, R., Chung, K.F., Barnes, P.J., Ashmore, M. and Newman-Taylor, A. 2000, "Airway inflammation after controlled exposure to diesel exhaust particulates", *Am J of Respir and Crit Care Med*, vol. 162, pp. 161-6.

Nordenhall, C., Pourazar, J., Blomberg, A., Levin, J.O., Sandstrom, T., and Adelroth E. 2000, "Airway inflammation following exposure to diesel exhaust: a study of time kinetics using induced sputum", *Eur.Respir.J.*, vol. 15, pp. 1046-1051.

Pietropaoli, A.P., Frampton, M.W., Hyde, R.W., Morrow, P.E., Oberdorster, G., Cox, C., Speers, D.M., Frasier, L.M., Chalupa, D.C., Huang, L.S., Utell, M.J.2004, "Pulmonary function, diffusing capacity and inflammation in healthy and asthmatic subjects exposed to ultrafine particles", *Inhal. Toxicol.* vol. 16, pp. 59-72.

Preining, O. 1998, "The physical nature of very, very small particles and its impact on their behaviour", *J Aerosol Sci*, vol. 29, pp. 481-495.

Rappaport, S.M. and Smith, T.J. eds. 1991, *Exposure Assessment for Epidemiology and Hazard Control*. Lewis Publishers, Chelsea, Michigan.

Rappaport, S.M. 1991, "Assessment of long-term exposures to toxic substances in air", *The Annals of Occupational Hygiene*, vol. 35, pp. 61-121.

Routledge, H.C., Manney, S., Harrison, R.M., Ayres, J., Townend, J.N. 2005, "The effect of inhaled sulphur dioxide and carbon particles on heart rate variability and markers of inflammation and coagulation in human subjects", *Heart*, vol. 92, no. 2, pp. 220 - 227.

Ruddell, B., Ledin, M-C., Hammarstrom, U., Stjernberg, N., Lundback, B., Sandstrom, T. 1996, "Effects of symptoms and lung function in humans experimentally exposed to diesel exhaust", *Occup and Environ Med*, vol. 53, pp. 658-662.

Salvi, S., Blomberg, A., Rudell, B., Kelly, F., Sandstrom, T., Holgate, S.T. and Frew A. 1999, "Acute inflammatory responses in the airways and peripheral blood after short-term exposure to

diesel exhaust in healthy human volunteers”, *Am J of Respir and Crit Care Med*, vol. 159, pp. 702-9.

Salvi, S., Nordenhall, C., Blomberg, A., Rudell, B., Pourazar, J., Kelly, F.J., Wilson, S., Sandstrom, T., Holgate, S.T. and Frew A.J. 2000, “Acute exposure to diesel exhaust increases IL-8 and GRO-alpha production in healthy human airways”, *Am J of Respir and Crit Care Med*, vol. 161, pp. 550-7.

Schwela, D., Morawska, W., Kotzias, D. eds. 2002, *Guidelines for concentration and exposure-response measurement of fine and ultrafine particulate matter for use in epidemiological studies*. World Health Organisation.

Schulte, P.A., Geraci, C.L., Schubauer-Berigan, M.K., Zumwalde, R., Candis, M., McKernan, J.L. 2009, “Issues in the Development of Epidemiologic Studies of Workers Exposed to Engineered Nanoparticles”, *Journal of Occupational and Environmental Medicine*, vol. 51, no. 3, pp. 1-12.

Sorahan, T., Hamilton, L., van Tongeren, M., Gardiner, K. and Harrington J.M. 2001, “A cohort mortality study of U.K. carbon black workers, 1951-1996”, *Am J Ind Med*, vol. 39, pp. 158-170.

Törnqvist, H, Mills, N.L., Gonzalez, M., Miller, M.R., Robinson, S.D., Megson, I.L., Macnee, W., Donaldson, K., Söderberg, S., Newby, D.E., Sandström, T. and Blomberg, A. 2007, “Persistent endothelial dysfunction in humans after diesel exhaust inhalation.”, *American journal of respiratory and critical care medicine*, vol. 176, no. 4, pp. 395-400.

Tran, C.L., Donaldson, K., Stone, V., Fernandez, T., Ford, A., Christofi, N., Ayres, J.G., Steiner, M., Hurley, J.F., Aitken, R.J. and Seaton, A. 2005, *A scoping study to identify hazard data needs for addressing the risks presented by nanoparticles and nanotubes*, Defra Research Report CB01072 , UK. Accessed at:
http://randd.defra.gov.uk/Document.aspx?Document=CB01072_3060_FRP.doc (15/10/09)

van Tongeren, M.J., Kromhout, H., Gardiner, K., Calvert, I.A., Harrington, J.M. 1999, “Assessment of the sensitivity of the relation between current exposure to carbon black and lung function parameters when using different grouping schemes”, *American Journal of Industrial Medicine*, vol. 36, pp. 548-556.

van Tongeren, M.J., Gardiner, K., Rossiter, C.E., Beach, J., Harber, P., Harrington, M.J. 2002, “Longitudinal analyses of chest radiographs from the European Carbon Black Respiratory Morbidity Study”, *The European Respiratory Journal*, vol. 20, pp. 417-425.

Wake, D. 2001, *Ultrafine aerosols in the workplace*, Report number IR/ECO/00/18, Health and Safety Laboratory, UK.

Wichmann, H.E., Spix, C., Tuch, T., Wolke, G., Peters, A., Heinrich, J., Kreyling, W.G., Heyder, J. 2000, “Daily mortality and fine and ultrafine particles in erfurt, germany part I: role of particle number and particle mass” *Research Report Health Effects Institute*, vol. 98, pp. 5-86.

8 ECOTOXICITY

8.1 INTRODUCTION

The ecotoxicological literature on nanomaterials has been reviewed several times since 2006 in a range of different journals. Although more than 15 reviews have been published (as noted in this chapter's references), the number of primary scientific papers is arguably still limited, although increasing at a rapid rate. The present review is intended to be a critical compilation and analysis of the existing literature, aimed at including all scientific papers published before 2009 dealing with ecotoxicity of engineered nanomaterials belonging to one of the four groups: fullerenes, CNT, metals, or metal oxides.

The ecotoxicological literature has been reviewed for each of the four groups of nanomaterials mentioned above with respect to aquatic toxicity, terrestrial toxicity, bioaccumulation, and degradability. Aquatic ecotoxicity is further sub-divided into studies dealing with fish, crustacean, algae and other taxa (covering studies on bacteria, non-crustacean invertebrates, and amphibians) with the view to providing data for risk assessment purposes. Due to the strong focus on regulatory use of the ecotoxicity data in the ENRHES review, a special effort has been put into translating the effects, found in the reviewed papers, into the terminology traditionally used in risk assessment, e.g. EC_x - and LC_x -values and NOEC/LOEC-values.

The present review contains descriptions of the majority of the 89 papers on ecotoxicity of nanomaterials published before 12 December 2008 which have been considered subsequently in the risk assessments of the four nanomaterial classes. Each individual study has been addressed by the material class and environmental compartment(s).

8.2 CARBON FULLERENES

8.2.1 Aquatic toxicity

8.2.1.1 Fullerene toxicity towards fish

Oberdörster (2004) observed a significant increase in lipid peroxidation in the brain of the juvenile largemouth bass (*Micropterus salmoides*) after exposure to uncoated C_{60} (99.5%) at concentrations of 0.5 and 1 ppm, after exposure for 48 hours. The C_{60} were suspended in THF which has since led to some discussion regarding the role of THF on the observed effects (Zhu *et al.* 2006a; Henry *et al.* 2007; Klaine *et al.* 2008). In the fathead minnow (*Pimephales promelas*) a 100% mortality was observed after exposure to 0.5 ppm THF-suspended C_{60} for 6-18 hours, whereas no obvious effects were observed in fathead minnow after exposure to 0.5 ppm water-stirred C_{60} for 48 hours. The THF-suspended C_{60} resulted in elevated lipid peroxidation in the brain and gill along with increased expression of CYP2 family isozymes in the liver (Zhu *et al.* 2006a; Oberdörster *et al.* 2006).

Henry *et al.* (2007) compared the effects on larval zebrafish, *Danio rerio*, of 1) 99.5% C_{60} that had been prepared in water through stirring and sonication and 2) 99.5% C_{60} that had been suspended in THF. No effect on survival was observed after exposure to stirred and sonicated C_{60} in concentrations up to 6.25 mg l^{-1} for 72 hours. For C_{60} dissolved in THF, mortality was observed within 60 minutes at concentrations above 1.25 mg l^{-1} and an $LC_{50,72 \text{ h}}$ of 0.78 mg l^{-1} [0.58;1.05]_{95%} was found. The animals exposed to concentrations over 1.25 mg l^{-1} C_{60} suspended in THF typically had arched backs, as well as severe yolk-sac and pericardial edema. Henry *et al.* (2007) also observed significant changes in expression of 271 genes when animals were exposed to C_{60} suspended in THF as compared to only 10 genes when exposed to stirred or sonicated C_{60} . Most of these genes (182) were also expressed when the zebrafish was only exposed to THF.

Henry *et al.* (2007) clearly demonstrated that compounds other than THF or C_{60} (e.g. γ -butylacetone) may be responsible for toxicity when THF was used for preparing suspensions of

C₆₀, therefore ecotoxicological studies carried out with C₆₀ suspended in THF and resuspended in water, after solvent removal, would need to be reconsidered.

Blickley and McClelland-Green. (2008) exposed embryos, larvae and female adults of the saltwater minnow *Fundulus heteroclitus* to aqueous suspensions of C₆₀ in concentrations up to 10 mg l⁻¹ with exposure periods of 12 days (embryos) or 96 hours (larvae and adults). Aggregation and sedimentation of nC₆₀ in the saline media were observed. In tests with embryos, it was found that nC₆₀ adhered to the membranes surrounding the embryos, and was also found in the fry/embryo tissue in low concentrations (µg l⁻¹ range). However, no significant mortality, developmental delays, or malformations were observed. In larval tests no significant mortality was observed, although total glutathione levels were found to increase with increasing nC₆₀ concentration in exposed larvae, combined with a (non-statistically significant) decreasing trend in lipid peroxidation. Similarly, no mortality was observed in exposed adult fish. In liver tissue (but not in gills) elevated glutathione levels were measured, but no significant lipid peroxidation was observed.

Zhu *et al.* (2008b) observed no unusual behaviours and no mortality of juvenile carp (*Carassius auratus*) after 32 days of exposure to between 0.04–1.0 mg l⁻¹ C₆₀ aggregates. C₆₀ was reported to be 99.5 % pure and prepared by suspending in water after long-term stirring described by Lyon *et al.* (2006). The average diameter of the C₆₀ aggregates were observed to be approximately 320 nm in MillQ water, but as high as 1394 nm in test solution (i.e. tap water). Excretion difficulty was observed and slender, orange fish faeces were found in the test tanks within 24 hours of initial exposure and within 24 hours of each test solution renewal (50% of the water volume was renewed every 24 hours). Colour and hardness of these faeces were different compared to controls. Zhu *et al.* (2008b) also observed a significantly lower mean total length after 32 d exposure to 0.2 mg l⁻¹ of C₆₀ and a significantly reduced body weight at 1.0 mg l⁻¹. No detectable effects were observed after exposure to 0.04 mg l⁻¹ for 32 days.

In terms of traditionally used endpoints in ecotoxicology these findings correspond to a NOEC of 0.04 mg l⁻¹ and a LOEC of 0.2 mg l⁻¹ for the length of juvenile carp and a LOEC of 1.0 mg l⁻¹ for the body weight after exposure to C₆₀ in water for 32 days.

Inhibition rates in mean total length and body weight were 12.3 and 27.3%, respectively, after exposure to 1.0 mg l⁻¹ of C₆₀. An inverse significant dose-dependent ($p < 0.01$) induction of superoxide dismutase (SOD) and catalase (CAT) activity were observed in the liver compared to the control, whereas a non-significant increase was observed in the gills. In the brain the levels of SOD and CAT were not significantly different from control levels after 32 days. The content of the antioxidant GSH in gill tissue was between 75-90% compared to control in the 0.04, 0.20, and 1.0 mg l⁻¹ C₆₀ exposures, whereas the percentage differences were 87-104% and 78-88% for the liver and brain tissue, respectively. A statistically significant ($p < 0.01$) decrease was observed in lipid peroxidation (LPO) levels in various tissues of juvenile *C. auratus* exposed to concentrations between 0.04-1 mg l⁻¹ C₆₀. Finally, a significant ($p < 0.05$) increase in thiobarbituric acid in the liver was observed, in contrast to the statistically significant decreases observed in gill tissue and in the brain. In summary, these findings indicate that oxidative stress may be a main mode of action for C₆₀ in juvenile carp.

Harper *et al.* (2008) observed a significant increase in the mortality of zebra fish embryos (120 hours post fertilisation; hpf) and the incidence of pericardial edema and fin malformations after exposure to C₆₀ and C₇₀ sonicated in dimethylsulfoxide (DMSO) and diluted to 1% DMSO in test concentrations, however exposure concentrations are not available in the paper. For C₆₀(OH)₂₄ the observed effects were found to be less pronounced than both of the non-hydroxylated fullerenes even at concentrations reported to be an order of magnitude higher.

Usenko *et al.* (2008) studied the effect of C₆₀ in zebrafish embryos (*Danio rerio*), 24 hpf on a range of endpoints, with a particular focus on the influence of light on any of the observed effects. In the stock solution 50 ppm C₆₀ was suspended in 100% DMSO and 1% (v/v) DMSO solutions were used as solvent controls. This solvent content corresponds to the volume of DMSO in the highest tested concentration (0.5 ppm C₆₀). Full concentration-response relationships were observed for mortality, fin malformations and pericardial edema in the range of 0.05-0.30 ppm after four days of incubation (120 hpf). The authors did not estimate effect

concentrations usually reported in the ecotoxicological literature for the three endpoints, however for fin malformations and pericardial edema $EC_{50,96h}$ -values of about 0.11 ppm (light conditions) can be extrapolated from analysis of Figure 1 (Usenko *et al.* 2008) and a $LC_{50,96h}$ of about 0.19 ppm, following a similar approach (that is analysis of Figure 1 of the same paper). Incubation in the dark for five days resulted in a significant reduction in fin malformations (40%), pericardial edema (85%) and mortality (30%) of zebrafish (*Danio rerio*) exposed to 0.2 ppm and 0.3 ppm C_{60} , when compared with similar light incubations. A mortality of 100% was observed within 24 hours at 0.5 ppm, regardless of light exposure. Overall light exposures resulted in more deleterious effects than dark exposures. Similarly to the studies of Zhu *et al.* (2008b), it was concluded that oxidative stress may be a major cause of the effects observed.

8.2.1.2 Fullerene toxicity towards crustaceans

In the pioneering study by Lovern and Klaper (2006) a comparison between water-sonicated C_{60} and C_{60} suspended in THF was made. Despite a great variation in mortality in *Daphnia magna*, they observed an $LC_{50,48h}$ of 7.9 mg l^{-1} for water-sonicated C_{60} . The lowest- and no observed effect concentrations (LOEC and NOEC) were reported to be 0.5 mg l^{-1} and 0.2 mg l^{-1} , respectively, in the lethality study (48 h) of water-sonicated C_{60} suspensions.

For C_{60} suspended in THF, a dose-dependent increase in mortality was observed after 48 hours, and LC_{50} , LOEC, and NOEC were found to be lower than for sonicated C_{60} , i.e. 460, 260 and $180 \mu\text{g l}^{-1}$, respectively. However, the data presented do not allow for statistical comparison of values obtained by the two methods of preparation. Zhu *et al.* (2006a) found an $LC_{50} > 35 \text{ mg l}^{-1}$ (48 hours) in *Daphnia magna* after exposure to hydroxylated 10-200 nm C_{60} of 99.5% purity. When C_{60} was suspended in THF an LC_{50} of 0.8 ppm (48 hours) was found for *Daphnia magna*. A series of behavioural changes in exposures to C_{60} suspended in THF have also been reported in the literature. After exposure to 0.26 mg l^{-1} C_{60} suspended in THF, Lovern *et al.* (2007) observed a significant increase in hopping rate, heart rate as well and the number of cycles per minute in appendage movement compared to controls. As mentioned above, ecotoxicological studies carried out with C_{60} suspended in THF and resuspended in water after solvent removal do not have a high credibility.

Very few studies of long-term effects on crustaceans have been carried out. Oberdörster *et al.* (2006) exposed *Daphnia magna* to 2.5 mg l^{-1} stirred C_{60} and found a significant reduction in the number of offspring after 21 days and delays in moulting of the carapace. However, up to 40% mortality was found at these exposures and this may have affected the lowering of the number of offspring. Furthermore, Oberdörster *et al.* (2006) investigated the effect of hydroxylated C_{60} on the reproduction and survival rate of *Daphnia magna* and observed an increased cumulative mortality and significant delay in moulting and reduced offspring after exposure to $1\text{-}5 \text{ mg l}^{-1}$ for 21 days. The study of Oberdörster *et al.* (2006) also included the benthic crustacean *Hyaella azteca* and a marine benthic harpacticoid copepod species (unidentified). For *H. azteca* no mortality was found in the exposure concentrations used (up to 7 mg l^{-1} for 96 hours) and also the copepod did not show any mortality in concentrations up to 22.5 mg l^{-1} (for 96 hours). Precipitation of C_{60} due to the salt content in the medium was reported.

8.2.1.3 Fullerene toxicity towards algae

A profound lack of data on algal toxicity of fullerenes was found when reviewing the literature published before 12 December 2008. Only the study by Baun *et al.* (2008) describes tests carried out on algae and they found up to 30% algal growth rate inhibition at C_{60} concentrations of 90 mg l^{-1} . The C_{60} was suspended by slow stirring. No concentration-response relationships could be established in the 48 hours algal growth inhibition tests.

8.2.1.4 Toxicity of fullerenes towards bacteria

A range of bacterial studies have been made with C_{60} . Most of these are focused at the anti-microbial effects of fullerenes as these maybe important e.g. for disinfection purposes. However, when reading through the following reviews it should be noted that most of the papers have used THF to prepare the solutions/suspensions of C_{60} in the growth media. As mentioned

above, studies carried out with C₆₀ suspended in THF and resuspended in water after solvent removal do not have a high credibility due to the risk of effects induced by the solvent, the solvent-nanoparticle interaction, or solvent degradation products.

For C₆₀ suspended in THF, Lyon *et al.* (2005, 2006) observed minimal inhibitory concentrations of 0.5-1 mg l⁻¹ and 1.5-3 mg l⁻¹ for *E. coli* and *B. subtilis*, respectively. However, in the paper by Lyon *et al.* (2006) a comparison between different preparation methods for C₆₀ in water was carried out. It was found that, irrespectively of preparation method, quite strong antibacterial activity of C₆₀ was observed. Thus, both suspensions prepared by sonication and by stirring without the presence of THF resulted in minimal inhibitory concentrations (i.e. the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation, which could be considered equivalent to LOEC) in the range of 0.4-0.6 mg l⁻¹ in *B. subtilis*.

In the studies by Fang *et al.* (2007) the effects of C₆₀ on gram positive (*Bacillus subtilis*) and gram negative (*Pseudomonas putida*) bacteria were investigated. The C₆₀ suspensions were prepared through stirring with THF followed by evaporation and filtration. A minimal inhibitory concentration (MIC) for bacterial growth was found to be between 0.5 and 0.75 mg l⁻¹ for *B. subtilis* and between 0.25 and 0.5 mg l⁻¹ for *P. putida*. Exposure of *P. putida* and *B. subtilis* to nC₆₀ in non-inhibitory concentrations (0.01 mg l⁻¹) resulted in changes in fatty acid profiles. This was interpreted as an adaptation response to the exposure to C₆₀ depending both on the exposure concentration and the cell membrane composition. For *P. putida*, the proportion of saturated fatty acids increased, the proportion of cyclopropane fatty acids increased slightly and the proportion of monounsaturated fatty acids decreased, however the presented data did not allow for a statistical evaluation. These changes are similar to effects observed for organic solvents, however this is only briefly discussed in the article. In the case of *B. subtilis*, levels of *iso*- and *anteiso*-branch fatty acids increased when bacteria were exposed to 0.01 mg l⁻¹ of nC₆₀, while the proportion of saturated and unsaturated fatty acids decreased (similar to the response of gram-positive bacteria to aromatic hydrocarbons). At 0.75 mg l⁻¹ the opposite was observed: levels of *iso*- and *anteiso*-branch fatty acids decreased dramatically, while the proportion of saturated and unsaturated fatty acids increased.

Microbial growth assays were carried out to test the effect of aqueous suspensions of C₆₀ on gram positive (*Bacillus subtilis*) and gram negative (*Escherichia coli*) bacteria by Fortner *et al.* (2005). The suspensions were again prepared through stirring with THF, followed by THF evaporation and filtration. In these suspensions C₆₀ formed water-stable colloidal aggregates with diameters of 20-500 nm. Particle size was affected by rate of mixing as well as the pH of the suspension. At higher pH the diameter of the colloids was smaller and at lower pH their diameter was larger. From the microbial growth assays it was found that effects depended on media. In Minimal Davis medium no growth was observed at concentrations > 0.4 mg l⁻¹. Less growth inhibition was seen in a rich medium (Luria broth). The pH of the two different media used is not listed, but the authors suggest that the lower effects observed in the Luria broth medium may be due to salt induced precipitation of the fullerenes out of solution, due to the higher ionic strength of the Luria broth compared to the Minimal Davis medium. Another explanation offered by the authors is that a coating of the fullerenes with excess protein occurs due to the presence of yeast extract in the Luria broth medium. Fortner *et al.* (2005) did not observe reduced growth of the bacteria when exposed to hydroxylated C₆₀ in the two types of growth media.

Li *et al.* (2008) observed a significant decrease in the respiration rate (measured as rate of CO₂ production) of *Escherichia coli* (*E. coli*) K12 (ATTC 25404) exposed to 0.5 mg l⁻¹ C₆₀ alone in Minimal Davis medium. C₆₀ was prepared using THF and the average size of C₆₀ aggregates was reported to be 108 nm. It was found that the toxic effect of C₆₀ was mitigated dose-dependently by powder activated carbon indicating that sorption to natural organic matter in the environment may diminish the antimicrobial effect of C₆₀ in the environment.

Using the luminescent marine bacterium *Vibrio fischeri* Velzeboer *et al.* (2008) did not observe any effects on the light emission on exposure to 1 mg l⁻¹ C₆₀ for up to 30 minutes in the saltwater medium.

Very few studies have been carried out with fullerenes using mixed cultures or focussed at potential inhibition of wastewater treatment processes. However, Nyberg *et al.* (2008) use biosolids from anaerobic wastewater treatment sludge exposed to: 1) C₆₀ suspended in methanol/ethanol: 0.321 mg kg⁻¹ of biomass dissolved in MeOH/EtOH; 2) aqueous suspended C₆₀: 8.6 mg kg⁻¹ of biomass; 3) C₆₀ plated on dried sludge from a toluene solution: 30000 mg kg⁻¹ of biomass plated on dried sludge (toluene) and; 4) C₆₀ plated on dried sludge from a o-xylene solution: 50000 mg kg⁻¹ of biomass. Nyberg *et al.* (2008) observed no statistically significant difference in gas production and microbial community function in anaerobic sludge from wastewater treatment after 89 days of exposure to C₆₀. Furthermore, they found no indication of biodegradation and no effect on the community structure of methanogenic, bacteria and *Eukarya* populations.

8.2.2 Terrestrial toxicity of fullerenes

Three studies focussed on the terrestrial toxicity of fullerenes. Two of these studied microbial communities (Tong *et al.* 2007; Johansen *et al.* 2008) and only one study assessed the effects of fullerenes on invertebrate taxa (earthworms) (Scott-Fordsmand *et al.* 2008).

Tong *et al.* (2007) observed no significant differences ($p > 0.05$) in basal respiration in soil (Drummer, silty clay loam, 4% organic matter, pH 6.9) between control and any treatments with granular C₆₀ (1000 µg g⁻¹ soil) and THF-C₆₀ (1 µg g⁻¹ soil) after 30 days. The average diameter of the aggregates in aqueous suspensions was reported to be 85 nm. No significant difference ($p > 0.05$) was observed on the microbial community's ability to respond to added nutrients. The level of ¹⁴C₂O₂ production over 3 hours ranged from 16.5 to 18% of the applied glucose after 30 days, whereas it was reduced an additional 3% after 180 days. Soil enzymatic activities were found to vary after 180 days, especially with respect to dehydrogenase, but only phosphatase activity was observed to be significantly ($p > 0.05$) different from control during the first month and only in soil treated with THF-C₆₀.

Johansen *et al.* (2008) observed a three- to four-fold inhibition of the number of bacterial colony-forming units (CFUs) in clay loam soil 3 hours after incorporation of 99.5% pure C₆₀ aggregates at concentrations of 5, 25 and 50 µg g⁻¹. Zeta-potential of the aggregates was reported to be -63.90 ± 0.06 mV for the stock solution. The effect was evident for the fast-growing bacteria but after 23 d of incubation the differences in the number of CFUs between treatments were not statistically significant different from the number of CFUs in the controls. The number of CFUs on two different media was compared after 14 days and no medium-related differences were observed. Exposure to 50 µg g⁻¹ C₆₀ resulted in a statistically significant decrease in the number of CFUs to approximately 60% of the CFUs in the control. No significant effect was observed on the estimated microbial biomass, although it did decline non-significantly with increasing concentrations of C₆₀ in incubations of soil sampled 3 hours after incorporation of the C₆₀. The number of protozoans was not unaffected by the C₆₀ exposure after 3 hours, but did seem to decrease in the highest concentration (50 µg g⁻¹), however this was not statistically significant.

Scott-Fordsmand *et al.* (2008) observed no significant mortality in the earthworm *Eisenia veneta* after consuming dry food spiked with C₆₀ concentrations of 0 and 1 g kg⁻¹ dry food for up to 28 days. C₆₀ was reported to be 99.5% pure and have a metal content of 0.4%, with an outer diameter of 11 nm as dry powder. Non-significant reductions in growth (20% compared to control) and in cocoon production (78% compared to control) were observed after exposure to C₆₀. The hatchability was not affected at the single exposure dose of 1 g kg⁻¹ dry food.

8.2.3 Bioaccumulation of fullerenes

No studies on bioaccumulation of fullerenes have been reported in the literature before 12 December 2008. However, Oberdörster *et al.* (2006) found an increase in the uptake of C₆₀ in *Daphnia magna* exposed to 30 ppm C₆₀ for up to five days. The C₆₀ content of the animals was measured in whole organism homogenates after a rinsing of the outer surface of the daphnids with bleach. The maximum uptake was measured after 48 hours of incubation with a C₆₀ content of 2.2-2.4 ppm per mg wet weight measured in the daphnid homogenates. After 96 hours of incubation a C₆₀ content of less than 2 ppm per mg wet weight was found, however the data presented do not allow for any statistical analysis.

8.2.4 Degradability of fullerenes

No studies on the degradability of fullerenes have been found in the literature. The cage-like structure of C₆₀ suggests very low biological degradability (ref), however functionalisation (e.g. hydroxylation) may alter the degradability behaviour significantly (ref). Schreiner *et al.* (2009) suggest that C₆₀ can be oxidised to C₆₀-fullerol through both abiotic- and biotic-mediated means, and in their experimental study they demonstrate that the ability of two white rot basidiomycete fungi (*Phlebia tremellosa* and *Trametes versicolor*) to metabolize and degrade an C₆₀ fullerol to CO₂ after 32 weeks of decay, with minor amounts of the fullerol carbon incorporated into lipid biomass.

8.2.5 Summary

Since 2004, a range of studies have been carried out with aquatic species and C₆₀. However, less than ten studies have been carried out on fullerene toxicity towards the base-set organisms used in the REACH risk assessment procedures for chemicals (fish, crustacean and microalgae). More studies are available using bacterial groups and, though they do not report the findings in traditional ecotoxicological endpoints, these studies may be of value for mechanistic interpretations of fullerene ecotoxicity in both the aquatic and the terrestrial environment.

Initial studies used different solvents to suspend C₆₀. However, more recent studies have avoided the use of any solvents since it has been demonstrated that not only C₆₀/solvent interactions may affect toxicity, but also solvent degradation products may be responsible for some of the effects observed.

For fish, the studies by Zhu *et al.* (2008b) resulted in NOEC of 0.04 mg l⁻¹ and a LOEC of 1 mg l⁻¹ in terms of reduced lengths and body weights, after 32 days of exposure. In the study by Usenko *et al.* (2008) an LC_{50, 96h} of 0.19 mg l⁻¹ can be proposed from their results. However, in this study the stock solutions of C₆₀ were prepared in pure DMSO and the solvent concentration in the tests was as high as 1% (vol/vol).

Less information is available for crustaceans and in fact the only reported LC₅₀-value is the LC_{50, 48h} of 7.9 mg l⁻¹ for sonicated C₆₀ found by Lovern and Klaper (2006). They also observed a LOEC of 0.45 mg l⁻¹ and a NOEC of 0.18 mg l⁻¹ in the 48h acute toxicity test for the sonicated suspensions of C₆₀. In a long-term study with *Daphnia magna*, Oberdörster *et al.* (2006) reported a LOEC of 2.5 mg l⁻¹ for the number of offspring after 21 days for water stirred C₆₀, but the validity of this result is questionable due to too high mortality in the exposed tests.

There is not enough data for other invertebrates or algal groups in regards to the toxicity of fullerenes to draw any further conclusions.

In the three studies currently published on the terrestrial toxicity of fullerenes, significant effects are only reported by Johansen *et al.* (2008) who found that exposure to 50 µg g⁻¹ yielded a three- to four-fold inhibition of the number of bacterial CFUs in clay loam soil 3 hours after incorporation of 99.5% pure C₆₀ aggregates.

Major knowledge gaps are identified within persistence and bioaccumulation of fullerenes since no structured studies aimed at addressing this area have been reported in the reviewed literature.

8.3 CARBON NANOTUBES

While not stated specifically in the reviews below, it should be emphasized that CNT cannot be regarded as one compound, and both SWCNT and MWCNT have many configurations (and lengths) that may influence any toxic responses. Furthermore, impurities stemming from the synthesis of CNT seem to be an issue that needs to be taken into account when interpreting results of ecotoxicity studies of CNT.

8.3.1 Aquatic toxicity

8.3.1.1 Toxicity of carbon nanotubes towards fish

Cheng *et al.* (2007) studied the effects of SWCNT and DWCNT (double-walled carbon nanotubes) on the development of zebrafish (*Danio rerio*). During an exposure period of up to 96 hpf, the hatching success, time-to-hatch, body length and head-trunk angle of the embryos was monitored. For SWCNT also cell death, potential effects on developmental regulatory factors and effects on the circulatory system (blood vessel formation) were investigated. The dispersion of SWCNT was carried out by stirring with a magnetic stir bar for 30 minutes in the filtered tap water. The 4 hpf zebrafish embryos were selected and exposed to six concentrations of SWCNT (20, 40, 60, 120, 240, and 360 mg l⁻¹) dispersed in filtered tap water and a filtered tap water control (pH 7, 28.5°C). The SWCNT had a diameter of approximately 11 nm and a length of 0.5-100 µm. Results showed that SWCNT agglomerates were in the micrometer range, and covered the egg membrane of the embryos. However the membrane pores (necessary for oxygen and nutrient transport) were in the nm size range and this was therefore found to be a protective barrier for the embryos to CNT agglomerates. Exposure to SWCNT induced a significant hatching delay in zebrafish embryos between 52 to 72 hpf at concentrations greater than 120 mg l⁻¹. LOECs for delayed hatching were found to be 120 mg l⁻¹ SWCNT and 240 mg l⁻¹ DWCNT. No embryo mortality or effect on hatching success rate were observed for any of the tested CNT. Head-trunk angle, body length, blood vessel formation and development regulatory factors were not affected by exposure to 240 mg l⁻¹ SWCNT. Also, no cell death was observed after SWCNT exposure. These results indicate that the outer membrane is likely to be protecting the embryos from CNT exposure. In this study the differences in hatching delay effects of SWCNT and DWCNT may be due to differences in metal (Ni and Co) impurities for the two different types of CNT.

Smith *et al.* (2007) tested the ecotoxicity of SWCNT suspended through a combination of sodium dodecyl sulphate (SDS) and sonication. These authors observed a dose-dependent rise in ventilation rate, gill pathologies (oedema, altered mucocytes, hyperplasia), and mucus secretion with SWCNT precipitation on the gill mucus of juvenile rainbow trout. Exposure concentrations were 0.1, 0.25 and 0.5 mg l⁻¹ and the fish were exposed for up to 10 days. Even at the lowest test concentration (0.1 mg l⁻¹) there was a statistically significant (p<0.05) effect on the ventilation rate of the fish compared to the control (solvent control was not found to be significantly different from the control). Smith *et al.* (2007) also observed dose dependent changes in brain and gill Zn or Cu, partly attributed to SDS, a significant increase in Na⁺K⁺-ATPase activity in the gills and intestine for all the concentrations tested, a significant dose dependent decrease in thiobarbituric acid reactive substances (TBARS), especially in the gill, brain and liver, and a significant increase in the total glutathione levels in the gills (28%) and livers (18%) for the concentrations tested, compared to the solvent control (15 mg l⁻¹ SDS). TBARS are indicators of lipid peroxidation and oxidative stress. Despite the observed lipid peroxidation in the liver and brain, it could not be confirmed that the fish were directly suffering from oxidative stress. The authors suggest that aqueous SWCNT act as respiratory toxicants, rather than an ionoregulatory toxicant. Finally, Smith *et al.* (2007) observed increasing aggressive behaviour, possible aneurisms or swellings on the ventral surface of the cerebellum in the brain and apoptotic bodies and cells in abnormal nuclear division in liver cells.

The findings of Smith *et al.* (2007) are very important since they indicate some new modes of toxicity that have not been identified in fish before, i.e. subtle neurotoxic or cardiovascular effects of SWCNT that may affect fish behaviour. Furthermore, the findings of cellular pathologies in the liver (which suggest genotoxicity or cell cycle defects), give rise to concerns regarding carcinogenicity potentially being observed after long-term exposure to SWCNT (Smith *et al.* 2007).

8.3.1.2 Toxicity of carbon nanotubes to crustaceans

Kennedy *et al.* (2008) observed no significant mortality in *Ceriodaphnia dubia* after 48 hours of exposure to 32 mg l⁻¹ of hydroxylated multiwalled carbon nanotubes (MWCNT-OH) dispersed into moderately hard reconstituted water containing 100 mg l⁻¹ of NOM. Raw MWCNT,

MWCNT–OH (1.76% hydroxylated by weight), and MWCNT–COOH (1.23% carboxylated by weight) were greater than 95% in purity. In moderately hard reconstituted water containing 100 mg l^{-1} of NOM the hydrodynamic diameter of aggregates was found to be 208.6 ± 2.2 to $223.3 \pm 0.8 \text{ nm}$, 181.5 ± 1.1 to $187.4 \pm 1.1 \text{ nm}$, 181.1 ± 0.3 to $185.1 \pm 2.0 \text{ nm}$ for raw MWCNT, MWCNT–OH and MWCNT–COOH, respectively. Zeta potentials were determined to be -22.7 ± 1.5 to $-24.6 \pm 1.0 \text{ mV}$, -23.0 ± 1.0 to $23.6 \pm 1.8 \text{ mV}$, and -19.7 ± 2.0 to $-23.2 \pm 1.5 \text{ mV}$ for raw MWCNT, MWCNT–OH, and MWCNT–COOH, respectively. The surface area was found to be 150 , 140 , and $115 \text{ m}^2 \text{ g}^{-1}$ for MWCNT, MWCNT–COOH and MWCNT–OH, respectively. No significant effect was observed on survival of *Ceriodaphnia dubia* after exposure to 120.2 mg l^{-1} MWCNT–OH and 88.9 mg l^{-1} MWCNT–COOH after 7 days of settling in 100 mg l^{-1} of NOM. Survival rates were found to be $80 \pm 20 \%$ and $100 \pm 0 \%$ for MWCNT–OH and MWCNT–COOH, respectively. A significantly lower survival rate of $7 \pm 12\%$ was observed after exposure to raw MWCNT at a concentration of 39.5 mg l^{-1} . An LC_{50} for *Ceriodaphnia dubia* was reported to be 50.9 [38.4 – 67.6] mg l^{-1} after 48 hours exposure to MWCNT.

Kennedy *et al.* (2008) did not observe reduced survival after exposures to MWCNT at concentrations up to 99 g kg^{-1} and 264 g kg^{-1} for the benthic crustaceans *Leptocheirus plumulosus* and *Hyaella azteca*, respectively, on exposures over 10 days. The LC_{50} for *L. plumulosus* was reported to be 68 (50 – 93) g kg^{-1} sediment for MWCNT. For *Hyaella azteca* the LC_{50} could not be determined (over the MWCNT concentration of 264 g kg^{-1} in sediment, the highest concentration tested).

Roberts *et al.* (2007) studied the interactions between *Daphnia magna* and a water-soluble, lysophosphatidylcholine coated single-walled carbon nanotubes. The authors observed peak survival in *Daphnia magna* at 0.5 mg l^{-1} whereas mortality of 20% and 100% were observed at 10 and 20 mg l^{-1} , respectively, after 96 hours of exposure.

Templeton *et al.* (2006) assessed how exposures to SWCNT at concentrations of 0 , 0.58 , 0.97 , 1.6 , and 10 mg l^{-1} , affected the development of the estuarine meiobenthic copepod *Amphiascus tenuiremis* by focussing on the different life-stages. Three microplate bioassays were performed to test the chronic toxicity of “as prepared” SWCNT (i.e., SWCNT after synthesis without prior purification), purified SWCNT, and the fluorescent nanocarbon byproduct fraction. Test periods for individual microplate bioassays ranged from 28 days to 35 days, to account for treatment specific developmental delays. An increasing average cumulative life-cycle mortality was observed with greater concentrations of the complex mixture of “as prepared” SWCNT up to 10 mg l^{-1} , where the mortality ($36 \pm 11 \%$) was significantly higher than in exposures to the control ($13 \pm 4\%$). At 10 mg l^{-1} they also found that the development success was reduced to 51% for the nauplius-to-copepodite stage and to 34% overall for the nauplius-to-adult period. Furthermore, the study showed a significantly lowered fertilisation rate, averaging only $64 \pm 13\%$ during 35 days of exposure to 10 mg l^{-1} SWCNT. Templeton *et al.* (2006) showed that the fluorescent byproduct fraction of nanocarbon was significantly more toxic than the “as prepared” SWCNT, and thus caused significant effects on the development at the lowest test concentration of 0.58 mg l^{-1} . Exposure to the purified SWCNT did not result in any negative effects in the concentrations up to 10 mg l^{-1} .

8.3.1.3 Toxicity of carbon nanotubes towards algae

No studies have been published in the scientific literature on this topic before 12 December 2008.

8.3.1.4 Toxicity of carbon nanotubes to other taxa

The toxicity of different carbon nanotubes have been tested on a range of different taxa (i.e. bacteria, protozoans, soil and sediment worms, terrestrial plants and amphibians).

Zhu *et al.* (2006b) observed a time- and dose-dependent growth inhibition and viability of the prokaryotic unicellular protozoan *Stylonychia mytilus* after up to 5 days of exposure to 95% pure MWCNT in concentrations ranging from 0.1 – 200 mg l^{-1} . After 5 days 53% survival was observed at a concentration of 10 mg l^{-1} and 43% survival at 50 mg l^{-1} . The MWCNT also contained 0.1% Ni, 0.2% Fe, and 0.39% N, but the effect of impurities cannot be evaluate based on the

information given in the paper. Zhu *et al.* (2006b) also observed damage to the macronucleus and external membrane of the cells when exposed to concentration above 1.0 mg l^{-1} .

Ghafari *et al.* (2008) observed four interrelated responses in the protozoan *Tetrahymena thermophila* on exposures to SWCNT after up to 72 hours of exposure to concentrations up to 17.2 mg l^{-1} . Suspended SWCNT were mostly individual or in small bundles or amorphous tangles with a length ranging from less than 100 nm to greater than 1000 nm, but predominantly less than 500 nm. Diameters ranged from 2 to 10 nm. SWCNT were observed to cause an initial cell aggregation of 5 to 50 cells and a slight and/or no mobility between 0–3 hours, whereas a recovery of cell mobility and movement out of the initial cell aggregates was observed after 3–12 hours exposure. After between 12–72 hours exposure an increased visibility of the matrix associated with the persisting aggregates was observed along with the appearance of dead cells. No loss of cell viability was observed after exposure to concentrations below 6.8 mg l^{-1} after 72 hours, even though initial cell aggregation could be observed. With increasing concentrations the size of aggregates increased, as well as loss of mobility and cell death. The lowest level of recovery of mobility occurred at the highest SWCNT concentrations maybe due to entrapment of cells in larger aggregates as shown in the supporting information to the paper, but not discussed by the authors. Ghafari *et al.* (2008) also observed that SWCNT had little effect on *E. coli*-green fluorescent protein (gfp) viability, but addition of SWCNT resulted in reduced bacterivory (the ingestion of bacteria as a food source). The decrease of bacterivory was the most pronounced effect after exposure to SWCNT, thus the ingestion of bacteria was partially affected at 3.6 mg l^{-1} SWCNT and completely blocked at 7.3 and 14.6 mg l^{-1} SWCNT exposures.

Petersen *et al.* (2008b) observed no increases in the mortality of the sediment dwelling oligochaeta *Lumbriculus variegatus* exposed to sediments spiked with radioactively-labelled (^{14}C) SWCNT and MWCNT at the tested concentrations and exposure durations of 28 days, when compared to unspiked sediments. Diameters of the MWCNT and SWCNT ranged from 30-70 nm and 1-2 nm, respectively, and the specific radioactivities were $0.122 \pm 0.004 \text{ mCi g}^{-1}$ and $1.35 \pm 0.03 \text{ mCi g}^{-1}$, respectively. *L. variegatus* were exposed to 0.003 and 0.03 mg SWCNT per gram of dry sediment or 0.037 and 0.37 mg MWCNT per gram of dry sediment, which had been dispersed by sonication in water prior to addition to the sediment.

Mouchet *et al.* (2008) investigated the effect of CNT on African clawed frog, *Xenopus laevis*, under static exposure conditions compared to air bubbling in test media. CNT were prepared in their laboratory and comprised a mixture of ca. 80% DWCNT, together with ca. 15% SWCNT and ca. 5% triple-walled carbon nanotubes. Based on Raman analysis the diameters of DWCNT were found to be in a range between 0.7-2.2 nm, whereas bundles of CNT had a diameter of 10-20 nm. Surface area of DWCNT was measured to be 800 and 900 $\text{m}^2 \text{ g}^{-1}$. Without added air in media a mortality of 85% and a dose-dependent size reduction was observed after 12 days of exposure to 500 mg l^{-1} of DWCNT. Abnormal behaviour was also observed after exposure to concentrations of 10, 100 and 500 mg l^{-1} . With air bubbling 15% mortality was observed after exposure to 10 mg l^{-1} whereas only 5% were observed after exposure to 100 and 500 mg l^{-1} . Dose-dependent size reduction and behavioural changes were observed for the two highest concentrations only (100 and 500 mg l^{-1}). Results showed no genotoxicity in erythrocytes of larvae exposed to DWCNT in water. Dissection of larvae revealed invasive black masses in the gills and in the digestive tract of larvae, particularly in the intestine. The gut seemed to be morphologically dystrophic and this effect seemed to be dose-dependent.

In a complementary study, Mouchet *et al.* (2007) observed no acute toxicity of a mixture of CNT, namely 80% DWCNT, 15 % SWCNT and ca. 5% triple walled CNT, to the salamander *Ambystoma mexicanum* exposed to concentrations ranging from 1-1000 mg l^{-1} . No genotoxicity was observed after exposure to concentrations of 500 and 1000 mg l^{-1} and levels of micronucleated erythrocytes were 8 and 9%, respectively.

Kang *et al.* (2008) compared the bacterial toxicity of commercially obtained MWCNT before and after physico-chemical modification via common purification and functionalisation routes, such as dry oxidation and acid treatment. These authors found no significant toxicity towards *Escherichia coli* K12 grown in LB medium after 60 minutes exposure to 20 mg l^{-1} of "As prepared"(AP)-MWCNT and "Dry oxidized" (DO)-MWCNT. Cell membrane damage was

assessed using a standard, fluorescence-based, nucleic acid assay, and the cell viability verified using a parallel test for cell metabolic activity. A percentage of inactivated cells of 13.6 ± 3.1 and $10.4 \pm 2.4\%$ were observed respectively for AP- and DO-MWCNT, however these effects were not significantly different from the control. AP- and DO-MWCNT had a diameter of 17 ± 9 and 20 ± 8 nm, respectively, whereas the length was reported to be 91 ± 21 and 84 ± 18 , respectively. "Acid treated" (AT)-MWCNT with a diameter of 17 ± 6 nm and a length of 77 ± 31 nm was found to exhibit significantly higher toxicity in cell membrane integrity inactivating $32.7 \pm 4.2\%$ of the cells. "Annealing" (AN)-MWCNT with a diameter of 21 ± 11 and a length of 82 ± 23 nm inactivated significantly $26.3 \pm 7.9\%$ of the cells whereas short-MWCNT (diameter 35 ± 20 nm, length 2.3 ± 0.6 nm) were found to significantly inactivate $28.7 \pm 3.8\%$ of the cells. Functionalised short and debundled MWCNT (f-MWCNT) with a diameter of 19 ± 7 and a length of 4.1 ± 3.7 nm were found to exhibit the highest rates of cytotoxicity with $41.6 \pm 3.7\%$ inactivated after 60 minutes exposure to 20 mg l^{-1} . The amount of amorphous carbon was found not to affect toxicity in *E. coli* and no correlation was found between catalytic metal contents and cell membrane damage.

Velzeboer *et al.* (2008) observed no effects on *V. fischeri* NRRL B-11177 after up to 30 minutes exposure to SWCNT suspensions of unknown concentration.

8.3.2 Terrestrial toxicity of carbon nanotubes

Scott-Fordsmand *et al.* (2008) observed no significant effect on the mortality of the earthworm *Eisenia veneta* after consuming dry food spiked with 0, 50, 100, 300 and 495 mg kg^{-1} DWCNT in dry food for up to 28 days. The purity of the DWCNT was 99.5% with a metal content of 0.4%, with the length and outer diameter of DWCNT reported to be 5-15 nm and 10-30 nm, respectively. A non-significant 20% reduction in growth was observed compared to control at the highest concentration and EC_{10} and EC_{50} were reported to be $94 \pm 45 \text{ mg kg}^{-1}$ and $> 500 \text{ mg kg}^{-1}$. Cocoon production was found to be significantly reduced by 60% at 495 mg kg^{-1} DWCNT in food, however, no clear concentration response pattern was observed in concentrations from 50- 495 mg kg^{-1} food. For reproduction $EC_{10, 28d}$ and $EC_{50, 28d}$ were reported to be $37 \pm 73 \text{ mg kg}^{-1}$ and $176 \pm 150 \text{ mg kg}^{-1}$ DWCNT in food, thus significant uncertainty on the EC-values was apparent. The hatchability was not affected at any exposure concentration.

In a study by Lin and Xing (2007) on phytotoxicity of MWCNT, effects on seed germination and root growth were investigated on six higher plant species. The MWCNT (purity >95%) were reported to have a diameter of 10-20 nm and lengths of 1.2 μm . No statistically significant effects on germination rates and root elongation were observed for seeds soaked in a suspension of 2000 mg l^{-1} MWCNT.

Cañas *et al.* (2008) studied the effect of functionalised and non-functionalised SWCNT on the root elongation of 6 crop species (cabbage, carrot, cucumber, lettuce, onion and tomato). The plants were exposed to SWCNT in concentrations up to 1750 mg l^{-1} and root growth was monitored during a period of 48 hours. The width of both types of SWCNT was approximately 8 nm, with a length between 200-2000 nm. Results showed that both SWCNT and functionalised SWCNT reduced the root growth of some crops, with the non-functionalised type causing more effects. However, no significant effect was observed on cucumber, lettuce, carrot or cabbage. Non-functionalised SWCNT reduced the root elongation of tomato but increased the root length of onion and cucumber. Functionalised SWCNT significantly ($p < 0.01$) reduced the root elongation of tomato (with considerable variation among replicates) as well as lettuce (in one out of two repeated tests). Both functionalised and non-functionalised SWCNT were found to attach to the surface of cucumber roots, forming a layer of varying thickness, often more pronounced around root hairs. Also adsorption onto secondary roots was seen. However no uptake into the inside of the roots was visible from cross-sections. The SWCNT layers on the roots might have the potential to cause effects on plant and root functions e.g., by changing surface chemistry, causing microbial toxicity or altering biochemical processes.

8.3.3 Bioaccumulation of carbon nanotubes

Ferguson *et al.* (2008) studied the bioaccumulation of xenobiotic organic chemicals in the presence of SWCNT in the copepod *Amphiascus tenuiremis* and the polychaete worm *Streblospio benedicti*. The organisms were exposed for 14 days, in the presence of sediment with addition of SWCNT (5 mg g⁻¹). A 14-days exposure of *S. benedicti* and *A. tenuiremis* to sediments with ¹⁴C-SWCNT did not result in detectable uptake into the tissues, however it was evidenced that *S. benedicti* ingested the SWCNT as worm faecal rods had similar content of radioactivity as the spiked sediments.

Petersen *et al.* (2008a) observed that bioaccumulation factors (BAF) of SWCNT (99±1% C) and MWCNT (91.1±0.2% C) by the earthworm *Eisenia foetida* were almost 2 orders of magnitude lower than those found for pyrene. SWCNT were 1-2 nm in diameter whereas MWCNT was reported to have a fishbone configurations consisting to 30-70 cylinders with an outer diameter commonly between 30 and 50 nm. Biological uptake of MWCNT after 14 days was higher than that for SWCNT in a soil with 5.5% organic carbon content (Chelsea soil) and lower in a soil with 1.7% organic carbon content (Ypsilanti soil). BAF in Chelsea soil was reported to be 0.016 ± 0.001 and 0.023 ± 0.01 after 14 days exposure to 0.03 and 0.3 mg g⁻¹ MWCNT uptake by *E. foetida*. In Ypsilanti soil BAF was found to be 0.014 ± 0.003 after exposure to 0.3 mg g⁻¹. BAF Chelsea soil was reported to be 0.0061 ± 0.002 and 0.0078 ± 0.005 after 14 days exposure to 0.03 and 0.1 mg g⁻¹ SWCNT whereas it was found to be 0.022 ± 0.003 after exposure to 0.3 mg g⁻¹ SWCNT in Ypsilanti soil. After 28 days, exposure was almost identical between SWCNT and MWCNT in Chelsea soil. No clear patterns were observed in regard to the depuration pattern of the SWCNT and MWCNT after exposure for 14 days and depuration for 1, 2 or 7 days. The authors state that no conclusion can be drawn on the differences in uptakes of SWCNT and MWCNT as the apparent differences observed may be related to the gut contents of the worms at the time of analysis.

Petersen *et al.* (2008b) exposed the sediment-dwelling oligochaete *L. variegatus* to SWCNT at concentrations of 0.003 and 0.03 mg g⁻¹ dry sediment or MWCNT of 0.037 and 0.37 mg g⁻¹ dry sediment, which had been dispersed by sonication in water prior to addition to the sediment. The biota-sediment accumulation factors (BSAFs) for *L. variegatus* were found to be almost an order of magnitude lower for SWCNT and MWCNT compared to pyrene, whereas there was no indications of any systematic differences between the SWCNT and MWCNT. BSAF values for worms exposed to sediments spiked with SWCNT, MWCNT, and pyrene for 28 days were 0.28 ± 0.03, 0.40 ± 0.1, and 3.6 ± 0.2, respectively. After reducing the organic carbon content by a factor of 8 a decrease in the BSAF value from 0.51 ± 0.09 to 0.035 ± 0.015 was observed after 14 days of exposure. Whereas decreasing by an order of magnitude the MWCNT and SWCNT concentrations initially added to the sediments did not significantly change the BSAF values. After 28 days of exposure and an initial 6 hours of depuration (to allow gut clearance), the worms were transferred to clean water. Here the organisms were observed to purge over 80% of single- or multiwalled nanotubes remaining after the initial 6 hours of depuration, while only 13% of the pyrene was excreted after the same interval. Concentrations of nanotubes detected in organisms were below background carbon concentration levels after two days of depuration in clean sediment dispersions in water and nanotubes were not observed to be attached to cellular tissues.

8.3.4 Degradability of carbon nanotubes

Only one paper refers to the possible degradation of SWCNT via enzymatic catalysis (Allen *et al.* 2008). These authors incubated SWCNT with a natural horseradish peroxidase (HRP) and low concentrations of H₂O₂ (40 µM) at 4°C over 12 weeks under static conditions, and results indicated degradation of nanotube structure. Allen *et al.* (2008) suggest that plant peroxidases may have a role in CNT degradation, which will be dependent on material type and physico-chemical conditions.

The study by Roberts *et al.* (2007) showed a biological modification of a water-soluble, lysophosphatidylcholine-coated SWCNT upon ingestion by *Daphnia magna*. Thereby the organisms decreased the solubility of the SWCNT, which was accounted for by the stripping of

the lysophosphatidylcholine from the particle surface. In the presence of *D. magna*, initial concentrations of 2.5-20 mg l⁻¹ were decreased by around 50%. Without *D. magna* the SWCNT remained in solution. This finding may be of importance for the evaluation of the fate of modified CNT after release to the environment.

8.3.5 Summary

Only a few ecotoxicological studies on the effects of CNT to aquatic species have been carried out. Until now there has not been a strong focus on taxa belonging to the base set of organisms used for risk assessment of chemicals (fish, crustacean, and algae). Only two studies have been carried out on fish and four studies on crustaceans. To date, no algal studies have been published. Several studies exist on other taxa (ranging from bacteria and protozoans to amphibians) but due to the high variability in these studies it is not possible to draw any common conclusion on the effects of CNT on this basis. It should however be noted that a number of studies do not find adverse effects after exposure to CNT in often very high concentrations.

For aquatic toxicity the findings of Smith *et al.* (2007) raise several concerns that need to be addressed by future studies. These findings indicate new modes of toxicity that have not been identified in fish before, i.e., subtle neurotoxic or cardiovascular effects of SWCNT that affect fish behaviour. Furthermore, the findings of cellular pathologies in the liver (which indicate genotoxicity or cell cycle defects) give rise to concerns whether carcinogenicity may be observed after long-term exposure to SWCNT (Smith *et al.* 2007).

So far only three studies have reported on the terrestrial toxicity of CNT. While one study finds no effects on seed germination and root growth after exposure to MWCNT at concentrations of up to 2000 mg l⁻¹ (Lin and Xing, 2007), another study found that addition of SWCNT significantly reduced the root elongation of tomato plants. However, this may be attributed to the very high exposure concentrations (up to 1750 mg l⁻¹) leading to a CNT attachment especially to root hairs (Cañas *et al.* 2008). In this study no decrease in root elongation was found for cabbage, carrot, cucumber, lettuce and onion. Dietary exposure to DWCNT resulted in EC_{50, 28d} values of 176±150 mg kg⁻¹ food for reproduction of earthworms (Scott-Fordsman *et al.* 2008). However, considering the large variability, this result is not likely to contribute to risk assessment of CNT in soil.

For worms living in soil and sediments, low BAF have been found for SWCNT and MWCNT. In soil the maximum BAF was 0.02 for *E. foetida* exposed to 0.03 and 0.3 mg g⁻¹ of MWCNT in soil for 14 days (Petersen *et al.* 2008a). In sediments it was observed that after two days of depuration in clean sediments CNT could not be detected in *L. variegatus* (Petersen *et al.* 2008b)

Further studies on the degradability of CNT are required, although indications of degradation mediated by plants peroxidases exist (Allen *et al.* 2008), as well as suggestions of biomodification of functionalised CNT have been reported by Roberts *et al.* (2007).

Testing difficulties in relation to obtaining, handling, purification and solubilisation are likely to have an influence in the very limited number of studies available for environmental risk assessment (i.e. ecotoxicity, persistency, and bioaccumulation).

8.4 METALS

Metal nanoparticles should not be considered as one group in terms of risk assessment and no general conclusion on the general ecotoxicity of metal nanoparticles can, or should be made. Just like "regular forms" of metals there are large differences in speciation, behaviour, fate and effects even in standardised test systems. The major part of the scientific literature published deals with effects of silver and copper nanoparticles. Therefore, the reviews below are separated into a specific section on the ecotoxicity of silver and copper nanoparticles and a section on the ecotoxicity of other metal nanoparticles reported in the scientific literature. Bioaccumulation and degradability will be addressed in separate sections covering all studies.

8.4.1 Ecotoxicity of silver nanoparticles

8.4.1.1 Toxicity of silver nanoparticles towards fish, crustaceans, and algae

Griffitt *et al.* (2008) reports on the toxicity of Ag nanoparticles in 48 hour static toxicity tests on adult and juvenile life stages of the zebrafish (*Danio rerio*), adult *Daphnia pulex*, *Ceriodaphnia dubia* neonates, and algae (*Pseudokirchneriella subcapitata*). Silver nanoparticles were found to be toxic to all organisms and life stages studied. Estimated LC₅₀-values for Ag nanoparticles ranged from 0.04 mg l⁻¹ for *D. pulex* to 7.2 mg l⁻¹ in juvenile *D. rerio*. An LC_{50, 48 h} of 7.07 (6.04-8.28) mg l⁻¹ was observed for adult *D. rerio* and 7.20 (5.9-8.6) mg l⁻¹ for juvenile *D. rerio*. LC_{50, 48h}-values for Ag nanoparticles towards *D. pulex* adults and *C. dubia* neonates were 0.040 (0.030-0.050) mg l⁻¹ and 0.067 mg l⁻¹, respectively. For the green algae *P. subcapitata* an EC_{50, 96h} of 0.19 mg l⁻¹ was found. Ag nanoparticles were reported to have a primary average particle size of 26.6 ± 8.8 nm whereas the average particle diameter in suspension was 44.5 nm. The specific surface area was 14.53 m² g⁻¹ and ζ-potential was -27 mV. Less than 1% by mass of the original dose of Ag nanoparticles was present in the dissolved form after 48 h.

In the study by Asharani *et al.* (2008), the toxicity of two types of silver nanoparticles on zebra fish was assessed. Using starch and bovine serum albumin (BSA) as capping agents, silver nanoparticles were synthesised and any effects on mortality, hatching, pericardial edema and heart rate recorded. The original particles had average sizes of 5-20 nm. The embryos were exposed to Ag nanoparticles in concentrations of 5-100 mg l⁻¹ for 72 hours. A concentration-dependent toxicity was observed. At a concentration of 5 mg l⁻¹ sub-lethal effects occurred, including slimy coating and nuclear staining of the embryos. An LC_{50,72h} of between 25-50 mg l⁻¹ was found, but varied depending on developmental stage. After exposure to Ag nanoparticles, severe phenotypic changes were observed in concentrations above 50 mg l⁻¹. Asharani *et al.* (2008) claimed that these changes were not due to release of Ag⁺ by comparing Ag nanoparticle results with exposures of embryos to Ag⁺ (added as AgNO₃), where no significant abnormalities were observed. However, since the exposure concentration to Ag⁺ was only 0.022 mg l⁻¹ the conclusion is not supported by the data presented in the paper. More information on the solubility of the Ag nanoparticles used in this study would be required in order to interpret the observed results.

Yeo and Kang (2008) observed abnormal notochord development after a 48 hour exposure of zebrafish embryos to 10 ppt and 20 ppt Ag nanoparticles. Silver nanoparticles were suspended in tap water and their size was reported to be 10-20 nm. After 72 hours of exposure almost all the individuals exposed to 10 and 20 ppt Ag nanoparticles had abnormal characteristics, including weak heart beats, edema and abnormal notochords. Dose-dependent hatching rates were also observed to be significantly decreased whereas the catalase activities increased for both exposure concentrations. At 20 ppt the increase was significantly different from the control. Gene expression was also found to decrease compared to the control group, but a statistical analysis is not provided in the paper.

Navarro *et al.* (2008) observed a time-dependent toxicity of carbonated coated Ag nanoparticles towards the green algae *Chlamydomonas reinhardtii*. EC₅₀ ranged from 0.355 mg l⁻¹ ± 0.062 mg l⁻¹ after 1 hour, to around 0.092 ± 0.011 mg l⁻¹ after 3-5 hours. Expressed as a function of free Ag⁺, EC₅₀ was estimated to range from 3.6 ± 0.5 µg l⁻¹ after 1 hour, to 0.9 ± 0.08 µg l⁻¹ after 5 hours. Particle size ranged between 10-200 nm, with 98% being within 25 ± 13 nm, and a ζ-potential of -36.6 ± 3.2 mV. In experiments where Ag nanoparticles were co-incubated with cysteine (in concentrations 10, 50, 100, and 500 nM) as a silver ligand, EC_{50, 1h} values was found to be 6.1 µg l⁻¹ and 6.6 µg l⁻¹, for 5 and 10 µM added respectively. Based on these experiments Navarro *et al.* (2008) concluded that the measured Ag⁺ in Ag nanoparticle suspensions could not explain the toxicity found. It is likely that nanoparticles contributed as a source for delivering the toxic Ag⁺ ion formed when algae interact with the Ag nanoparticles.

8.4.1.2 Toxicity of silver nanoparticles towards other organisms

Choi and Hu (2008) used microbial growth inhibition tests with nitrifying bacteria to study the effects of Ag nanoparticles of different sizes (9-21 nm). The effects of Ag nanoparticles were

compared to those of colloid AgCl and free Ag⁺ ions (added as AgNO₃) in Ag concentrations of 0.05-1 mg l⁻¹. Results from respirometry tests showed no correlation between nanoparticle size and bacteria inhibition. However, a correlation was found between inhibition and the fraction of nanoparticles with sizes less than 5 nm, indicating that this fraction might have a stronger effect on respiration than larger size fractions. Furthermore it was found that, at the same total concentration of Ag, Ag nanoparticles caused a greater inhibition than the free Ag⁺. It was found that intracellular ROS concentrations (measured by a fluorescence assay with dichlorodihydrofluorescein diacetate) but not photocatalytic ROS concentrations (measured using 3-(p-aminophenyl) fluorescein) could be correlated with inhibition, but that these correlations differed for all three Ag types (Ag nanoparticles, colloid AgCl and free ions).

A substantial part of the results presented by Choi *et al.* (2008) are the same as reported by Choi and Hu (2008). Besides the results for the autotrophic (nitrifying bacteria), heterotrophic (*E. coli*) bacterial growth was also included in the paper by Choi *et al.* (2008). Respirometry tests with nitrifying bacteria exposed to Ag concentrations of 1 mg l⁻¹ (9.34 µM) in the bacterial suspensions, showed significant inhibition by Ag nanoparticles, Ag⁺ ions, and AgCl colloids of 86 ± 3%, 42 ± 7%, and 46 ± 4%, respectively. For inhibition of heterotrophic growth, an IC₅₀ value for *E. coli* for Ag of 0.43 mg l⁻¹ (4 µM) was estimated for Ag nanoparticles. Ag⁺ was found to be more toxic to *E. coli* than Ag nanoparticles and AgCl colloids. In the tests with nitrifying bacteria attachment of Ag nanoparticles to the nitrifying bacterial cells was observed. Live/Dead assays showed that cell integrity was not affected. However, Choi *et al.* (2008) mention that the attachment of Ag nanoparticles to the cells might affect the cell membrane by creating holes or by interfering with ammonia oxidation enzymes.

8.4.1.3 Terrestrial toxicity of silver nanoparticles

No tests on terrestrial toxicity of silver nanoparticles were published in peer-reviewed journals before 12 December 2008.

8.4.2 Ecotoxicity of Cu nanoparticles

8.4.2.1 Aquatic toxicity of Cu nanoparticles

Griffitt *et al.* (2008) investigated the toxicity of nanosized Cu towards adult and juvenile life stages of the zebrafish (*Danio rerio*), adult *Daphnia pulex*, *Ceriodaphnia dubia* neonates, and algae (*Pseudokirchneriella subcapitata*). Cu nanoparticles were found to be toxic to both adult and juvenile zebrafish (*D. rerio*), adult *D. pulex*, *C. dubia* neonates, and algae (*P. subcapitata*). Estimated LC_{50, 48h} for Cu nanoparticles were found to be 0.94 (0.75-0.17) mg l⁻¹ and 0.71 (0.54-0.93) mg l⁻¹ for adult and juvenile *D. rerio*, respectively. LC_{50, 48h} of Cu nanoparticles towards *D. pulex* adults was reported to be 0.060 (0.050-0.070) mg l⁻¹ and for and *C. dubia* neonates an LC_{50, 48h} of 0.419 mg l⁻¹ was found. For *P. subcapitata* an EC_{50, 48h} of 0.54 mg l⁻¹ was found for Cu nanoparticles. Primary particle size distribution and the major particle diameter of Cu particles was 26.7 ± 7.1 and 216 nm, respectively, and the specific surface area was 30.77 m² g⁻¹ and the ζ-potential was -0.69 mV. Less than 1% by mass of the original amount of Cu was present in the dissolved form after 48 hours and 50% of added Cu nanoparticles had disappeared from the water column within 48 hours.

Griffitt *et al.* (2007) observed that Cu nanoparticles were toxic to adult female zebrafish (*Danio rerio*) with a LC_{50, 48h} of 1.56 mg l⁻¹ (95% CI 0.79 – 3.08 mg l⁻¹) whereas the LC_{50, 48h} for copper sulphate (used as positive control) was 0.25 mg l⁻¹ of Cu (95% CI 0.16-0.33 mg l⁻¹). In these concentration-response experiments 10 concentrations in the range from 0.75-12.5 mg l⁻¹ was used. Copper nanoparticles were found to have a size of 80 nm in filtered aquatic medium, although agglomerates greater than 1 µm in diameter were observed. Particle density, specific surface area and ζ-potential in deionized water were measured to be 8.96 g cm⁻³, 30.77 m² g⁻¹ and -0.69 mV, respectively. In studies to determine the influence of soluble copper Griffitt *et al.* (2007) exposed zebrafish to two concentration of Cu nanoparticles: 0.25 and 1.5 mg l⁻¹. It was found that “rapid aggregation of particles occurred after suspension in water, resulting in 50–60% of added mass leaving the water column”. Griffitt *et al.* (2007) observed a dose-dependent proliferation of epithelial cells and edema of primary and secondary gill filaments after 48 hours

exposure to Cu nanoparticle suspensions. These effects were significant at the high concentration (1.5 mg l^{-1}). Besides some eosinophilic vacuolation of the liver, no significant histological evidence of injury was observed in internal organs and there was no hepatocellular necrosis. Exposure to Cu nanoparticles decreased Na^+/K^+ -ATPase activity by 29 and 58% in the low (0.25 mg l^{-1}) and high (1.5 mg l^{-1}) Cu nanoparticle exposures, respectively, whereas high Cu nanoparticle exposure elevated plasma blood urea nitrogen levels, although this was not statistically significant. Plasma alanine aminotransferase was not affected in any exposures. An increased level of expression of all genes surveyed after exposure to higher levels of Cu nanoparticles when compared to copper sulphate exposed fish was also observed.

8.4.2.2 Terrestrial toxicity of Cu nanoparticles

Lee *et al.* (2008) observed that the seedling lengths of two terrestrial plants *Phaseolus radiatus* (mung bean) and *Triticum aestivum* (wheat) were negatively related to exposure concentration ($0\text{-}1,000 \text{ mg l}^{-1}$ Cu expressed as total Cu) of Cu nanoparticles for concentrations exceeding 48 mg l^{-1} . An adverse effect was also observed on the seedling growth of both plants. Lee *et al.* (2008) observed $\text{EC}_{50,48\text{h}}$ values for Cu nanoparticles of 335 mg l^{-1} (251-447) and 983 mg l^{-1} (908-1,064) on *P. radiatus* seedling and shoot growth, respectively. NOAEC and LOAEC were reported to be $<200 \text{ mg l}^{-1}$ and 200 mg l^{-1} for seedling growth and 800 mg l^{-1} and 1000 mg l^{-1} for shoot growth. An observed EC_{50} of 570 (450-722) mg l^{-1} was recorded for *T. aestivum* seedling growth. Concentrations used were: 200, 400, 600, 800, and 1000 mg l^{-1} . The amount of cupric ion released during Cu nanoparticle preparation was measured and found not to be toxic to the plants and even enhanced plant growth. Individual and aggregated particles were observed in the cytoplasm and cell wall of the root cell of both *P. radiatus* and *T. aestivum*.

8.4.3 Ecotoxicity of Al, Au, Co and Ni nanoparticles

Griffitt *et al.* (2008) investigated the toxicity of nanosized Al, Ni and Co towards adult and juvenile life stages of the zebrafish (*Danio rerio*), adult *Daphnia pulex*, *Ceriodaphnia dubia* neonates, and algae (*Pseudokirchneriella subcapitata*). The $\text{LC}_{50,48\text{h}}$ -values for Al, Co, Ni were greater than 10 mg l^{-1} for both adult and juvenile *D. rerio*. Nickel nanoparticles showed toxicity towards adult *D. pulex*, *C. dubia* neonates and *P. subcapitata* with $\text{LC}_{50,48\text{h}}$ values of 3.89 (95% CI 1.93-7.43) mg l^{-1} estimated for *D. pulex* and 0.674 mg l^{-1} (no 95% CI listed) for *C. dubia*. For *P. subcapitata* an $\text{EC}_{50,96\text{h}}$ of 0.35 mg l^{-1} (no 95% CI listed) was found. Nickel nanoparticles were reported to have an average primary particle size of $6.1 \pm 1.4 \text{ nm}$, whereas an average diameter of 446.1 nm in suspension was determined. The specific surface area was $50.56 \text{ m}^2 \text{ g}^{-1}$ and the ζ -potential was 21.9 mV .

For Al nanoparticles, Griffitt *et al.* (2008) found $\text{LC}_{50,48\text{h}}$ values of $> 10 \text{ mg l}^{-1}$ for *D. pulex* adults, 3.99 mg l^{-1} (no 95% CI listed) for *C. dubia* neonates and $\text{EC}_{50,96\text{h}}$ for *P. subcapitata* of 8.30 mg l^{-1} (no 95% CI listed). Aluminium nanoparticles were reported to have a average primary particle size distribution of $41.7 \pm 8.1 \text{ nm}$ with a larger average particle diameter of 4442 nm in suspension. The specific surface area was $27.26 \text{ m}^2 \text{ g}^{-1}$ and the ζ -potential was 18.2 mV .

Griffitt *et al.* (2008) showed that Co nanoparticles had $\text{LC}_{50,96\text{h}}$ values for *D. pulex* adults and *C. dubia* neonates of over 10 mg l^{-1} and 1.67 mg l^{-1} (no 95% CI listed), respectively. Cobalt nanoparticles were reported to have an average primary particle size of $10.5 \pm 2.3 \text{ nm}$ with a larger average particle diameter of 742 nm in suspension. The specific surface area was $36.39 \text{ m}^2 \text{ g}^{-1}$ and the ζ -potential was 17.8 mV .

Harper *et al.* (2008) investigated the effect of exposing *Danio rerio* embryonic zebrafish (AB strain) to 0.8 and 1.5 nm gold (Au) nanoparticles with three different surface groups: 2-(2-mercaptoethoxy)ethanol (MEE), N,N,N-trimethylammoniummethanethiol (TMAT) and 2-mercaptoethanesulfonate (MES) with neutral, positive and negative charge, respectively. These authors observed that Au-TMAT caused significantly higher cumulative mortality 120 hpf in embryonic zebrafish compared to Au-MES. Mortality was observed at 0.40 mg l^{-1} and 10 mg l^{-1} for 0.8 nm and 1.5 nm Au-TMAT particles, respectively. Though clear concentration-response relationships are shown for the Au-TMAT nanoparticles, the data presented in the paper do not allow for estimation of LC_{50} values. From their statistical analysis it can be concluded that NOEC and LOEC 120 hpf for 0.8 nm Au-TMAT are 0.08 mg l^{-1} and 0.4 mg l^{-1} , respectively and for the

1.5 Au-TMAT nanoparticles 2 mg l^{-1} and 10 mg l^{-1} , respectively. The other types of surface modified Au nanoparticles did not cause statistically significant mortality in concentrations up to 250 mg l^{-1} . Exposures were started at 8 hpf and morphological malformations were observed after exposure to 0.08 mg l^{-1} and 50 mg l^{-1} of 0.8 nm and 1.5 nm Au-TMAT particles, respectively. Au-MES did not cause increased mortality after exposure to concentrations above 250 mg l^{-1} , however 2 and 50 mg l^{-1} did result in an increased incidence of morphological malformations at 1.5 and 0.8 nm, respectively. Thus, the study by Harper *et al.* (2008) demonstrated that the 0.8 nm Au-nanoparticles with the N,N,N-trimethylammoniummethanethiol (TMAT) surface group attached is the most toxic of the three Au nanoparticles towards zebrafish embryos.

8.4.4 Bioaccumulation of metal nanoparticles

Oughton *et al.* (2008) investigated the dietary uptake and excretion of neutron-activated Co nanoparticles in earthworm *Eisenia fetida*, expressed as the radioactivity measured in kilo bequerels per gram (kBq g^{-1}). The average particle size of Co nanoparticles was $3.9 \pm 0.8 \text{ nm}$ and the surface area was reported to be $59.07 \pm 0.24 \text{ m}^2 \text{ g}^{-1}$. After 7 days of exposure to Co nanoparticles at an activity of $13.7 \pm 0.5 \text{ kBq g}^{-1}$ wet wt, corresponding to a concentration of $87 \pm 3 \text{ } \mu\text{g g}^{-1}$ horse manure, the concentration ratios of activity of assimilated Co nanoparticles in the worms: activity in horse manure ranged from 0.16 and 0.20 relative to the particle concentration in horse manure and equivalent to approximately $5 \text{ } \mu\text{g}$ Co nanoparticles per worm. After additional 60 hours of gut clearance in clean media, the activity levels of Co particles in the worms ranged from 2245 ± 598 to $2768 \pm 640 \text{ Bq g}^{-1}$ wet wt. After eight weeks of depuration, less than 20% of the absorbed Co was excreted. Radioactivity of Co particles was incorporated in all body parts sampled, including spermatogenic cells, cocoons and blood.

In the study by Lee *et al.* (2008) the bioaccumulation factors of Cu nanoparticles in *P. radiatus* (mung bean) and *T. aestivum* (wheat) were calculated to be 8 and 32 l kg^{-1} , respectively, when they were exposed to a very high Cu nanoparticle concentration of 1000 mg l^{-1} in growth media for 48 hours.

8.4.5 Degradability of metal nanoparticles

By definition metal nanoparticles are not degradable. However, changes in the metal speciation can occur depending on redox conditions, salt content etc. These changes in speciation are as complex as they are for conventional metal forms and no general conclusion can be made in this regard.

8.4.6 Summary

Metal nanoparticles should not be considered as one group in terms of risk assessment and no general conclusion on the general ecotoxicity of metal nanoparticles can, or should be made. Just like “regular forms” of metals there are large differences in speciation, behaviour, fate and effects even in standardised test systems. The major part of the scientific literature published deals with toxic effects of silver and copper nanoparticles and some general conclusions on the toxicity of these are listed below.

Only a very few studies have dealt with bioaccumulation of metal nanoparticles. However, accumulation of metal has to be of high concern when looking at past experiences with “regular” metal. By definition metals, and hence also metal nanoparticles, are not degradable. However, changes in the metal speciation can occur depending on redox conditions, salt content etc. These changes in speciation are as complex as they are for conventional metal forms and no general conclusion can be made in this regard. Also functionalisation of metal nanoparticles is an issue of high relevance for the effects of metal nanoparticles, but so far the number of studies is too limited to draw conclusion on the influence of functionalisation on ecotoxicity, speciation, and accumulation.

Silver is known as a highly ecotoxic metal. A range of studies with fish, crustaceans and algae confirms that also when silver is tested as Ag nanoparticles, low effect concentrations are

found. For fish and crustaceans the lowest reported $LC_{50, 48h}$ values are 7 mg l^{-1} (*Danio rerio*) and 0.040 mg l^{-1} (*Daphnia pulex*) (Griffitt *et al.* 2008). In the fish studies by Yeo and Kang (2008) exposure to 10 ppt Ag nanoparticles resulted in adverse effects. For algae, an $EC_{50, 5h}$ of 0.092 mg l^{-1} was found for *Chlamydomonas reinhardtii* (Navarro *et al.* 2008). For silver the issue of dissolution is crucial to understanding the mechanisms of ecotoxicity since toxic effects could be linked to the concentration of the free mono-valent silver ion. However, both the studies by Asharani *et al.* (2008) and Navarro *et al.* (2008) showed that higher effect levels, than those stemming from the free Ag^+ , were found for fish and algae, respectively. This was also found in for nitrifying bacteria in the studies by Choi and Hu (2008). Neither degradability nor bioaccumulation of Ag nanoparticles have been addressed in the literature published before 12 December 2008.

In terms of the ecotoxicity of copper nanoparticles, the study by Griffitt *et al.* (2008) provides evidence that Cu nanoparticles are highly toxic to fish, daphnids, and algae. The 50%-effect levels are below 1 mg l^{-1} , with a $LC_{50,48h}$ of 0.060 mg l^{-1} towards adult *Daphnia pulex* as the lowest reported effect value. However, Griffitt *et al.* (2007) found that copper sulphate was six times more toxic towards adult female zebrafish (*D. rerio*) than Cu nanoparticles, when comparing $LC_{50,48h}$ -values. It was found that aggregation and sedimentations significantly reduced the exposure concentration of Cu nanoparticles. For terrestrial plants, Lee *et al.* (2008) found high $EC_{50, 48h}$ values ($>300 \text{ mg l}^{-1}$) for seedling and shoot growth of mung beans (*P. radiatus*) and wheat (*T. aestivum*) when using very high exposure concentrations (from 200-1000 mg l^{-1}). A single study of bioaccumulation of Cu nanoparticles in plants has been reported (Lee *et al.* 2008). However, due to a very high exposure concentration (1000 mg l^{-1}), further studies are needed to make conclusions on the accumulation behaviour of Cu nanoparticles.

Ecotoxicity studies of nanoparticles of aluminium, gold, cobalt, and nickel have also been reported. However, the literature on these metals can best be described as extremely limited. In fact, only one study has dealt with aquatic toxicity of Al, Co, and Ni (Griffitt *et al.* 2008), one study focussed on the importance of Au nanoparticle-functionalisation for fish toxicity (Harper *et al.* 2008), and one study documented accumulation of Co nanoparticles in earthworms (Oughton *et al.* 2008). Nevertheless no general conclusion on the ecotoxicity or accumulation of these metals can be drawn based on these studies, though all three studies deal with important issues related to environmental effects of metal nanoparticles

8.5 METAL OXIDES

Metal oxide nanoparticles should not be considered as one group in term of risk assessment and as stated for the metals, no general conclusion on the ecotoxicity of metal oxide nanoparticles can, or should, be made. Just like conventional forms of metal oxides there are large differences in speciation, behaviour, fate and effects even in standardised test systems. The major part of the scientific papers published compare different metal oxide nanoparticles. Therefore, the reviews below are not separated into specific sections for ecotoxicity of each metal oxide nanoparticle, however to ease reading the individual metal oxides are indicated in bold.

8.5.1 Ecotoxicity toxicity of metal oxide nanoparticles

8.5.1.1 Toxicity of metal oxide nanoparticles towards fish

Federici *et al.* (2007) exposed rainbow trout (*Oncorhynchus mykiss*) to suspensions of Degussa P25 TiO_2 nanoparticles (average diameter 21nm) in concentrations between 0.1-1 mg l^{-1} in a semi-static test for a period of 14 days. The suspensions were prepared using sonication, and without the use of solvents. Concentrations of TiO_2 in fish tanks were measured by use of UV/VIS spectroscopy at a wavelength of 329nm, and were found to be close to nominal concentrations. In order to investigate the effects of TiO_2 , the following analysis were performed: haematology and blood plasma analysis, tissue ion (Ti, Zn, Cu, Mn, Ca, Na and K) analysis (in gill, liver, muscle and brain), and histological observations of gill filaments, intestine, liver, spleen, a transverse section of the body (including kidney and bone section) and the brain. The results of the tests showed that TiO_2 did not cause mortality in the exposed rainbow trout at the

tested concentrations (up to 1 mg l^{-1}). However, fish exposed to $0.1 \text{ mg l}^{-1} \text{ TiO}_2$ for 14 days showed gill pathologies and mucus secretion, indicating respiratory toxicity. Furthermore changes in levels of Cu and Zn in the tissue were measured, as well as decreases in Na^+K^+ -ATPase activity in gills and intestine. Increase of thiobarbituric acid reactive substances (TBARS) in gill, intestine and brain indicated oxidative stress, despite unaffected/increased levels of levels of total glutathione in these organs. Histology of the brain indicated biochemical disturbances, which might be of importance in case of longer exposure times.

Vevers *et al.* (2008) observed varying levels of DNA strand breakage in rainbow trout (*Oncorhynchus mykiss*) gonadal tissue cells after 24 hours of exposure to TiO_2 nanoparticles under UVA (2.25 kJ m^{-2}) in minimal essential medium (MEM). Particle mean diameter was reported to be $24.4 \pm 0.5 \text{ nm}$ with a minimum of 11.8 nm and a maximum of 38.5 nm . Using the Comet assay a significantly higher response was observed when initially exposed in H_2O compared to MEM and PBS (phosphate buffered saline). The potentiating effects of UVA irradiation were observed across the different media used, with the maximum response observed in MEM, followed by H_2O and PBS. An overall increase in DNA strand breaks was observed following incubation of the nucleoids with formamidopyrimidine DNA glycosylase (Fpg), but only the highest concentration of $50 \text{ mg l}^{-1} \text{ TiO}_2$ nanoparticles in combination with 3.0 kJ m^{-2} UVA, produced significant ($p < 0.001$) damage. No significant media-dependent (MEM, distilled H_2O and PBS) difference in micronuclei induction was observed when compared to the MEM control. A decrease in the frequencies of micronucleus was observed after TiO_2 nanoparticle treatments. For TiO_2 nanoparticles suspended in MEM or PBS significant cytotoxic effects were observed after 24 hours when exposed to the highest dose of TiO_2 only (50 mg l^{-1}). These effects were observed both in the presence and the absence of UVA excitation (3 kJ m^{-2} , 40 minutes exposure time).

Zhu *et al.* (2008a) observed no significant effect compared to control on the survival rate of zebrafish embryos and larvae after 96 hour of exposure to $>99.5\%$ pure spindle shaped TiO_2 and $>99.9\%$ pure Al_2O_3 nanoparticles in concentrations up to 500 and 1000 mg l^{-1} , respectively. Similar observations were made for bulk TiO_2 and Al_2O_3 . Published size, actual size range in suspension and actual mean size in suspension were reported to be ≤ 20 , $100\text{-}550$, and 230 nm for TiO_2 nanoparticles and 80 , $285\text{-}2450$, and 930 nm for Al_2O_3 , respectively. No significant effect was observed on the hatching rate of zebrafish embryos exposed to nanosized or bulk Al_2O_3 and TiO_2 for 96 hours even after exposure to the highest concentration. No significant malformations on zebrafish embryos and larvae were observed for nano or bulk TiO_2 and Al_2O_3 .

Zhu *et al.* (2008a) observed a 96 hpf dose-dependent decrease in survival rates of zebrafish (*Danio rerio*) embryos or larvae after exposure ZnO nanoparticles in concentrations of 0 , 0.1 , 0.5 , 1 , 5 , 50 mg l^{-1} . The ZnO nanoparticles formed irregularly shape aggregates in water suspensions. Reported purity was reported to be $> 99.9\%$ and the published size, actual size range in suspension and actual mean size in suspension was 20 nm , $50\text{-}360 \text{ nm}$, and 180 nm for ZnO nanoparticles, respectively. In concentrations $\leq 0.5 \text{ mg l}^{-1}$ no toxicity towards zebrafish embryos or larvae was found, whereas no survival was observed after exposure to 50 mg l^{-1} . $\text{LC}_{50, 96\text{h}}$ for ZnO nanoparticles was found to be 1.793 mg l^{-1} (95% CI, $1.498\text{-}2.145 \text{ mg l}^{-1}$) for zebra fish embryos. A similar concentration-dependent relationship was observed for bulk ZnO. Zhu *et al.* (2008a) also observed a concentration-dependent effect on the hatching rates of zebrafish embryos 84 hpf exposed to ZnO nanoparticles. No significant effect was observed 84 hpf after exposure to $\leq 0.5 \text{ mg l}^{-1}$ ZnO nanoparticles. $\text{EC}_{50, 84\text{h}}$ for the hatching rate of zebrafish was estimated to be 2.065 mg l^{-1} (95% CI, $1.687\text{-}2.529 \text{ mg l}^{-1}$) for ZnO nanoparticles and 2.066 mg l^{-1} (95% CI, $1.472\text{-}2.897 \text{ mg l}^{-1}$) for bulk ZnO.

Harper *et al.* (2008) investigated the toxicity of various metal oxides towards *Danio rerio* embryos (AB strain) through three different routes of exposure, i.e., dermal, ingestion and microinjection. These included positively charged Al_2O_3 , TiO_2 , ZrO_2 , Gd_2O_3 , Dy_2O_3 , Ho_2O_3 , Sm_2O_3 , Er_2O_3 , and negatively charged Y_2O_3 , $\text{SiO}_2/\text{Al}_2\text{O}$ (alumina doped SiO_2), and CeO_2 . TiO_2 , CeO_2 , Dy_2O_3 , Sm_2O_3 and Er_2O_3 were found to form large aggregates. After 5 days of waterborne oral and dermal exposure at concentrations ranging from 0.016 mg l^{-1} and 250 mg l^{-1} , a significant mortality was observed at 50 mg l^{-1} for Er_2O_3 and Sm_2O_3 and at 250 mg l^{-1} for Ho_2O_3 and Dy_2O_3 . Significant morphological malformations were observed after exposure to Er_2O_3 , Sm_2O_3 and Dy_2O_3 at concentrations of 10 , 50 , and 250 mg l^{-1} , respectively.

Exposure to Sm_2O_3 caused a significant increase in the incidence of jaw, heart, eye, and snout malformations at 50 mg l^{-1} . Exposure to $\text{SiO}_2/\text{Al}_2\text{O}_3$ resulted in a significant incidence of jaw malformations at 250 mg l^{-1} . At 10 mg l^{-1} , ErO_2 exposure elicited jaw malformations in 40% of embryos after 5 days. Exposure to 50 mg l^{-1} Er_2O_3 significantly increased the incidence of jaw, heart, snout, trunk, and body axis malformations. DyO_3 exposure significantly affected the jaw and eyes at 250 mg l^{-1} . Embryonic exposure to Y_2O_3 significantly increased the incidence of jaw malformations at 10 mg l^{-1} and the incidence of jaw and heart malformations of embryos to 250 mg l^{-1} . After microinjection of Sm_2O_3 and Y_2O_3 into 8 hpf embryos, morphological malformations were observed after 120 hpf, whereas no significant morbidity or mortality was observed for any metal oxide after injection of nanoparticles at a dose of 0.5 ng g^{-1} body weight.

8.5.1.2 Toxicity of metal oxide nanoparticles towards crustaceans

Adams *et al.* (2006) reported the effects of three different types of metal oxide nanoparticles (ZnO , TiO_2 and SiO_2) on the freshwater crustacean *Daphnia magna*. The nanoparticles were tested in concentrations between $1\text{-}20 \text{ mg l}^{-1}$ (TiO_2 and SiO_2 nanoparticles) or $0.2\text{-}1 \text{ mg l}^{-1}$ ZnO nanoparticles. The duration of the test was 8 days. No concentration–response relationships were established, but it was found that ZnO nanoparticles were the most toxic of the three particle types. Concentrations of 0.5 and 0.2 mg l^{-1} resulted in 100% and 73% mortality, respectively. SiO_2 concentrations of 10 mg l^{-1} were found to cause 70% mortality, whereas 20 mg l^{-1} TiO_2 caused 40% mortality.

Hund-Rinke and Simon (2006) studied the effect of TiO_2 nanoparticles on the immobilisation of *Daphnia magna*. The immobilisation after exposure to $1\text{-}3 \text{ mg l}^{-1}$ after 48 hours was found to be concentration dependent, however with high standard deviations. Pre-illumination seemed to increase toxicity towards daphnids.

Griffitt *et al.* (2008) tested the toxicity of TiO_2 nanoparticles on *D. pulex* adults and *C. dubia* neonates after 48 hours of exposure. Titanium dioxide nanoparticles were reported to be 20% rutile and 80% anatase and have a primary particle size distribution of $20.5 \pm 6.7 \text{ nm}$ whereas the major particle diameter was 687.5 nm in suspension. The specific surface area was $45.41 \text{ m}^2 \text{ g}^{-1}$ and the zeta-potential was -25.1 mV . LC_{50} of TiO_2 nanoparticles towards *D. pulex* adults, *Ceriodaphnia dubia* neonates was reported to be more than 10 mg l^{-1} after 96 hours.

Velzeboer *et al.* (2008) observed no measurable effects on *Chydorus sphaericus* exposed to a concentration of 100 mg l^{-1} TiO_2 , Al_2O_3 , CeO_2 nanoparticles and tap water for 48 hours.

In 48 hours tests with *Daphnia magna* Heinlaan *et al.* (2008) found $\text{LC}_{50, 48\text{h}}$ values to be 3.2 ± 1.3 (2.6 ± 1.04), 3.2 ± 1.6 (2.6 ± 1.3) and $\sim 2000 \text{ mg l}^{-1}$ for ZnO , CuO , and TiO_2 nanoparticles respectively, and the NOEC was found to be 0.5 mg l^{-1} nominal concentration (or 0.4 mg l^{-1}) for ZnO and 50.0 mg l^{-1} nominal concentration (or 40.0 mg l^{-1}) for CuO nanoparticles. NOEC for TiO_2 could not be established. $\text{LC}_{50, 24\text{h}}$ for *Thamnocephalus platyurus* was found to be $0.18 \pm 0.03 \text{ mg l}^{-1}$ ($0.14\text{-}0.02$) for ZnO , $2.1 \pm 0.5 \text{ mg l}^{-1}$ (1.7 ± 0.4) for CuO , and $> 2000 \text{ mg l}^{-1}$ for TiO_2 particles and the NOEC was found to be $0.05(0.04)$, $0.5(0.4)$ and over 2000 mg l^{-1} for ZnO , CuO , and TiO_2 respectively.

8.5.1.3 Toxicity of metal oxide nanoparticles towards algae

Wang *et al.* (2008) observed no significant differences compared to control in the growth of *Chlamydomonas reinhardtii* after up to 5 days of exposure to TiO_2 nanoparticles in concentrations $\leq 1 \text{ mg l}^{-1}$ (concentrations used: 0.001 , 0.01 , 0.1 , 1 , 10 , 100 mg l^{-1}) The initial size of the TiO_2 nanoparticles was 21 nm and they had a surface area of $50 \text{ m}^2 \text{ g}^{-1}$. The median diameter of the particles increased over time in buffer solution with the median size of TiO_2 nanoparticles aggregations at 48 hours measured as 892 nm . Reduction in growth was observed at the end of day one and growth was completely inhibited on day three for exposure concentrations of 10 and 100 mg l^{-1} . After day 3, growth resumed and was found to be 80% of the control on day five. Growth inhibition was significant on day two and three. $\text{EC}_{50, 72\text{h}}$ was found to be 10 mg l^{-1} . A significant increase in malondialdehyde (MDA) level compared with the control was measured 6 hours and 12 hours after exposure to 10 and 100 mg l^{-1} TiO_2

nanoparticles, respectively, following which MDA content declined.

Aruoja *et al.* (2008) used the green algae *Pseudokirchneriella subcapitata* to study the growth related effects of the metal oxide nanoparticles ZnO, TiO₂ and CuO. Also the reasons for the observed effects were investigated. EC₅₀ values as well as NOEC values were determined. For ZnO, the toxicity of bulk and nanoparticles were found to be similar (EC₅₀ values were not statistically significantly different) and toxicity was attributed to dissolved Zn²⁺. TiO₂ and CuO nanoparticles were found to be more toxic than the corresponding bulk particles. The reasons for this were believed to be the entrapping of algal cells by particle aggregates (TiO₂ nanoparticles) and increased metal bioavailability (CuO nanoparticles). The following EC_{50,72h} values were determined for the soluble salts, nano and bulk particles: 0.04 mg l⁻¹ Zn (added as ZnSO₄), 0.037 mg l⁻¹ Zn (bulk ZnO), 0.042 mg l⁻¹ Zn (nano ZnO), 35.9 mg l⁻¹ Ti (bulk TiO₂), 5.83 mg l⁻¹ Ti (TiO₂ nanoparticles), 0.02 mg l⁻¹ Cu (added as CuSO₄), 11.55 mg l⁻¹ Cu (bulk CuO) and 0.71 mg l⁻¹ Cu (nano CuO). NOEC values were found to be ~0.02 mg l⁻¹ Zn for both bulk and ZnO nanoparticles, 0.42 mg l⁻¹ Cu (CuO nanoparticles) and 8.03 mg l⁻¹ Cu (bulk Cu), 0.98 mg l⁻¹ Ti (TiO₂ nanoparticles) and 10.1 mg l⁻¹ Ti (bulk TiO₂).

Hund-Rinke and Simon (2006) assessed the growth inhibition of the algae, *Desmodesmus subspicatus*, exposed to 25 nm anatase TiO₂ nanoparticles in concentrations ranging from 0 to 50 g l⁻¹ and observed that it was concentration-dependent with an EC_{50,72h} of 44 mg l⁻¹, (95 % CI 30-94 g l⁻¹). No difference was observed between 25 nm and 100 nm size TiO₂ particles and pre-illumination was found not to influence the growth of algae.

Velzeboer *et al.* (2008) observed no measurable effects on the green alga *Pseudokirchneriella subcapitata* when exposed to 100 mg l⁻¹ TiO₂, ZrO₂, Al₂O₃, CeO₂ nanoparticles for 4.5 hours.

Franklin *et al.* (2007) compared the toxicity of sonicated ZnO nanoparticles with sonicated ZnO nanoparticles in the presence of a surfactant (nonylphenol etoxylate) to the freshwater green algae *Pseudokirchneriella subcapitata* after 72 hours of exposure to Zn at concentrations of between 25-600 µg l⁻¹. Sonicated ZnO nanoparticles ranged from 180–360 nm and were near-spherical to ellipsoidal in shape whereas the same nanoparticles were 100–400 nm when in the presence of a surfactant. The observed inhibition effects could be attributed to dissolved Zn(II) originating from the ZnO nanoparticles, and IC_{50,72h} expressed as the dissolved Zn was 68 (61–76) for sonicated ZnO and 44 (36–62) µg l⁻¹ Zn for sonicated ZnO in the presence of the surfactant.

Fujiwara *et al.* (2008) observed a size-dependent, but non-linear growth inhibition of *Chlorella kessleri* after 96 hour of incubation with spherical SiO₂ nanoparticles. IC_{50,96h} was found to be 0.8 ± 0.6, 7.1 ± 2.8 and 9.1 ± 4.7 weight/volume % for 5, 26 and 78 nm particles, respectively. SiO₂ nanoparticles at concentrations of less than 1% did not show any effect, whereas adverse effects were observed after exposure to a concentration of 0.5% of 78 nm SiO₂ nanoparticles. The size of cells increased in the presence of SiO₂ nanoparticles as evidenced by flow cytometry, an effect that was higher for 5 nm particles than for 26 and 708 nm particles. The authors ascribe this effect to obstruction of cell division since coagulation of cells was observed after exposure to 5 nm SiO₂ nanoparticles.

Van Hoecke *et al.* (2008) observed a EC_{20, 72h} (growth rate) of *Pseudokirchneriella subcapitata* of 20.0 ± 5.0 mg l⁻¹ and 28.8 ± 3.2 mg l⁻¹ after exposure to commercial LUDOX[®] suspensions of 12.5±0.2 and 27.0±0.5 nm SiO₂ nanoparticles. Test vessels were incubated with concentrations of nanoparticles ranging from 2.2 to 460 mg l⁻¹ for 72 hours under continuous illumination of 70 µE/[m².s]. No aggregation was observed and dissolution of the nanoparticles was negligible and the observed effects were attributable to the solid nanospheres of a specific surface area of 236 and 135 m² g⁻¹ SiO₂ for 12.5 and 27.7 nm SiO₂ nanoparticles, respectively. EC_{10, 72h} was found to be 10.9 ± 4.4 and 15.0 ± 4.3 mg l⁻¹ for 12.5 nm and 27.7 nm SiO₂ nanoparticles, respectively. Expressed as a surface area, mean EC_{10, 72h} values were 2.6 ± 1.0 and 2.0 ± 0.6 m² l⁻¹, and EC_{20, 72h} values were 4.7 ± 1.2 and 3.9 ± 0.4 m² l⁻¹ for 12.5 and 27.0 nm nanoparticles, respectively. Bulk silica material was found to be non-toxic up to 1 g l⁻¹. A clear dose-response relationship was observed when expressed both by mass and surface area, and a clear plateau was observed at concentrations of 200 mg l⁻¹ which was independent of nanoparticle size. NOEC and LOEC were observed to be 4.6 and 10.0 mg l⁻¹, respectively, for both 12.5 and 27.0

nm nanoparticles. Expressed in surface area, NOEC and LOEC were 1.09 and 2.36 $\text{m}^2 \text{l}^{-1}$ for 12.5 nm nanoparticles and 0.62, and 1.35 $\text{m}^2 \text{l}^{-1}$ for 27 nm particles. When expressed as mass a significant difference was observed in the growth rate of algae when exposed to small and larger particles ($p = 0.0019$ and 0.0352) for EC_{20} and EC_{10} , respectively, whereas this difference was not significant when expressed in surface area ($p=0.0904$ and 0.1522) for EC_{20} and EC_{10} . Nanoparticles were observed to adhere to the outer cell surface, but there was no evidence of particle uptake and no significant changes in shape or cell morphology were noted after 96 hours.

8.5.1.4 Toxicity of metal oxide nanoparticles towards other organisms

Heinlaan *et al.* (2008) observed not acute toxicity of TiO_2 nanoparticles towards *Vibrio fischeri* after 30 minutes exposure to concentrations from 0.5 to 20,000 mg l^{-1} . $\text{EC}_{50, 30\text{min}}$ for *Vibrio fischeri* was reported to be 1.9 ± 0.2 (1.5 ± 0.16), 79 ± 27 (63 ± 22), $> 20,000 \text{ mg l}^{-1}$ for 50–70 nm ZnO, ~30 nm CuO, and 25–70 nm TiO_2 , respectively. NOECs of 0.75(0.6), 16(12), $> 2000 \text{ mg l}^{-1}$ were reported for ZnO, CuO, TiO_2 nanoparticles, respectively.

Mortimer *et al.* (2008) tested the effects of ZnO (particle size 50–70 nm) and CuO (particle size 30 nm) on *Vibrio fischeri* (strain NRRL-B-11177) using the Flash Assay performed in cuvettes and microplates, and Microtox® tests. Mortimer *et al.* (2008) observed a concentration-dependent effect towards *V. fischeri* after 30 minutes of exposure. Compared to bulk CuO, CuO nanoparticles were found to be 20-fold more toxic in microplates and 60-fold in Flash Assay performed in cuvettes and microplates. The 30-min EC_{50} values were reported to be 68.1 ± 4.3 and $204 \pm 42 \text{ mg l}^{-1}$ in cuvettes and microplates, respectively. Zinc oxide nanoparticle exposures were found to result in 30-min EC_{50} values of 4.8 ± 1.1 and $3.9 \pm 1.8 \text{ mg l}^{-1}$ in Flash Assays performed in microplates and cuvettes, respectively. No toxic effect was observed for CuO and ZnO assessed via the Microtox® test at the maximum concentration studied.

Velzeboer *et al.* (2008) observed no measurable effects on *V. fischeri* NRRL B-11177 after a 30 minute exposure to TiO_2 , ZrO_2 , Al_2O_3 , or CeO_2 nanoparticles at concentrations of 1, 10 and 100 mg l^{-1} . Titanium dioxide nanoparticles were found to be between 50 and 150 nm in a 10% (w/w) TiO_2 suspension.

Thill *et al.* (2006) observed aggregation and sedimentation of the *Escherichia coli* wild strain RR1 after 1 hour of exposure to 1.2–37 mg l^{-1} of 7 nm CeO_2 nanoparticles in 0.1 M KNO_3 buffer. Particles had a positive surface charge (at neutral pH), consisted of ellipsoidal monocrystallites and had a surface area of 400 $\text{m}^2 \text{g}^{-1}$. These authors also observed a concentration-dependent decrease in the percentage of colony forming units. A 50 % decrease was observed at 5 mg l^{-1} and no survival was observed in exposures at concentrations above 230 mg l^{-1} CeO_2 nanoparticles. In contrast, the presence of particles in the LB growth medium did not influence the growth of the cells when exposed to up to 500 mg l^{-1} in exposures tests ranging from 1–5 hours.

Stoimenov *et al.* (2002) observed that 4 nm MgO polyhedral shaped nanoparticles with a surface area of 600 $\text{m}^2 \text{g}^{-1}$ killed *Escherichia coli* strain C3000 and *Bacillus megaterium* strain ATCC14581 completely after 20 min. Spores were observed to be less sensitive with a mortality of 36% and 48% after 20 and 60 min, respectively. MgO doped with Cl_2 and Br_2 (MgO/Cl_2 and MgO/Br_2) had a significant effect on the viability of spores varying from 61 to 98% after 20 and 60 minutes, respectively. From TEM images it was observed that the MgO/Cl_2 and MgO/Br_2 nanoparticles penetrated the cells and damaged the cell membranes of *E. coli*. Similar effects were observed for MgO nanoparticles, but to a lesser extent. At an ionic strength of 0.01 NaCl, MgO/Br_2 , MgO/Cl_2 and MgO had a zeta-potential of 27.0 mV, 33.0 mV and 35.2 mV, respectively.

Huang *et al.* (2008) observed a concentration-dependent decrease in the number of *Streptococcus agalactiae* and *Staphylococcus aureus* colonies after overnight incubations at concentrations of up to 0.12 M PVA-coated ZnO nanoparticles. The ZnO nanoparticles were synthesised in an EG aqueous system, and were reported to be rodlike with around 150 nm in average size. A concentration of 0.12 M ZnO caused more than 95% inhibition of bacterial growth, whereas concentrations between 0.0012 and 0.006 M ZnO caused about 30%

inhibition. Furthermore observed damage and integrity loss in *Streptococcus agalactiae* cell walls were observed as well as cell penetration of nanoparticles and membrane damage.

Adams *et al.* (2006) studied the effects of three different types of metal oxide nanoparticles (ZnO, TiO₂ and SiO₂) on gram positive (*Bacillus subtilis*) and gram negative (*Escherichia coli*) bacteria. Antibacterial assays were carried out using nanoparticle suspensions with concentrations of 10 – 5000 ppm and incubation overnight. The study found that the antibacterial effects increased with increasing particle concentration, and that the effects differed for the three particle types with ZnO being the most toxic, followed by TiO₂ and SiO₂. A higher degree of effect was observed for *B. subtilis* than for *E. coli*. Even though three different particle sizes were tested for each of the three material types, no effect of primary particle size was observed. This was believed to be due to aggregation of the particles in the media. An interesting discovery from this study is that antibacterial effects also occurred under dark conditions, indicating that other mechanisms besides photocatalytic ROS are responsible for the effects.

The cytotoxicity of maghemite ($\gamma\text{Fe}_2^{\text{III}}\text{O}_3$), magnetite ($\text{Fe}_3^{\text{II/III}}\text{O}_4$), and zerovalent iron (ZVI) nanoparticles towards gram negative bacteria *E. coli* was investigated by Auffan *et al.* (2008). The bacteria were incubated overnight at 37°C in nanoparticle concentrations between 7-700 mg l⁻¹. In order to obtain stable suspensions the pH in these experiments was adjusted to 5-5.5. Due to some inhibitory effect of this lower pH value, compared to the standard physiological conditions, the results of the toxicity tests were normalized to the number of bacterial colonies in control replicates incubated at pH 5.5. A dose-dependent toxicity was observed for magnetite nanoparticles and ZVI nanoparticles between concentrations of 70 and 700 mg l⁻¹. In contrast, maghemite did not cause toxicity. Tests with a double mutant strain of *E. coli* (sodA sodB) showed higher toxicity. This mutant does not have the iron and manganese superoxide dismutase (SOD) enzymes, which are antioxidant enzymes responsible for defending the cells against oxidative stress. Hence, the increased toxicity in these mutant strains is thought to indicate that at least some of the effects are linked to ROS generation. No evidence of internalisation of nanoparticles by the bacteria was found, but strong adherence of the nanoparticles onto the bacteria cell walls was observed. As a result of this other modes of toxicity, including disruption of cell membrane integrity and electron/ionic transport chains, are also possible explanations for the observed effects. The shape of magnetite and ZVI nanoparticles were found to change when incubated with bacteria, whereas this was not the case for maghemite nanoparticles.

Brayner *et al.* (2006) studied ZnO nanoparticles synthesised in di(ethylene glycol) using different molecules as a template during the forced hydrolysis reaction, resulting in ZnO nanoparticles of different shapes (spherical, cubic, rods) and with different sizes and size distributions (broad or narrow). ZnO particles prepared without the use of molecule template had diameters of 10.8 ± 2.2 nm, and were found to inhibit growth of *E. coli* (85% growth reduction at 1.7x10⁻³ M ZnO, 100% growth reduction at 3.4x10⁻³M ZnO). It was found that di(ethylene glycol), used in the particle synthesis, did not reduce bacterial growth. However, *E. coli* exposed to di(ethylene glycol) showed changes in cell morphology as well as cell wall damage and loss of cell integrity. When cells were exposed to ZnO (14 ± 0.9 nm, 10⁻³M) that had been synthesised in di(ethylene glycol) these types of damage were also observed, leading to increased cell permeability. This indicates that di(ethylene glycol) is a confounding factor in the assessment of these results.

8.5.2 Metal oxide nanoparticles tested in a biotest battery

The study by Blaise *et al.*(2008) covers a range of metal-containing nanoparticles (CuZnFe₄O₄, NiZnFe₂O₄, Y₃Fe₅O₁₂, TiO₂, SrFe₁₂O₁₉, (In₂O₃)_{0.9}(SnO₂)_{0.1}, SmO₃, Er₂O₃, Ho₂O₃) and two carbon based nanoparticles (C₆₀ and SWCNT). Ecotoxicological tests were carried out on organisms of different trophic levels, including bacteria (*Vibrio fischeri*), algae (*Pseudokirchneriella subcapitata*), crustacea (*Thamnocephalus platyurus*), cnidaria (*Hydra attenuate*) and fish (*Oncorhynchus mykiss*). Additional assays were carried out (Luminotox and Microbial Array for Risk Assessment). A large variation in aggregation was observed, with 13.8-100% of the particles being larger than 220nm in size, depending on material type. A large variation in effects were seen for the 11 nanoparticle types and different test assays. All nanoparticle types

were found to be harmful, toxic or very toxic in at least one of the test assays. However, $Y_3Fe_5O_{12}$, $SrFe_{12}O_{19}$ and C_{60} appeared to be less toxic than the others with effects concentrations over 10 mg l^{-1} . The nanoparticles with lowest effect concentrations ($0.1\text{-}1\text{ mg l}^{-1}$) were found to be $CuZnFe_2O_3$, $NiZnFe_2O_3$, Sm_2O_3 and Ho_2O_3 . The toxicity responses were found to span over three orders of magnitude.

Based on the MARA assay, a cluster analysis was carried out to compare the 'toxic fingerprints' of the different nanoparticle types with regards to their effects on 11 different microbial species. The result indicated similar modes of toxic action for $SrFe_{12}O_{19}$ and $NiZnFe_2O_3$.

By mixing the nanoparticles with a certified reference material (a naturally-contaminated sediment) in a 1:1 ratio, it was found that five of the nanoparticle types resulted in increased sediment toxicity of the certified reference material as well as its elutriate toxicity.

8.5.3 Terrestrial toxicity of metal oxide nanoparticles

8.5.3.1 Toxicity of metal oxide nanoparticles towards soil invertebrates

Jemec *et al.* (2008) observed no significant effects on the feeding rate, defecation rate, food assimilation efficiency, weight change, and mortality of the woodlouse *Porcellio scaber* after 3 days of exposure to TiO_2 nanoparticles through the ingestion of hazelnut tree leaves (*Corylus avellana*) treated with non-sonicated and sonicated TiO_2 nanoparticles in concentrations of 1000, 2000, and 3000 $\mu\text{g g}^{-1}$ leaf. Enzyme activities of both CAT and GST were, however, found to be reduced at 2000 and 3000 $\mu\text{g g}^{-1}$ leaf of non-sonicated TiO_2 , but not for sonicated TiO_2 nanoparticles. This effect was found not to be concentration-dependent, although enzymic activity was observed to decrease after exposure to concentrations as low as 0.5 $\mu\text{g g}^{-1}$ leaf after 3 days of exposure.

8.5.3.2 Toxicity of metal oxide nanoparticles towards terrestrial plants

Zheng *et al.* (2005) observed that treatment with rutile TiO_2 nanoparticles significantly increased the germination rate, the germination index, the dry weight of single seedling, and the vigour index of aged spinach (*Spinacia oleracea*) seeds. The treatment included nanoparticle suspensions at concentrations of 0-6.0‰ for 48 hours at 15°C, under illumination with natural light. Spinach growth (fresh weight) was greatly improved when compared to control and dry weights were both significantly higher after exposure to 0.25-4‰ TiO_2 nanoparticles. The photosynthetic rate and the chlorophyll content of spinach plants increased when treated with TiO_2 nanoparticle concentrations of 0.25-4‰, whereas it greatly decreased above 4‰. In contrast, larger sized TiO_2 particles did not have significant effects. Possible explanations for these effects proposed by Zheng *et al.* (2005) include the photocatalytic effect of these particles, which resulted in antimicrobial effects, which resulted in increased strength and resistance to stress.

Yang *et al.* (2007) observed that soaking of spinach seeds in 0.25% TiO_2 nanoparticles for 48 hours along with spraying of spinach seedlings with 0.25% TiO_2 nanoparticles caused an increase in the leaf area, as well as an 86.14% and 88.32 % increase in fresh and dry weight of spinach, respectively, after 35 days of growth in Hoagland solution. Fresh and dry weights of treated spinach were 3.7 and 2.0 times higher, respectively, than the control after 35 days of growth in N-deficient Hoagland solution. Titanium dioxide nanoparticles were reported to be anatase and have an original average diameter of 5 nm. The total nitrogen of spinach increased by 23.35% in Hoagland solution and increased 2.03 times in N-deficient Hoagland solution, although the total nitrogen of seeds was found to be the same. $NH_4^+ -N$ of treated spinach were increased by 2.39% in Hoagland solution and increased 17.51% in N-deficient Hoagland solution. TiO_2 also increased the oxygen-evolving rate of spinach chloroplast significantly by 43.41% in Hoagland medium, however it was inhibited by 85.61% in N-deficient Hoagland solution. The chlorophyll content of spinach was found to increase by 37.48% in Hoagland solution, whereas it was inhibited by 31.81% in N-deficient Hoagland solution. Finally, the protein content was found to be enhanced by 17.55% in Hoagland solution, whereas a 31.77% reduction was observed in N-deficient Hoagland solution.

Lin and Xing (2008) investigated the toxicity of 99.5% pure ZnO nanoparticles and Zn²⁺ ions (added as ZnSO₄) to ryegrass seedlings in bulk nutrient and rhizosphere solution. Zinc oxide nanoparticles were initially observed to be near spherical and cuboid in shape, have a mean size of 19 ± 7 nm and a surface area 58 m² g⁻¹. In bulk nutrient and rhizosphere solution the size of the ZnO aggregates was reported to be 900 ± 300 and 500 ± 80 nm, respectively, and the ζ-potential was measured to be -1.8 ± 0.2 mV and -2.9 ± 0.3 mV, respectively. Test material was added to medium one hour before being planted and experiments lasted 12 days. Independent of the test concentration, seedling growth was observed to be retarded with shorter roots and shoots, especially in concentrations of ZnO nanoparticles or Zn²⁺ higher than 50 mg l⁻¹, although symptoms seemed more severe after exposure to Zn²⁺ than for ZnO nanoparticles. The threshold of Zn²⁺ for both shoot and root of ryegrass was ca. 20 mg l⁻¹, whereas it was around 10 and 50 mg l⁻¹ of ZnO nanoparticles for ryegrass shoots and roots, respectively. A concentration-dependent decrease in seedling biomass was observed for both ZnO nanoparticles and Zn²⁺ treated ryegrass and the 50% biomass inhibitory concentrations (IC₅₀) were estimated to be ca. 64 mg l⁻¹ for ZnO nanoparticles and ca. 38 mg l⁻¹ for Zn²⁺.

Zhu *et al.* (2008c) observed no toxicological effects or visual difference compared to the control in the growth of pumpkin plants (*Cucurbita maxima*) after plants had been grown for 20 days in an aqueous medium containing 5 g l⁻¹ magnetite (Fe₃O₄) nanoparticles. Magnetite nanoparticles of 20 nm, as well as agglomerates of various sizes (up to 2 μm), existed in the suspension, and particle surface possessed a slightly negative charge (-7 to -9 mV). Whereas no magnetisation signal was detected from the control plants, strong magnetic signals (>1.0 memu g⁻¹) were detected in all leaf specimens and much weaker signals from the stem tissue samples. The strongest magnetisation (3.26 memu g⁻¹) was detected right above the roots, which might be due to nanoparticle agglomeration. When grown in sand, reduced but measurable levels of magnetic signals were detected in all tissues of pumpkin plants ranging from <0.1 memu g⁻¹ in most tissues to 0.4–1.2 memu g⁻¹ in leaves and stems. No magnetic signal was observed when pumpkin plants were grown in soil. No magnetic signal was detected in any parts of lima bean plants (*Phaseolus limensis*) grown hydroponically in a Fe₃O₄ nanoparticle suspension.

In a study by Lin and Xing (2007) the phytotoxicity of five types of nanoparticles were investigated, namely MWCNT, Al₂O₃, ZnO, Al and Zn. Effects on seed germination and root growth were investigated for six higher plant species. Low or no toxic effects were observed on exposures to Al₂O₃ and MWCNT at the tested concentrations. Three different exposures were investigated: 1) seed soaking and incubation in nanoparticle suspensions, 2) seed soaking in nanoparticle suspension followed by incubation in water, and finally 3) soaking in water followed by incubation in nanoparticle suspension. It was found that alumina nanoparticles affected root elongation of lettuce and ryegrass. Furthermore, the phytotoxicity of Zn and ZnO was evident with IC₅₀ values for growth inhibition at 50 mg l⁻¹ for radish and 20 mg l⁻¹ for rape and ryegrass for both Zn and ZnO.

Yang and Watts (2005) studied the effects of alumina nanoparticles on root elongation of five higher plants. Only at the highest test concentration of alumina nanoparticles (2 mg l⁻¹) did the suspensions show statistically significant effects on root elongation. Results showed that the phytotoxicity of alumina decreased when the nanoparticles were loaded with a 10 or 100 % monomolecular layer of phenanthrene. The study also shows that, as phenanthrene loading increased, the phenanthrene loaded particles showed increased inhibitory effects. The increased inhibition exceeded the effect that the corresponding concentration of phenanthrene (0.28 mg l⁻¹) would have on plant growth, indicating possibly increased bioavailability of phenanthrene attached to the particles.

Furthermore effects of sub-μm alumina particles on root growth were investigated by the same authors to determine if the effect was caused by the chemical composition (Al₂O₃) rather than particle size. It was found that sub-μm particles did not have any detectable effect on root growth - whether or not they were loaded with phenanthrene.

This result has subsequently been commented by Murashov (2006), who expressed the view that increased toxicity of nanoscale alumina, compared to microscale particles, can be expected due to increased dissolution of alumina.

8.5.3.3 Toxicity of metal oxide nanoparticles towards soil bacteria

Velzeboer *et al.* (2008) observed no measurable effects of TiO₂, Al₂O₃, CeO₂ nanoparticles on a mix of soil bacteria up to 7 days after incubation for 24 hours with 100 mg l⁻¹ of TiO₂ nanoparticles.

8.5.4 Bioaccumulation of metal oxide nanoparticles

No studies on the bioaccumulation of metal oxide nanoparticles have been found in the refereed scientific literature published before 12 December 2008. However, a couple of studies have focused on the potential carrier effect of engineered nanoparticles for other contaminants. These studies are described below.

The effect of the presence of TiO₂ nanoparticles (Degussa P25) on the accumulation of cadmium in carp (*Cyprinus carpio*) was studied by Zhang *et al.* (2007). The results were compared to the effects in the presence of natural sediment particles and combined with sorption studies. Results of this work indicate that TiO₂ nanoparticles have a stronger sorption capacity for Cd than the natural soil particles and that the presence of the TiO₂ nanoparticles greatly enhanced Cd accumulation in carp (total whole body BCF was 9.4 times higher when compared to that in the absence of TiO₂ nanoparticles). It was found that Cd sorbed onto the TiO₂ nanoparticles and accumulated in the carp together with the accumulation of TiO₂. Compared to the scenario with an absence of TiO₂ the largest increase in BCF in the presence of TiO₂ occurred in the muscles (1026% increase in BCF) as well as in the viscera (internal organs, including the intestine, showed 688% increase in BCF). The increase in BCF in the skin/scale and gills was 170% and 372%, respectively. Since this study does not include transfer of test animals into clean media there is a possibility that the increase in BCF in the presence of TiO₂ compared to Cd alone (841% increase) is over estimated. This increase may partly reflect the presence of TiO₂ with the sorbed Cd and the increase in Cd concentration could be partly reversible through excretion and desorption of TiO₂ from the intestine, skin and scale, respectively. However, the increase in muscle BCF indicates actual uptake. Not only the accumulation of Cd but also the accumulation of TiO₂ was also measured. The measured accumulation of TiO₂ corresponded well with the accumulation of Cd. Zhang *et al.* (2007) is one of the first studies to show that not only the fate and toxicity of nanoparticles themselves should be investigated, but also in association with other chemicals. In order to estimate the potential risks of nanoparticle exposure the facilitated transport of other chemical compounds should also be taken into account.

Sun *et al.* (2007) investigated the accumulation of arsenic (As) in *Cyprinus carpio* after co-exposure of As and 21 nm TiO₂ nanoparticles in dechlorinated tap water. Titanium dioxide nanoparticle were found to aggregate at around 50–400 nm and As aqueous concentrations were reported to be 150.0 ± 7.6 µg l⁻¹ in the presence of TiO₂ due to sorption. Concentration in the carp increased sharply in the presence of TiO₂ nanoparticles and reached 6.86 µg g⁻¹ after 25 days exposure to As (200.0 ± 10.2 µg l⁻¹) and TiO₂ (10.0 ± 1.3 mg l⁻¹). The increase was 132% compared to accumulation of As without TiO₂ nanoparticles. The BCF were reported to be 55.6 and 22.67, in the presence and the absence of TiO₂ nanoparticles. Accumulation of TiO₂ nanoparticles in carp was observed, with the TiO₂ concentration in carp reaching 4.95 mg g⁻¹ on day 10. As and TiO₂ were observed to accumulated in intestine, stomach and gills of the fish, and in the muscle to a lower extent, and TiO₂ nanoparticles did not change the distribution of As. After 20 days of exposure, As concentration in liver and muscle of carp increased by 80% and 126% in the presence of TiO₂ nanoparticles.

8.5.5 Persistence

By definition metal oxide nanoparticles are not degradable. However, changes in the metal speciation can occur depending on redox conditions, salt content etc. These changes in speciation are as complex as they are for traditional forms of metals and no general conclusion can be made in this regard.

8.5.6 Summary

Metal oxide nanoparticles should not be considered as one group in term of risk assessment and again no general conclusion on the ecotoxicity of metal oxide nanoparticles can, or should be made. Just like conventional forms of metal oxides there are large differences in speciation, behaviour, fate and effects even in standardised test systems. When going through the literature, it is apparent that the major part of the scientific papers published compare different metal oxide nanoparticles. While this may be important in terms of hazard ranking and benchmarking, it is of limited relevance when it comes to actual application of nanoparticles in products. This is due to the fact that substitution of a toxic nanoparticle with a less toxic one only is possible if the two nanoparticles have similar beneficial properties for the product (SiO₂ will for instance not be a potential substitute for TiO₂ in sunscreens). However, the data produced for each individual nanoparticles are of high importance for establishing ecotoxicity dossiers for risk assessment purposes.

While a number of toxicity tests have been carried out with metal oxide nanoparticles, no studies focussed specifically on bioaccumulation have been described in the literature published before 12 December 2008. For TiO₂ nanoparticles a carrier effect has however been observed in bioaccumulation of cadmium and arsenic in fish (Zhang *et al.* 2007; Sun *et al.* 2007) indicating that the bioavailability of other contaminants may be affected by the presence of TiO₂ nanoparticles. Both these studies also demonstrate an accumulation of TiO₂ in different parts of the carp, with highest concentrations detected in the viscera. Lower concentrations were found in skin, scales and muscles.

As mentioned above, general conclusions on metal oxide ecotoxicity are hampered by the large diversity of materials. However, based on the review above, it was found that for three individual types of metal oxide (TiO₂, ZnO and SiO₂) a number of trends could be outlined:

Titanium dioxide nanoparticles are among the most frequently tested nanoparticles in ecotoxicological tests. Thus, tests are available for the whole range of base set organisms (fish, crustaceans, and algae) and for a number of other species. However, the properties of the tested TiO₂ nanoparticles (e.g. size, crystallinity, surface coating) differ from study to study. Comparisons between different types of TiO₂ may therefore not be valid. While a number of studies find low or no effects of TiO₂ (e.g. Hund-Rinke and Simon 2006; Adams *et al.* 2006; Griffith *et al.* 2008; Heinlaan *et al.* 2008; Jemec *et al.* 2008), the results of Federici *et al.* (2007), showed that fish exposed to 0.1 mg l⁻¹ TiO₂ (P25) for 14 days showed signs of respiratory toxicity (as evidenced by gill pathologies and mucus secretion). Histological examination of the brain of exposed fish indicated biochemical disturbances at this relatively low exposure concentration. For algae (*P. subcapitata*) an EC_{50,72h} of 5.83 mg l⁻¹ was found by Aruoja *et al.* (2008). Since TiO₂ is an effective photo-catalyst, a number of studies have investigated the influence of light on the toxicity response of TiO₂. Vevers *et al.* (2008) found that UVA irradiation of TiO₂ increased the DNA strand breakage in gonadal tissue cells of rainbow trout (*O. mykiss*) when exposed to 50 mg l⁻¹ TiO₂ nanoparticles for 24 hours. However, also significant cytotoxicity was observed at this concentration and this may have influenced the results of the Comet assay. Hund-Rinke and Simon (2006) did not observe any effects of pre-illumination of TiO₂ in algal tests and Adams *et al.* (2006a) found that antibacterial effects also occurred under dark conditions (cell death was less pronounced under dark compared to light conditions). Therefore photocatalytic production of ROS cannot alone be responsible for the inhibition observed and additional modes of action for TiO₂ nanoparticles remain to be elucidated.

As it is the case for silver nanoparticles, dissolution of zinc is one of the major issues addressed in the studies of ZnO nanoparticles. For ZnO nanoparticles Zhu *et al.* (2008a) found hatching and survival of zebrafish embryos to be affected with an LC_{50, 96h} of 1.79 mg l⁻¹. However, the effect levels were not different from results obtained with bulk ZnO. For crustaceans, Heinlaan *et al.* (2008) found an LC_{50, 48 h} value of 3.2 mg l⁻¹ ZnO nanoparticles and a NOEC of 0.5 mg l⁻¹ for *Daphnia magna*. In the same study, it was found that the LC_{50,24h} for *Thamnocephalus platyurus* was 0.18 mg l⁻¹ and the NOEC 0.05 mg l⁻¹. In the studies of algal toxicity of ZnO nanoparticles, bulk ZnO and ZnSO₄ no statistically significant difference in EC₅₀-values (expressed as Zn mg l⁻¹) for *P. subcapitata* could be observed (Aruoja *et al.* 2008). In accordance with this, Franklin *et al.* (2007) concluded that the toxicity of ZnO nanoparticles to *P.*

subcapitata was due to dissolved zinc. Thus, in contrast to what was found for silver nanoparticles, the toxicity of zinc oxide nanoparticles seems to be equivalent to that of the released free ion (in this case Zn^{2+}).

The aquatic toxicity of SiO_2 has only been addressed by a two studies of growth inhibition of algae. For the freshwater green algae *Pseudokirchneriella subcapitata*, van Hoecke *et al.* (2008) reported $EC_{10, 72h}$ -values of 10.9 mg l^{-1} and 15.0 mg l^{-1} for 12.5 nm and 27.7 nm SiO_2 nanoparticles, respectively. For both particle types the NOEC was 4.6 mg l^{-1} and LOEC was 10 mg l^{-1} . In the study by Fujiwara *et al.* (2008) effect concentrations for the inhibition of *Chlorella kessleri* were in the same range for 5 nm SiO_2 nanoparticles with an $IC_{50, 96h}$ of 8 mg l^{-1} . Effect concentrations were significantly higher for 26 nm and 78 nm SiO_2 nanoparticles with $IC_{50, 96h}$ -values of 71 mg l^{-1} and 91 mg l^{-1} , respectively.

8.6 CONCLUSIONS

8.6.1 Ecotoxicity of fullerenes

Since 2004, a range of studies have been carried out with aquatic species and C_{60} . However, in total less than ten studies have been carried out on fullerene toxicity towards the base-set organisms used in the REACH risk assessment procedures for chemicals (fish, crustacean and algae). More studies are available using bacterial groups and, though they do not report the findings in traditional ecotoxicological endpoints, these studies may be of value for mechanistic interpretations of fullerene ecotoxicity in both the aquatic and the terrestrial environment.

Initial studies used different solvents to suspend C_{60} , but more recent studies have avoided the use of any solvents since it has been demonstrated that not only C_{60} /solvent interactions may affect toxicity, but also solvent degradation products may be responsible for some of the observed effects.

For fish, the studies by Zhu *et al.* (2008b) resulted in a NOEC of 0.04 mg l^{-1} and a LOEC of 1 mg l^{-1} , in terms of reduced lengths and body weights, after 32 days of exposure. In the study by Usenko *et al.* (2008) an $LC_{50, 96h}$ of 0.19 ppm can be proposed from their results, however in this study the stock solutions of C_{60} were prepared in pure DMSO and the solvent concentration in the tests were as high as 1% (vol/vol).

Less information is available for crustaceans: the only reported LC_{50} -value is the $LC_{50, 48h}$ of 7.9 ppm for sonicated C_{60} found by Lovern and Klaper (2006). They also observed a LOEC of 0.45 mg l^{-1} and a NOEC of 0.18 mg l^{-1} in the 48-h acute toxicity test for the sonicated suspensions of C_{60} . In a long-term study with *Daphnia magna*, Oberdörster *et al.* (2006) reported a LOEC of 2.5 mg l^{-1} for the number of offspring after 21 days for water stirred C_{60} , but the validity of this result is questionable due to too high mortality in the exposed organisms.

There is not enough data for other invertebrates and primary producers on the effects of fullerenes to draw any conclusions for these taxa.

In the three studies published on the terrestrial toxicity of fullerenes significant effects were only reported by Johansen *et al.* (2008) who found that exposure to $50 \text{ } \mu\text{g g}^{-1}$ yielded a three- to four-fold inhibition of the number of bacterial CFUs in clay loam soil 3 hours after incorporation of 99.5% pure C_{60} aggregates.

Major knowledge gaps are identified within the literature regarding persistence and bioaccumulation of fullerenes since no structured studies, aimed to investigate this, have been reported in the reviewed literature.

8.6.2 Ecotoxicity of carbon nanotubes

Only a few ecotoxicological studies of the effects of CNT to aquatic species have been carried out. Until now there has not been a strong focus on taxa belonging to the base set of organisms used for risk assessment of chemicals (fish, crustacean, and algae). Only two studies have

been carried out on fish and four studies on crustaceans. To date, no algal studies have been published. Several studies exist on other taxa (ranging from bacteria and protozoans to amphibians) but due to the high variability in these studies it is not possible to draw any common conclusion on the effects of CNT on this basis. It should however be noted that a number of studies do not find adverse effects after exposure to CNT in often very high concentrations. Nevertheless, given the large variation in types of MWCNT and SWCNT, making extrapolation to all CNT inappropriate.

For aquatic toxicity the findings of Smith *et al.* (2007) raise important concerns that need to be addressed by future studies. These findings indicate new modes of toxicity that have not been identified in fish before, i.e. subtle neurotoxic or cardiovascular effects of SWCNT that affect fish behaviour. Furthermore, the findings of cellular pathologies in the liver (which indicate genotoxicity or cell cycle defects) give rise to concerns regarding whether carcinogenicity may be observed after long-term exposure to SWCNT (Smith *et al.* 2007).

So far only three studies have reported on the terrestrial toxicity of CNT. While one study finds no effects on seed germination and root growth after exposure to MWCNT at up to 2000 mg l⁻¹ (Lin and Xing 2007), another study found that addition of SWCNT significantly reduced the root elongation of tomato plants. However, this may be attributed to the very high exposure concentrations (up to 1750 mg l⁻¹) leading to a CNT attachment, especially to root hairs (Cañas *et al.* 2008). In this study no decrease in root elongation was found for cabbage, carrot, cucumber, lettuce and onion. Dietary exposure to DWCNT resulted in EC_{50,28d} values of 176±150 mg kg⁻¹ food for reproduction of earthworms (Scott-Fordsman *et al.* 2008). However, considering the large variability, this result is not likely to contribute to risk assessment of CNT in soil.

For worms living in soil and sediments low BAF have been found for SWCNT and MWCNT. In soil the maximum BAF was 0.02 for *E. foetida* exposed to MWCNT at 0.03 and 0.3 mg g⁻¹ soil for 14 days (Petersen *et al.* 2008a). In sediments it was observed that after two days of depuration in clean sediments CNT could not be detected in *L. variegates* (Petersen *et al.* 2008b)

There is very little information on the biodegradation of CNT and this should be further addressed in the future.

Testing difficulties in relation to obtaining, handling, purification and solubilisation are likely to have an influence in the very limited number of studies available for environmental risk assessment (i.e. ecotoxicity, persistency, and bioaccumulation).

8.6.3 Ecotoxicity of metal nanoparticles

Only very few studies have dealt with bioaccumulation of metal nanoparticles. However, accumulation of metals is a topic of high concern when looking at past experiences with “regular” metals. By definition metals, and hence also metal nanoparticles, are not degradable. However, changes in the metal speciation can occur depending on redox conditions, salt content etc. These changes in speciation are as complex as they are for conventional metal forms and no general conclusion can be made in this regard. Also functionalisation of metal nanoparticles is an issue of high relevance for the effects of metal nanoparticles, but so far the number of studies is too limited to draw conclusion on the influence of functionalisation on ecotoxicity, speciation, and accumulation.

8.6.3.1 Ecotoxicity of Silver nanoparticles

Silver is known as a highly ecotoxic metal. A range of studies with fish, crustaceans and algae confirms that also when silver is tested as Ag nanoparticles, low effect concentrations are found. For fish and crustaceans the lowest reported LC_{50, 48h} values are for Ag concentrations of 7 mg l⁻¹ (*Danio rerio*) and 0.040 mg l⁻¹ (*Daphnia pulex*), respectively (Griffitt *et al.* 2008). In the fish studies by Yeo and Kang (2008) exposure to 10 ppt Ag nanoparticles resulted in adverse effects. For algae, an EC_{50,5h} of 0.092 mg l⁻¹ was found for *Chlamydomonas reinhardtii* (Navarro *et al.* 2008). For silver the issue of dissolution is crucial to understanding the

mechanisms of ecotoxicity since toxic effects usually can be linked to the concentration of the free mono-valent silver ion. However, both the studies by Asharani *et al.* (2008) and Navarro *et al.* (2008) show that higher effect levels than those stemming from the free Ag^+ , were found for fish and algae, respectively. This was also found for nitrifying bacteria in the studies by Choi and Hu (2008). Neither degradability nor bioaccumulation of Ag nanoparticles has been addressed in the literature published before 12 December 2008.

8.6.3.2 Ecotoxicity of Copper nanoparticles

The study by Griffitt *et al.* (2008) provides evidence that Cu nanoparticles are highly toxic to fish, daphnids, and algae. The 50%-effect levels are below 1 mg l^{-1} , with a $\text{LC}_{50,48\text{h}}$ of 0.060 mg l^{-1} towards adult *Daphnia pulex* as the lowest reported effect value. However, Griffitt *et al.* (2007) found that copper sulphate was six times more toxic towards adult female zebrafish (*D. rerio*) than Cu nanoparticles, when comparing $\text{LC}_{50,48\text{h}}$ -values. It was found that aggregation and sedimentation significantly reduced the exposure concentration of Cu nanoparticles. For terrestrial plants, Lee *et al.* (2008) found high $\text{EC}_{50, 48\text{h}}$ values ($> 300 \text{ mg l}^{-1}$) for seedling and shoot growth of mung beans (*P. radiatus*) and wheat (*T. aestivum*) when using very high exposure concentrations (from 200-1000 mg l^{-1}).

A single study of bioaccumulation of Cu nanoparticles in plants has been reported (Lee *et al.* 2008). However, due to a very high exposure concentration (1000 mg l^{-1}) further studies are needed to make conclusions on the accumulation behaviour of Cu nanoparticles.

In relation to the ecotoxicity of other metal nanoparticles studies of aluminium, gold, cobalt, and nickel nanoparticles have been reported. However, the literature on these metals can best be described as extremely limited. In fact, only one study has dealt with aquatic toxicity of Al, Co, and Ni (Griffitt *et al.* 2008), one study focussed on the importance of Au nanoparticle-functionalisation for fish toxicity (Harper *et al.* 2008), and one study documented accumulation of Co nanoparticles in earthworms (Oughton *et al.* 2008). No general conclusion on the ecotoxicity or accumulation of these metals can be drawn based on these studies, though all three studies deal with important issues related to environmental effects of metal nanoparticles

8.6.4 Ecotoxicity of metal oxide nanoparticles

Analysis of the literature indicates that the major part of the scientific papers published compare different metal oxide nanoparticles. While this may be important in terms of hazard ranking and benchmarking, it is of limited relevance when it comes to actual application of nanoparticles in products. This is due to the fact that substitution of a toxic nanoparticle with a less toxic one only is possible if the two nanoparticles have similar beneficial properties for the product (SiO_2 will for instance not be a potential substitute for TiO_2 in sunscreens). However, the data produced for each individual nanoparticles are of high importance for establishing ecotoxicity dossiers for risk assessment purposes.

While a number of toxicity tests have been carried out with metal oxide nanoparticles, no studies focussed specifically on bioaccumulation have been described in the literature published before 12 December 2008. For TiO_2 nanoparticles a carrier effect has however been observed in bioaccumulation of cadmium and arsenic in fish (Zhang *et al.* 2007; Sun *et al.* 2007) indicating that the bioavailability of other contaminants may be affected by the presence of TiO_2 nanoparticles. Both of these studies also demonstrate an accumulation of TiO_2 in different parts of the carp, with highest concentrations detected in the viscera. Lower concentrations were found in skin, scales and muscles.

As mentioned above, general conclusions on metal oxide ecotoxicity are hampered by the large diversity of materials. However, based on the review above, it was found that for three individual types of metal oxides (TiO_2 , ZnO and SiO_2) a number of trends could be outlined:

8.6.4.1 Ecotoxicity of titanium dioxide

Titanium dioxide nanoparticles are among the most frequently tested nanoparticles in ecotoxicological tests. Thus, tests are available for the whole range of base set organisms (fish, crustaceans, and algae) and for a number of other species.

However, the properties of the tested TiO₂ nanoparticles (e.g. size, crystallinity, surface coating) differ from study to study. Comparisons between different types of TiO₂ may therefore not be valid. While a number of studies find low or no effects of TiO₂ (e.g., Zhu *et al.* (2008); Hund-Rinke and Simon (2006); Adams *et al.* (2006); Griffitt *et al.* (2008); Heinlaan *et al.* (2008); Jemec *et al.* (2008)), the results of Federici *et al.* (2007), showed that fish exposed to 0.1 mg l⁻¹ TiO₂ (P25) for 14 days showed signs of respiratory toxicity (as evidenced by gill pathologies and mucus secretion). Histological examination of the brain of exposed fish indicated biochemical disturbances at this relatively low exposure concentration. For algae (*P. subcapitata*) an EC_{50,72h} of 5.83 mg l⁻¹ was found by Aruoja *et al.* (2008). Since TiO₂ is an effective photocatalyst, a number of studies have investigated the influence of light on the toxicity response of TiO₂. Vevers *et al.* (2008) found that UVA irradiation of TiO₂ increased the DNA strand breakage in gonadal tissue cells of rainbow trout (*O. mykiss*) when exposed to 50 mg l⁻¹ TiO₂ nanoparticles for 24 hours. However, also significant cytotoxicity was observed at this concentration and this may have influenced the results of the Comet assay. Hund-Rinke and Simon (2006) did not observe any effects of pre-illumination of TiO₂ in algal tests and Adams *et al.* (2006) found that antibacterial effects also occurred under dark conditions (cell death was less pronounced under dark compared to light conditions). Therefore photocatalytic production of ROS cannot alone be responsible for the inhibition observed and additional modes of action for TiO₂ nanoparticles remain to be elucidated.

8.6.4.2 Ecotoxicity of zinc oxide

As it is the case for silver nanoparticles, dissolution of zinc is one of the major issues addressed in the studies of ZnO nanoparticles. For ZnO nanoparticles, Zhu *et al.* (2008a) found hatching and survival of zebrafish embryos to be affected with an LC_{50, 96h} of 1.79 mg l⁻¹. However, the effect levels were not different from results obtained with bulk ZnO. For crustaceans, Heinlaan *et al.* (2008) found an LC_{50,48 h} value of 3.2 mg l⁻¹ ZnO nanoparticles and a NOEC of 0.5 mg l⁻¹ for *Daphnia magna*. In the same study, it was found that the LC_{50,24h} for *Thamnocephalus platyurus* was 0.18 mg l⁻¹ and the NOEC 0.05 mg l⁻¹. In the studies of algal toxicity of ZnO nanoparticles, bulk ZnO and ZnSO₄ no statistically significant difference in EC₅₀ values (expressed as Zn mg l⁻¹) for *P. subcapitata* could be observed (Aruoja *et al.* 2008). In accordance with this, Franklin *et al.* (2007) concluded that the toxicity of ZnO nanoparticles to *P. subcapitata* was due to dissolved zinc. Thus, in contrast to what was found for silver nanoparticles, the toxicity of zinc oxide nanoparticles seems to be equivalent to that of the released free ion (in this case Zn²⁺).

8.6.4.3 Ecotoxicity of silicon dioxide

The aquatic toxicity of SiO₂ has only been addressed by two studies of growth inhibition of algae. For the freshwater green algae *Pseudokirchneriella subcapitata*, van Hoecke *et al.* (2008) reported EC_{10,72h} values of 10.9 mg l⁻¹ and 15.0 mg l⁻¹ for 12.5 nm and 27.7 nm SiO₂ nanoparticles, respectively. For both particle types the NOEC was 4.6 mg l⁻¹ and LOEC was 10 mg l⁻¹. In the study by Fujiwara *et al.* (2008) effect concentrations for the inhibition of *Chlorella kessleri* were in the same range for 5 nm SiO₂ nanoparticles with an IC_{50,96h} of 8 mg l⁻¹. Effect concentrations were significantly higher for 26 nm and 78 nm SiO₂ nanoparticles with IC_{50,96h} values of 71 mg l⁻¹ and 91 mg l⁻¹, respectively.

8.6.5 General conclusions and recommendations

Attention should be drawn to the fact, that while many ecotoxicity studies are directed towards the core-particles, most real-world applications of engineered nanoparticles require a surface functionalisation (e.g. titanium dioxide in sunscreens should be functionalised to reduce photocatalytic activity). The effect of functionalisation on bioavailability and hence toxicity and

bioaccumulation of nanoparticles remains to be studied. As a range of nanoparticles are non-miscible with aquatic medium, solvents have been used for dispersing nanoparticles in aqueous media. This has especially been the case in a range of the “early studies” (i.e. studies carried out before 2007) e.g. Oberdörster (2004) and Lyon *et al.* (2005). While this may result in higher throughput of test (as long and tedious mixing procedures can be avoided) and may ensure a more uniform distribution throughout the water phase, it also raises serious problems with the validity of the results obtained due to testing artefacts introduced by solvent-medium-nanoparticle interactions (Henry *et al.* 2007). Due to the risk of producing testing artefacts, and keeping an eye on the environmental relevance of the tests carried out, the use of solvents are therefore at present not recommended.

While some studies have carried out some characterisation of the nanoparticles tested, most studies only report on chemical composition, sizes of nanoparticles (as purchased), and in some cases sizes of nanoparticles in suspension. It is evident that much more research is needed before specific properties, or combinations of properties, can be linked to the effects observed in ecotoxicity tests. For the time being only a few studies have documented links between characteristics and toxicity, e.g. van Hoecke *et al.* (2008) who found for SiO₂ nanoparticles that when results were expressed in terms of surface area instead of mass units, the apparent differential toxicity related to nanoparticle size was eliminated. The characterisation and quantification of nanoparticles in stock solutions, in media, and in biological tissues remains one of the biggest challenges in nanoecotoxicology. This is not only needed for linking characteristics with toxicity, but also for determination of actual exposure levels and for quantification of uptake, depuration and decay of nanoparticles. Going through the literature on environmental effects of nanomaterials, the lack of studies addressing degradability and accumulation is indeed striking. While these two properties, along with ecotoxicity, are fundamental for determining how environmentally hazardous a chemical is, it seems that most research efforts have been directed towards the potential toxicity of engineered nanomaterials.

In the reviewed ecotoxicological literature, it is obvious that the majority of the studies have used concentrations far above what is believed to be environmentally realistic. However, since we are in the beginning of ecotoxicological testing of nanoparticles this type of information is valid for risk assessment purposes in terms of ranking and benchmarking. Still it is important to emphasize that at present there are no studies supporting extrapolations from the effect levels documented in laboratory tests to environmental scenarios. Since some types of nanoparticles are not truly dissolved (and may not even be evenly dispersed in the test systems), it may be questioned whether dilution will always lead to lower effects and/or lower potential for accumulation. Dilution might result in a break of the agglomerates to smaller particulate sizes for which neither toxicity nor uptake is known.

Furthermore, most of the studies reviewed have focussed at short-term effects while long-term exposures to lower concentrations aimed at chronic endpoints have been far less studied. In a few cases (e.g. C₆₀ and TiO₂) data is now available for the test species in the base set for risk assessment in REACH (i.e., fish, crustacean, algae). However, even in these cases there is a need for replication of the test results obtained due to testing difficulties with regards to preparation, handling, and quantification of nanoparticle exposure. A range of environmentally relevant species have been used, but, due to the large number of different nanoparticles tested no clear pattern on species sensitivity, suitability as test organism in nanoecotoxicity or relevance of endpoints is seen. A number of studies are published on bacterial effects, mainly in pure cultures of either *E. coli* or *B. subtilis*. While these studies may be of interest from a mechanistic point of view, e.g. in relation to nanoparticles' interaction with cell membranes, they are at present of limited value for ecotoxicological effects assessment for other taxa. If future studies succeed in disclosing the mechanism of nanoparticle ecotoxicity, bacterial studies along with cellular studies may however be important screening tools.

8.7 REFERENCES

Adams, L.K., Lyon, D.Y. and Alvarez, P.J.J. 2006, "Comparative eco-toxicity of nanoscale TiO₂, SiO₂, and ZnO water suspensions", *Water Res.*, vol. 40, no. 19, pp. 3527-3532.

Aruoja, V., Dubourguier, H.C., Kasemets, K. and Kahru, A. 2008, "Toxicity of nanoparticles of CuO, ZnO and TiO₂ to microalgae *Pseudokirchneriella subcapitata*", *Science of the Total Environment*, vol. 407, no. 4, pp. 1461-1468.

Asharani, P.V., Wu, Y.L., Gong, Z., Valiyaveetil, S. (2008). Toxicity of silver nanoparticles in zebrafish models. *Nanotechnology*, 19, 255102 (8pp). doi:10.1088/0957-4484/19/25/255102.

Auffan, M., Achouak, W., Rose, J., Roncato, M.A., Chaneac, C., Waite, D.T., Masion, A., Woicik, J.C., Wiesner, M.R. and Bottero, J.Y. 2008, "Relation between the redox state of iron-based nanoparticles and their cytotoxicity toward *Escherichia coli*", *Environ.Sci.Technol.*, vol. 42, no. 17, pp. 6730-6735.

Baun, A., Sorensen, S.N., Rasmussen, R.F., Hartmann, N.B. and Koch, C.B. 2008, "Toxicity and bioaccumulation of xenobiotic organic compounds in the presence of aqueous suspensions of aggregates of nano-C(60)", *Aquat.Toxicol.*, vol. 86, no. 3, pp. 379-387.

Blaise, C., Gagne, F., Ferard, J.F. and Eullaffroy, P. 2008, "Ecotoxicity of selected nano-materials to aquatic organisms", *Environ.Toxicol.*, vol. 23, no. 5, pp. 591-598.

Blickley, T.M. and McClellan-Green, P. 2008, "Toxicity of Aqueous Fullerene in Adult and Larval *Fundulus heteroclitus*", *Environ.Toxicol.Chem.*, vol. 27, no. 9, pp. 1964-71.

Brayner, R., Ferrari-Iliou, R., Brivois, N., Djediat, S., Benedetti, M.F. and Fievet, F. 2006, "Toxicological impact studies based on *Escherichia coli* bacteria in ultrafine ZnO nanoparticles colloidal medium", *Nano Letters*, vol. 6, no. 4, pp. 866-870.

Canas, J.E., Long, M.Q., Nations, S., Vadan, R., Dai, L., Luo, M.X., Ambikapathi, R., Lee, E.H. and Olszyk, D. 2008, "Effects of functionalised and nonfunctionalised single-walled carbon nanotubes on root elongation of select crop species", *Environmental Toxicology and Chemistry*, vol. 27, no. 9, pp. 1922-1931.

Cheng, J.P., Flahaut, E. and Cheng, S.H. 2007, "Effect of carbon nanotubes on developing zebrafish (*Danio rerio*) embryos", *Environmental Toxicology and Chemistry*, vol. 26, no. 4, pp. 708-716.

Choi, O. and Hu, Z.Q. 2008, "Size dependent and reactive oxygen species related nanosilver toxicity to nitrifying bacteria", *Environ.Sci.Technol.*, vol. 42, no. 12, pp. 4583-4588.

Choi, O., Deng, K.K., Kim, N.J., Ross, L., Surampalli, R.Y. and Hu, Z.Q. 2008, "The inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth", *Water Res.*, vol. 42, no. 12, pp. 3066-3074.

Fang, J., Lyon, D.Y., Wiesner, M.R., Dong, J. and Alvarez, P.J. 2007, "Effect of a fullerene water suspension on bacterial phospholipids and membrane phase behavior", *Environ.Sci.Technol.*, vol. 41, no. 7, pp. 2636-2642.

Federici, G., Shaw, B.J. and Handy, R.D. 2007, "Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): Gill injury, oxidative stress, and other physiological effects", *Aquatic Toxicology*, vol. 84, pp. 415-430.

Ferguson, P.L., Chandler, G.T., Templeton, R.C., DeMarco, A., Scrivens, W.A. and Englehart, B.A. 2008, "Influence of sediment-amendment with single-walled carbon nanotubes and diesel soot on bioaccumulation of hydrophobic organic contaminants by benthic invertebrates", *Environ.Sci.Technol.*, vol. 42, no. 10, pp. 3879-3885.

Fortner, J.D., Lyon, D.Y., Sayes, C.M., Boyd, A.M., Falkner, J.C., Hotze, E.M., Alemany, L.B., Tao, Y.J., Guo, W., Ausman, K.D., Colvin, V.L. and Hughes, J.B. 2005, "C₆₀ in water: nanocrystal formation and microbial response", *Environ.Sci.Technol.*, vol. 39, no. 11, pp. 4307-4316.

Franklin, N.M., Rogers, N.J., Apte, S.C., Batley, G.E., Gadd, G.E. and Casey, P.S. 2007, "Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and ZnCl₂ to a freshwater microalga (*Pseudokirchneriella subcapitata*): The importance of particle solubility", *Environ.Sci.Technol.*, vol. 41, no. 24, pp. 8484-8490.

Fujiwara, K., Suematsu, H., Kiyomiya, E., Aoki, M., Sato, M. and Moritoki, N. 2008, "Size-dependent toxicity of silica nano-particles to *Chlorella kessleri*", *Journal of Environmental Science and Health Part A-Toxic/hazardous Substances and Environmental Engineering*, vol. 43, no. 10, pp. 1167-1173.

Ghafari, P., St-Denis, C.H., Power, M.E., Jin, X., Tsou, V., Mandal, H.S., Bols, N.C. and Tang, X.W. 2008, "Impact of carbon nanotubes on the ingestion and digestion of bacteria by ciliated protozoa", *Nature Nanotechnology*, vol. 3, no. 6, pp. 347-351.

Griffitt, R.J., Luo, J., Gao, J., Bonzongo, J.C. and Barber, D.S. 2008, "Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms", *Environmental Toxicology and Chemistry*, vol. 27, no. 9, pp. 1972-1978.

Griffitt, R.J., Weil, R., Hyndman, K.A., Denslow, N.D., Powers, K., Taylor, D. and Barber, D.S. 2007, "Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (*Danio rerio*)", *Environ.Sci.Technol.*, vol. 41, no. 23, pp. 8178-8186.

Harper, S., Usenko, C., Hutchinson, J.E., Maddux, B.I.S., Tanguay, R.L. (2008). In vivo biodistribution and toxicity depends on nanomaterial composition, size, surface functionalisation and routes of exposure. *J. Experimental Nanoscience*, vol. 3, no. 3, 195-206.

Heinlaan, M., Ivask, A., Blinova, I., Dubourguier, H.C. and Kahru, A. 2008, "Toxicity of nanosized and bulk ZnO, CuO and TiO₂ to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*", *Chemosphere*, vol. 71, no. 7, pp. 1308-1316.

Henry, T.B., Menn, F.M., Fleming, J.T., Wilgus, J., Compton, R.N. and Sayler, G.S. 2007, "Attributing effects of aqueous C₆₀ nano-aggregates to tetrahydrofuran decomposition products in larval zebrafish by assessment of gene expression", *Environ.Health Perspect.*, vol. 115, no. 7, pp. 1059-1065.

Huang, Z.B., Zheng, X., Yan, D.H., Yin, G.F., Liao, X.M., Kang, Y.Q., Yao, Y.D., Huang, D. and Hao, B.Q. 2008, "Toxicological effect of ZnO nanoparticles based on bacteria", *Langmuir*, vol. 24, no. 8, pp. 4140-4144.

Hund-Rinke, K. and Simon, M. 2006, "Ecotoxic effect of photocatalytic active nanoparticles (TiO₂) on algae and daphnids", *Environ.Sci.Pollut.Res.Int.*, vol. 13, no. 4, pp. 225-232.

Jemec, A., Drobne, D., Remskar, M., Sepcic, K. and Tisler, T. 2008, "Effects of Ingested Nano-Sized Titanium Dioxide on Terrestrial Isopods *Porcellio Scaber*", *Environ.Toxicol.Chem.*, 2:1, pp. 1.

Johansen, A., Pedersen, A., Karlson, U., Hansen, B.M., Scott-Fordsmann, J. and Winding, A. 2008, "Effects of C₆₀ fullerene nanoparticles on soil bacteria and protozoans", *Environ.Toxicol.Chem.*, 1:1. pp. 1.

Kang, S., Mauter, M.S. and Elimelech, M. 2008, "Physicochemical determinants of multiwalled carbon nanotube bacterial cytotoxicity", *Environ.Sci.Technol.*, vol. 42, no. 19, pp. 7528-7534.

Kennedy, A.J., Hull, M.S., Steevens, J.A., Dontsova, K.M., Chappell, M.A., Gunter, J.C., Weiss, C.A. (2008). Factors influencing the partitioning and toxicity of nanotubes in the aquatic

environment. *Environ. Toxicol. Chem.*, vol. 27, no. 9, 1932–1941.

Lee, W.M., An, Y.J., Yoon, H., Kweon, H.S. (2008). Toxicity and bioavailability of copper nanoparticles to the terrestrial plants mung bean (*Phaseolus radiatus*) and wheat (*Triticum aestivum*): plant agar test for water-insoluble nanoparticles. *Environ. Toxicol. Chem.*, vol. 27, no. 9, 1915–1921.

Li, D., Lyon, D.Y., Li, Q, Alvarez, P.J.J. (2008). Effect of soil sorption and aquatic natural organic matter on the Antibacterial activity of a fullerene water suspension. *Environ. Toxicol. Chem.*, vol. 27, no. 9, 1888–1894.

Lin, D.H. and Xing, B.S. 2007, "Phytotoxicity of nanoparticles: Inhibition of seed germination and root growth", *Environmental Pollution*, vol. 150, no. 2, pp. 243-250.

Lin, D.H. and Xing, B.S. 2008, "Root uptake and phytotoxicity of ZnO nanoparticles", *Environ.Sci.Technol.*, vol. 42, no. 15, pp. 5580-5585.

Lovern, S.B., Klaper, R. (2006). *Daphnia magna* mortality when exposed to titanium dioxide and fullerene (C₆₀) nanoparticles. *Environ. Toxicol. Chem.*, vol. 25, pp. 1132-1137.

Lovern SB, Strickler JR, Klaper R., 2007, "Behavioral and physiological changes in *Daphnia magna* when exposed to nanoparticle suspensions (titanium dioxide, nano-C₆₀, and C₆₀H_xC₇₀H_x)", *Environ Sci Technol.*, vol. 4, no. 12, pp. 4465-70.

Lyon, D.Y., Adams, L.K., Falkner, J.C. and Alvarez, P.J. 2006, "Antibacterial activity of fullerene water suspensions: effects of preparation method and particle size", *Environ.Sci.Technol.*, vol. 40, no. 14, pp. 4360-4366.

Lyon, D.Y., Fortner, J.D., Sayes, C.M., Colvin, V.L. and Hughe, J.B. 2005, "Bacterial cell association and antimicrobial activity of a C₆₀ water suspension", *Environ.Toxicol.Chem.*, vol. 24, no. 11, pp. 2757-2762.

Mortimer, M., Kasemets, K., Heinlaan, M., Kurvet, I. and Kahru, A. 2008, "High throughput kinetic *Vibrio fischeri* bioluminescence inhibition assay for study of toxic effects of nanoparticles", *Toxicol.In.Vitro.*, vol. 22, no. 5, pp. 1412-1417.

Mouchet, F., Landois, P., Flahaut, E., Pinelli, E. and Gauthier, L. 2007, "Assessment of the potential in vivo ecotoxicity of Double-Walled Carbon Nanotubes (DWNTs) in water, using the amphibian *Ambystoma mexicanum*", *Nanotoxicology*, vol. 1, no. 2, pp. 149-156.

Mouchet, F., Landois, P., Sarremejean, E., Bernard, G., Puech, P., Pinelli, E., Flahaut, E. and Gauthier, L. 2008, "Characterisation and in vivo ecotoxicity evaluation of double-wall carbon nanotubes in larvae of the amphibian *Xenopus laevis*", *Aquatic Toxicology*, vol. 87, no. 2, pp. 127-137.

Navarro E, Piccapietra F, Wagner B, Marconi F, Kaegi R, Odzak N, Sigg L and Behra R, 2008, Toxicity of Silver Nanoparticles to *Chlamydomonas reinhardtii*, *Environ. Sci. Technol.*, vol. 42, no. 23, pp. 8959–8964.

Nyberg, L., Turco, R.F. and Nies, L. 2008, "Assessing the impact of nanomaterials on anaerobic microbial communities", *Environ. Sci. Technol.*, vol. 42, no. 6, pp. 1938-1943.

Oberdorster, E. 2004, "Manufactured nanomaterials (Fullerenes, C-60) induce oxidative stress in the brain of juvenile largemouth bass", *Environ.Health Perspect.*, vol. 112, no. 10, pp. 1058-1062.

Oberdorster, E., Zhu, S.Q., Blickley, T.M., McClellan-Green, P. and Haasch, M.L. 2006, "Ecotoxicology of carbon-based engineered nanoparticles: Effects of fullerene (C-60) on aquatic organisms", *Carbon*, vol. 44, no. 6, pp. 1112-1120.

Engineered Nanoparticles: Review of Health and Environmental Safety

Oughton, D.H., Hertel-Aas, T., Pellicer, E., Mendoza, E., Joner, E.J. (2008). Neutron activation of engineered nanoparticles as a tool for tracing their environmental fate and uptake in organisms. *Environ. Toxicol. Chem.*, vol. 27, no. 9, pp. 1883–1887.

Petersen, E.J., Huang, Q.G. and Weber, W.J. 2008a, "Bioaccumulation of radio-labeled carbon nanotubes by *Eisenia foetida*", *Environ.Sci.Technol.*, vol. 42, no. 8, pp. 3090-3095.

Petersen, E.J., Huang, Q.G. and Weber, W.J. 2008b, "Ecological uptake and depuration of carbon nanotubes by *Lumbriculus variegatus*", *Environ.Health Perspect.*, vol. 116, no. 4, pp. 496-500.

Roberts, A.P., Mount, A.S., Seda, B., Souther, J., Qiao, R., Lin, S.J., Ke, P.C., Rao, A.M. and Klaine, S.J. 2007, "In vivo biomodification of lipid-coated carbon nanotubes by *Daphnia magna*", *Environ.Sci.Technol.*, vol. 41, no. 8, pp. 3025-3029.

Schreiner, K M; Filley, TR; Blanchette, RA; Bowen, BB; Bolskar, RD; Hockaday, WC; Masiello, CA; Raebiger, JW. 2009. White-Rot Basidiomycete-Mediated Decomposition of C₆₀ Fullerol. *Environ Sci Technol*; vol 43, no 9, pp. 3162-8.

Scott-Fordsmand, J.J., Krogh, P.H., Schaefer, M. and Johansen, A. 2008, "The toxicity testing of double-walled nanotubes-contaminated food to *Eisenia veneta* earthworms", *Ecotoxicol.Environ.Saf.*, vol. 71, no. 3, pp. 616-619.

Smith, C.J., Shaw, B.J. and Handy, R.D. 2007, "Toxicity of single-walled carbon nanotubes to rainbow trout, (*Oncorhynchus mykiss*): Respiratory toxicity, organ pathologies, and other physiological effects", *Aquatic Toxicology*, vol. 82, no. 2, pp. 94-109.

Stoimenov, P.K., Klinger, R.L., Marchin, G.L. and Klabunde, K.J. 2002, "Metal oxide nanoparticles as bactericidal agents", *Langmuir*, vol. 18, no. 17, pp. 6679-6686.

Sun, H.W., Zhang, X.Z., Niu, Q., Chen, Y.S. and Crittenden, J.C. 2007, "Enhanced accumulation of arsenate in carp in the presence of titanium dioxide nanoparticles", *Water Air and Soil Pollution*, vol. 178, no. 1-4, pp. 245-254.

Templeton, R.C., Ferguson, P.L., Washburn, K.M., Scrivens, W.A. and Chandler, G.T. 2006, "Life-cycle effects of single-walled carbon nanotubes (SWNTs) on an estuarine meiobenthic copepod", *Environ.Sci.Technol.*, vol. 40, no. 23, pp. 7387-7393.

Thill, A., Zeyons, O., Spalla, O., Chauvat, F., Rose, J., Auffan, M. and Flank, A.M. 2006, "Cytotoxicity of CeO₂ nanoparticles for *Escherichia coli*. Physico-chemical insight of the cytotoxicity mechanism", *Environ.Sci.Technol.*, vol. 40, no. 19, pp. 6151-6156.

Tong, Z., Bischoff, M., Nies, L., Applegate, B. and Turco, R.F. 2007, "Impact of fullerene (C₆₀) on a soil microbial community", *Environ.Sci.Technol.*, vol. 41, no. 8, pp. 2985-2991.

Usenko, C.Y., Harper, S.L. and Tanguay, R.L. 2008, "Fullerene C-60 exposure elicits an oxidative stress response in embryonic zebrafish", *Toxicol.Appl.Pharmacol.*, vol. 229, no. 1, pp. 44-55.

Van Hoecke, K., De Schamphelaere, K.A.C., Van der Meeren, P., Lucas, S. and Janssen, C.R. 2008, "Ecotoxicity of silica nanoparticles to the green alga *Pseudokirchneriella subcapitata*: Importance of surface area", *Environmental Toxicology and Chemistry*, vol. 27, no. 9, pp. 1948-1957.

Velzeboer, I., Hendriks, A.J., Ragas, A.M.J. and Van de Meent, D. 2008, "Aquatic ecotoxicity tests of some nanomaterials", *Environmental Toxicology and Chemistry*, vol. 27, no. 9, pp. 1942-1947.

Vevers, W.F. and Jha, A.N. 2008, "Genotoxic and cytotoxic potential of titanium dioxide (TiO₂) nanoparticles on fish cells in vitro", *Ecotoxicology*, vol. 17, no. 5, pp. 410-420.

Engineered Nanoparticles: Review of Health and Environmental Safety

Wang J., Zhang X., Chen Y., Sommerfield M. and Hu, Q. 2008, "Toxicity assessment of manufactured nanomaterials using the unicellular green alga *Chlamydomonas reinhardtii*", *Chemosphere*, vol. 73, no. 7.

Yang, F., Liu, C., Gao, F., Su, M., Wu, X., Zheng, L., Hong, F. and Yang, P. 2007, "The improvement of spinach growth by nano-anatase TiO₂ treatment is related to nitrogen photoreduction", *Biol.Trace Elem.Res.*, vol. 119, no. 1, pp. 77-88.

Yang, L. and Watts, D.J. 2005, "Particle surface characteristics may play an important role in phytotoxicity of alumina nanoparticles", *Toxicol.Lett.*, vol. 158, no. 2, pp. 122-132.

Yeo, M.K. and Kang, M. 2008, "Effects of nanometer sized silver materials on biological toxicity during zebrafish embryogenesis", *Bulletin of the Korean Chemical Society*, vol. 29, no. 6, pp. 1179-1184.

Zhang, X., Sun, H., Zhang, Z., Niu, Q., Chen, Y., Crittenden, J.C. (2007). Enhanced bioaccumulation of cadmium in carp in the presence of titanium dioxide nanoparticles. *Chemosphere*, vol. 67, pp. 160-166.

Zheng, L., Hong, F.S., Lu, S.P. and Liu, C. 2005, "Effect of nano-TiO₂ on strength of naturally and growth aged seeds of spinach", *Biol.Trace Elem.Res.*, vol. 104, no. 1, pp. 83-91.

Zhu, H., Han, J., Xiao, J.Q. and Jin, Y. 2008c, "Uptake, translocation, and accumulation of manufactured iron oxide nanoparticles by pumpkin plants", *Journal of Environmental Monitoring*, vol. 10, no. 6, pp. 713-717.

Zhu, S.Q., Oberdorster, E. and Haasch, M.L. 2006a, "Toxicity of an engineered nanoparticle (fullerene, C-60) in two aquatic species, *Daphnia* and fathead minnow", *Mar.Environ.Res.*, vol. 62, pp. S5-S9.

Zhu, X.S., Zhu, L., Duan, Z.H., Qi, R.Q., Li, Y. and Lang, Y.P. 2008a, "Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to Zebrafish (*Danio rerio*) early developmental stage", *Journal of Environmental Science and Health Part A-Toxic/hazardous Substances and Environmental Engineering*, vol. 43, no. 3, pp. 278-284.

Zhu, X.S., Zhu, L., Lang, Y.P. and Chen, Y.S. 2008b, "Oxidative stress and growth inhibition in the freshwater fish *Carassius auratus* induced by chronic exposure to sublethal fullerene aggregates", *Environmental Toxicology and Chemistry*, vol. 27, no. 9, pp. 1979-1985.

Zhu, Y., Zhao, Q.F., Li, Y.G., Cai, X.Q. and Li, W. 2006b, "The interaction and toxicity of multi-walled carbon nanotubes with *Stylomychia mytilus*", *Journal of Nanoscience and Nanotechnology*, vol. 6, no. 5, pp. 1357-1364.

Selected Ecotoxicology Reviews

Baun, A., Hartmann, N.B., Grieger, K. and Kusk, K.O. 2008, "Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing", *Ecotoxicology*, vol. 17, no. 5, pp. 387-395.

Behra, R. and Krug, H. 2008, "Nanoecotoxicology - Nanoparticles at large", *Nature Nanotechnology*, vol. 3, no. 5, pp. 253-254.

Crane, M., Handy, R.D., Garrod, J. and Owen, R. 2008, "Ecotoxicity test methods and environmental hazard assessment for engineered nanoparticles", *Ecotoxicology*, vol. 17, no. 5, pp. 421-437.

Handy, R.D., Henry, T.B., Scown, T.M., Johnston, B.D. and Tyler, C.R. 2008, "Manufactured nanoparticles: their uptake and effects on fish-a mechanistic analysis", *Ecotoxicology*, vol. 17, no. 5, pp. 396-409.

Handy, R.D. and Shaw, B.J. 2007, "Ecotoxicity of nanomaterials to fish: challenges for ecotoxicity testing", *Integr. Environ. Assess. Manag.*, vol. 3, no. 3, pp. 458-460.

Handy, R.D., von der Kammer, F., Lead, J.R., Hasselov, M., Owen, R. and Crane, M. 2008, "The ecotoxicology and chemistry of manufactured nanoparticles", *Ecotoxicology*, vol. 17, no. 4, pp. 287-314.

Hannah, W. and Thompson, P.B. 2008, "Nanotechnology, risk and the environment: a review", *Journal of Environmental Monitoring*, vol. 10, no. 3, pp. 291-300.

Helland, A., Wick, P., Koehler, A., Schmid, K. and Som, C. 2007, "Reviewing the environmental and human health knowledge base of carbon nanotubes", *Environ. Health Perspect.*, vol. 115, no. 8, pp. 1125-1131.

Hinton, D.E., Kullman, S.W., Hardman, R.C., Volz, D.C., Chen, P.J., Carney, M. and Bencic, D.C. 2005, "Resolving mechanisms of toxicity while pursuing ecotoxicological relevance?", *Mar. Pollut. Bull.*, vol. 51, no. 8-12, pp. 635-648.

Kennedy, A.J., Hull, M.S., Steevens, J.A., Dontsova, K.M., Chappell, M.A., Gunter, J.C. and Weiss, C.A. 2008, "Factors influencing the partitioning and toxicity of nanotubes in the aquatic environment", *Environmental Toxicology and Chemistry*, vol. 27, no. 9, pp. 1932-1941.

Klaine, S.J., Alvarez, P.J.J., Batley, G.E., Fernandes, T.F., Handy, R.D., Lyon, D.Y., Mahendra, S., McLaughlin, M.J. and Lead, J.R. 2008, "Nanomaterials in the environment: Behavior, fate, bioavailability, and effects", *Environmental Toxicology and Chemistry*, vol. 27, no. 9, pp. 1825-1851.

Kolosnjaj, J., Szwarc, H. and Moussa, F. 2007, "Toxicity studies of fullerenes and derivatives", *Adv. Exp. Med. Biol.*, vol. 620, pp. 168-180.

Lam, C.W., James, J.T., McCluskey, R., Arepalli, S. and Hunter, R.L. 2006, "A review of carbon nanotube toxicity and assessment of potential occupational and environmental health risks", *Crit. Rev. Toxicol.*, vol. 36, no. 3, pp. 189-217.

Navarro, E., Baun, A., Behra, R., Hartmann, N.B., Filser, J., Miao, A.J., Quigg, A., Santschi, P.H. and Sigg, L. 2008, "Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi", *Ecotoxicology*, vol. 17, no. 5, pp. 372-386.

Neal, A.L. 2008, "What can be inferred from bacterium-nanoparticle interactions about the potential consequences of environmental exposure to nanoparticles?", *Ecotoxicology*, vol. 17, no. 5, pp. 362-371.

Scheringer, M. 2008, "Nanoecotoxicology - Environmental risks of nanomaterials", *Nature Nanotechnology*, vol. 3, no. 6, pp. 322-323.

Warheit, D.B., Borm, P.J., Hennes, C. and Lademann, J. 2007, "Testing strategies to establish the safety of nanomaterials: conclusions of an ECETOC workshop", *Inhal. Toxicol.*, vol. 19, no. 8, pp. 631-643.

9 RISK ASSESSMENT

9.1 INTRODUCTION

Nanotechnology represents a promising industry that can revolutionise several economic and social sectors. Particles at nano-size have a wide variety of applications which range from industrial production efficiency, energy production, environment remediation, consumer products to medicine and many others. Besides the promising benefits there are concerns about possible risks for humans and more recently also for the environment.

Very few publications up to now have been dealing with risk assessment methodologies for nanomaterials. Some analyses were carried out to evaluate the applicability of existing approaches to nanomaterials, in particular the assessment methodologies as described in the Guidance Documents of the EU's chemicals legislation (REACH). A SCENHIR opinion (SCENHIR 2007) stated that the current methodologies are generally likely to be able to identify the hazards associated with the use of nanomaterials. However, Franco *et al.* (2007) reported that the application of EC legislation based on the 'incremental approach' (adaptation of existing legislation requirements to deal with nanomaterials) is still not achievable with sufficient reliability due to consistent data gaps. The main data gaps, as reported by Handy *et al.* (2008), concern nanomaterial characterisation, detection methods, nanomaterial (and adsorbed chemicals) fate and transport, standardised *in vivo*, *in vitro*, and *in silico* testing development, and the identification of mechanisms of action.

Other risk assessment approaches have been proposed in the literature, such as: i) the DuPont initiative framework integrating life cycle reasoning with the classical risk assessment framework (ED 2007); ii) a control banding approach evaluating the effectiveness of risk management measures in working environments (Paik *et al.* 2008); and iii) the application of multi-criteria decision analysis as alternative ranking tool (Linkov *et al.* 2007). Some of these approaches can be used to rank nanomaterials in a screening phase of the risk assessment.

This chapter presents basic risk assessments inspired by the REACH Guidance for the four types of nanomaterials under review based on the information provided by other parts of the review. It includes an assessment for both the human health and the environment to the extent data allows. For each nanomaterial uncertainties and additional work needed to complete the assessment are described.

Each of the four groups of nanomaterials under review - fullerenes, carbon nanotubes, metals and metal oxides – include different forms of the substances, e.g. fullerenes with different functionalisation or single and multi wall carbon nanotubes. In particular, neither the metals nor the metal oxides nanoparticles - like those in the conventional/'bulk' form - can and should not be considered as one group in terms of risk assessment due to the chemical, toxicological and ecotoxicological diversity between substances of one group. Therefore for metals and metal oxides the most data rich substance(s) were chosen as case studies in the development of a simple risk assessment approach.

The case studies are a purely scientific exercise which allows the exploration of key questions associated with the risk assessment of nanomaterials, and should not be used in any other way. These case studies do not reflect any opinion of the European Commission.

9.2 APPLIED RISK ASSESSMENT METHODOLOGY

The basic risk assessments carried out in this report are inspired by the REACH Guidance on Information Requirements and Chemicals Safety Assessment (http://guidance.echa.europa.eu/docs/guidance_document/information_requirements_en.htm?time=1252482386 accessed 16th October 2009), following its general methods and structure. However, given the availability of information (which does not comply comprehensively with the REACH requirements in terms of significant lack of knowledge of use, exposure, and data on inherent properties), the detailed assessments have been adapted to the available information and data, and also taking into account the overall structure of the ENRHES review. The

assessments therefore are more akin to a risk assessment as carried out under the old chemicals legislation (for "existing substances") where the authorities did an assessment based on the available information. Consequently, the scope of the assessments adopted is what would normally be encountered in a "regulatory chemicals risk assessment". In order to follow this format, information is extracted from previous sections of the report and assimilated into a risk assessment, which therefore introduces some repetition between this chapter and previous chapters of the review.

On the basis of the identified information, the risk assessments are carried out following both a quantitative and a qualitative approach. For human health, the quantitative approach requires establishing exposure values for the various routes of exposure (inhalation, dermal and oral) for consumers and workers and the establishment of a Derived-no-Effect Level (DNEL), typically based on extrapolation of animal data to the human situation by using appropriate assessment factors. For the environmental assessment, the quantitative approach requires the determination of the Predicted Exposure Concentration (PEC) and the Predicted No Effect Concentration (PNEC) for each environmental compartment. PEC and PNEC are then compared to identify any risk for environmental compartments. For both human health and environment, the application of assessment factors is based on the REACH guidance (http://guidance.echa.europa.eu/docs/guidance_document/information_requirements_en.htm?time=1252482386 Accessed 16th October 2009). Qualitative risk characterisation was carried out in case no exposure value and/or no dose descriptors were available or estimated.

Detailed consideration of physico-chemical properties in the risk assessments were limited to the information made available in the exposure and (eco)toxicological chapters of the review.

9.3 CARBON FULLERENES

9.3.1 Identity

Fullerenes are carbon allotropes which can be considered as a rolled up sheet of graphene to form a closed shell molecular structure. They are molecules (60 or more carbon atoms) and as such should not really be considered nanoparticles, although in the related literature it has become a convention to include them in this category.

Fullerenes often crystallise into larger particles. There are a number of different fullerene derivatives available, which stem from the different number of carbon atoms used to generate fullerenes, the diverse array of moieties that can be attached to the fullerene surface, and the different preparations processes utilised. Surface modifications are often used to render fullerenes water soluble e.g. for pharmaceutical purposes. Dispersants used to enhance dispersion and to minimise cluster/crystal size can also influence the (eco)toxicity.

9.3.2 Manufacturing and use

Fullerenes are generally produced as functionalised forms, and the functional groups are the key determinants of the properties (e.g. water solubility, cell membrane crossing, etc.). Examples of functional groups are perfluorinated groups, carboxylic groups, hydroxyl groups, phenol groups, amidic groups. The different physico-chemical properties influence the biological activity which renders it therefore difficult to make generalisations with regard to (eco)toxicological effects and consequently for a risk assessment.

The production and uses of fullerenes in the market are limited at the moment, but expected to grow significantly. The current applications are focused on targeted drug delivery, lubricants, the energy sector (fuel cells, solar cells, and batteries), catalysis, and polymers modifications (as highlighted in chapter 2 of the ENRHES review).

9.3.3 Exposure assessment of fullerenes

9.3.3.1 Occupational exposure

In an occupational setting, exposure to fullerenes could in principle occur for workers at all phases of the material life cycle. During the development stage it is probable that the material will be produced under tightly controlled conditions, typically in very small quantities. Accidental releases due to spills and accidents are also a possibility. In commercial production, exposures can potentially occur during synthesis of the material or in downstream activities such as recovery, packaging, transport, and storage. In these circumstances, the quantities of materials being handled will typically be much larger.

Depending on the specific properties of the fullerene, it may be incorporated subsequently in a range of other products or may be used in other processes as chemical feedstock or precursor.

Various downstream applications of fullerenes have been suggested or reported. These downstream applications also have the potential to result in exposure to workers involved in them. Applications suggested include in the chemical industry as catalysts. Other applications include as lubricants, or as components in composites and for drug delivery. All of these applications offer the possibility of occupational exposure. Inclusion of fullerenes in composites also may lead to exposure when that composite is machined, cut or drilled. The use of fullerenes in drug delivery systems may give rise to occupational exposure to those who manufacture or administer them.

For workers fullerene exposure measurements are available from two factories (Fujitani et al. 2008; Yeganeh et al. 2008). With respect to exposure assessment, airborne concentrations are mainly reported as particles per cm³ which makes it difficult to use these data for a risk assessment. Only one measurement of mass/volume is identified, i.e. PM_{2.5} of 50 – 125 µg m⁻³; these values will be used for a quantitative risk characterisation exercise. However there is no knowledge whether these particles are only fullerenes as also carbon nanotubes were produced at the same facility.

No information on dermal exposure has been identified.

9.3.3.2 Consumer exposure

There is no published data on how or if consumers are exposed to fullerenes. Use of fullerenes in drug delivery has been speculated but is not yet a commercial reality. Fullerenes have been reported as being present in skin creams although probably at low levels. This would lead to dermal exposure which could occur over a long period, dependant on usage patterns.

The use of fullerenes in medical devices is not within the scope of this risk assessment.

9.3.3.3 Exposure of humans via the environment

No information has been identified about exposure to fullerenes via the environment. At current production levels, it is unlikely that manufactured fullerenes are entering the environment at levels which would cause detectable exposure to humans.

9.3.4 Human health effects assessment

The potential toxicology relating to fullerenes exposure comprises respiratory and dermal effects due to their propensity to be exposed during their manufacture or utilisation. Exposure via the gastrointestinal tract can occur as a consequence of hand-to-mouth contact and due to mucociliary clearance from the lungs.

The toxicity of fullerenes in biomedical applications (targeted drug delivery) and their risk were outwith the scope of the ENRHES review.

Different fullerene derivatives may have different biological activities which makes it difficult to make generalisations with regard to human health effects.

9.3.4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

Absorption

Inhalation:

In a study by Baker *et al.* (2008) fullerenes were not detected in blood following inhalation by rats, suggesting that they do not translocate from their exposure site. A half life of 26 days for fullerene nanoparticles was determined which is similar to microparticles (29 days) suggesting that similar elimination processes are involved during the removal from the lungs. It is necessary to note that the preparation method and therefore the form of the fullerene dispersion could influence this data and therefore additional studies are required before this finding can be considered universal.

Oral:

C₆₀ was shown not to be effectively absorbed following oral administration to rats and mice, for a period up to 160 hours post exposure, but instead the majority was excreted in the faeces within 48 hours. However, trace amounts of fullerene were observed within urine, therefore implying that some fullerenes were able to pass through the gut wall (Yamago *et al.* 1995).

Dermal:

No information identified.

Distribution

No information on distribution following inhalation, oral or dermal absorption has been identified. Following intraperitoneal injection into rats fullerenes were transported via blood, accumulating in liver, spleen and kidney, with evidence of toxicity manifesting at sites of accumulation (Chen *et al.* 1998b). Following intravenous injection fullerenes were rapidly removed from the blood and accumulated primarily in liver, but also, presumably depending on their water solubility in kidney, lungs, spleen, heart and brain (Yamago *et al.* 1995, Bullard-Dillard *et al.* 1996).

Metabolism

The metabolism of fullerenes has been suggested to occur, following their accumulation within the liver of rats (Gharbi *et al.* 2005), with the metabolites, as yet, unspecified.

Elimination

The elimination of fullerenes in urine (Yamago *et al.* 1995) and faeces (Mori *et al.* 2006, Yamago *et al.* 1995) has been demonstrated in rat and mouse, suggesting that they may be eliminated, in part, from the body following exposure via a number of routes.

In summary, from the little information identified it appears that fullerenes are often not absorbed and remain at the deposition site (specifically within the lungs and gut). Particles can be eliminated from the lung through alveolar macrophages or mucociliary escalator. It is not known if fullerenes are able to cross cell barriers and are transported within the blood.

No fullerenes have been detected in blood following inhalation, which suggests a potential clearance from or biotransformation within the lung but could also be due to the insensitivity of the detection method. It has to be confirmed by more sensitive methods to which extent (and under which conditions) fullerenes could be absorbed from the lung.

After oral exposure fullerenes are found primarily in the faeces suggesting that they are not effectively absorbed. A small but unspecified proportion of the fullerene dose was found to be able to pass through the gut wall, and thereby enter the circulation.

No information on dermal uptake has been identified.

Following intravenous injection and intraperitoneal exposure fullerenes were found to be transported and rapidly removed from the blood. Fullerenes accumulated predominantly within the liver, but also in the kidney, lungs, spleen, heart and brain. This information however is only relevant if absorption via primary exposure routes takes place. Metabolism of fullerenes in the liver has been suggested but no metabolites could be identified. Elimination of fullerenes within the faeces and urine has been demonstrated.

To date it is not possible to make general conclusions on the ADME profile of fullerenes and their derivatives due to the limited amount of information. Further investigations are needed to clarify and confirm if fullerenes can be absorbed via the relevant primary exposure routes and reach organs apart from the deposition site. This information is important to interpret the effects seen following intraperitoneal and intravenous application and those seen *in vitro*. The use of radioisotope or fluorescent labelling is recommended to allow for the better detection of fullerenes.

9.3.4.2 Acute toxicity

Acute toxicity: oral

Two studies were identified that show a very low toxicity of fullerenes subsequent to oral exposure.

No lethality, or other signs of toxicity in terms of behaviour or body weight were evident in rats after oral exposure at a dose of 2000 mg kg⁻¹ of fullerite (a mixture of C₆₀ and C₇₀), during the observation period (up to 14 days) (Mori *et al.* 2006).

Chen *et al.* (1998b) demonstrated that polyalkylsulfonated (water soluble) C₆₀ showed no effects subsequent to oral exposure of rats in acute (50 mg kg⁻¹, single administration) or subacute (50 mg kg⁻¹ daily for 12 days) exposure set ups, and as a consequence it was considered to be not acutely toxic.

Acute toxicity: inhalation

In vivo:

Many studies have demonstrated that a range of nanoparticles induce pro-inflammatory effects in the lung (see for example Donaldson and Stone, 2003 for a review) however this has not always been seen with fullerenes.

No inflammatory potential was observed in rats exposed to fullerenes following nasal inhalation at concentrations of 2.22 mg m⁻³ (nanoparticle, 55 nm diameter) and 2.35 mg m⁻³ (microparticle, 0.93 µm diameter) for 3 hours per day for 10 consecutive days (Baker *et al.* 2008) and following a single intratracheal instillation at concentrations between 0.2 and 3 mg kg⁻¹ C₆₀ or C₆₀(OH)₂₄, for a period of up to 3 months following exposure (Sayes *et al.* 2007).

A study by Roursgaard *et al.* (2008) which exposed mice via intratracheal instillation to doses of 0.02 to 200 µg per mouse for 24 hours showed that at low concentrations (20 µg per mouse), fullerols (i.e. hydroxylated fullerenes) may have protective, anti-inflammatory properties probably due to the ability of fullerols to reduce ROS mediated inflammation, but at higher concentrations (200 µg/mouse) they exhibit a pro-inflammatory response.

In vitro:

Within the mouse FE1-Muta epithelial cell line it was demonstrated that C₆₀ exposure (0-200 µg ml⁻¹, 24 or 576 hours) was associated with a slight increase in ROS production, but no impact on cell viability was observed (Jacobsen *et al.* 2008).

Acute toxicity: dermal

In vivo:

No detrimental outcome was found in a human patch test to assess the skin irritant potential of fullerene soot (Huczko *et al.* 1999). No further information has been identified.

In vitro:

Several dermal models have shown that fullerenes were internalised by keratinocytes, however the reactions observed were different in nature. No effects on cell proliferation at concentrations between 20 nM and 2 µM were observed by Scrivens *et al.* (1994), whereas Bullard-Dillard *et al.* (1996) observed that C₆₀ elicited a decrease in cell proliferation that was evident at high concentrations (2µM) and over an extended period of time of 8 days. An inflammatory response and a dose dependent cytotoxicity via a necrotic mechanism after exposure to phenylalanine derivatised C₆₀ (up to 0.4 mg ml⁻¹) was observed in HEK keratinocytes (Rouse *et al.* 2006). It was observed in one study that penetration of the particles did not occur via their direct transport through cells, but indirectly between skin cells via intercellular spaces (Rouse *et al.* 2007). Sayes *et al.* (2004) found that the cytotoxic potential (mediated by lipid peroxidation) of different forms of derivatised fullerenes to human dermal fibroblasts (HDF), HepG2 hepatocytes and NHA astrocytes, was dependent on the type and level of functionalisation.

Acute toxicity: other routes

Intraperitoneal exposure:

Following intraperitoneal injection in mice and rats fullerenes induced antigenic behaviour by stimulating the generation of antibodies (Chen *et al.* 1998a) which were also able to interact with SWCNT, (Erlanger *et al.* 2001). An LD₅₀ of 600 mg kg⁻¹ was determined via intraperitoneal injection to rats with water soluble, polyalkylsulfonated C₆₀, in an acute (up to 1000 mg kg⁻¹, for 24 hours) or subacute setting (up to 60 mg kg⁻¹, with daily exposures for 12 consecutive days). The kidney was recognised as a primary site of fullerene elimination and toxicity (nephropathy) (Chen *et al.* 1998b). Subsequent to intraperitoneal administration fullerenes have also been observed to accumulate within Kupffer cells in the liver (Gharbi *et al.* 2005).

Human information:

The only identified information on humans, a patch testing of fullerene soot within 30 volunteers (Huczko *et al.* 1999) for 96 hours to assess the skin irritant potential, suggested that no detrimental outcome on the skin is apparent, but this requires more extensive investigation.

*In summary, different fullerene types have been shown in two studies to have a very low toxicity after oral exposure as no signs of toxicity have been described for the doses tested. From the identified data it might be possible to derive a NOAEL of 2000 mg kg⁻¹ bw for fullerite (mixture of C₆₀ and C₇₀) (Mori *et al.* 2006) and of 50 mg kg⁻¹ for polyalkylsulfonated (water soluble) C₆₀ (Chen *et al.* 1998b). As only one dose was tested and no dose with an effect has been determined (reported) it might be possible that a higher NOAEL could be determined, especially for the polyalkylsulfonated C₆₀. However as not more information from the study and on the influence of different fullerene types on their toxic behaviour is available, DNELs will be derived from those data. No risk characterisation however will be performed, as there are no data on oral exposure available.*

Following pulmonary exposure fullerenes have shown no or low ability to induce inflammation or even anti-inflammatory responses. It is not known whether these different responses were solely dependent on the dose administered or also on other factors such as the fullerene type or the exposure method. More information is needed where different fullerene derivatives are tested at different doses to make definitive conclusions about what drives the pro- and anti-inflammatory responses observed.

*For a risk characterisation exercise the concentration of 2.22 mg m⁻³ (nanoparticle) from the inhalation study performed by Baker *et al.* 2008 will be used as a NOAEC for deriving a DNEL_{acute} as no inflammatory potential was seen at this concentration. The results from the other studies that showed no inflammation (Sayes *et al.* 2007) or only at high doses (Roursgaard *et al.* 2008) were obtained following intratracheal exposure which confirms the low toxicity of fullerenes for the lung. However they will not be used for the risk characterisation.*

The only identified study investigating effects following dermal exposure (human patch test with fullerene soot) found no detrimental outcome. In vitro tests with fullerenes showed inflammation and dose dependent cytotoxicity. However cytotoxicity was also dependent on the type and level of functionalisation of fullerenes. Therefore no clear conclusion regarding uptake potential or toxicity can be generated for skin at this time, and it is possible that different fullerenes will behave differently. More information on possible local effects and dermal uptake is needed.

Following intraperitoneal injection kidney, liver and spleen have been demonstrated to be a target of fullerene toxicity. An LD₅₀ of 600 mg kg⁻¹ was determined. Mice have shown to be able to generate antibodies against the C₆₀ derivatives, which were also active against other nanoparticles (SWCNT). The relevance of the findings following intraperitoneal injection for primary routes of exposure (inhalation, dermal and oral) has to be further examined in light of the questionable uptake via these routes.

9.3.4.3 Irritation/ Corrosivity

Skin:

The only identified investigation was a patch test model that was used to assess the skin irritant potential of fullerene soot within 30 volunteers (Huczko *et al.* 1999) for 96 hours which suggested that there is no detrimental outcome.

Eye:

A Draize rabbit eye irritation test was performed to reveal the potential toxicity of fullerenes to the eye. Instillation of a fullerene soot suspension (for up to 72 hours) was observed to have no toxicity within the eye (Huczko *et al.* 1999).

In summary, no irritating effects were observed in a human patch testing and in a Draize rabbit eye irritation test with fullerene soot, suggesting that fullerenes may not have an irritating potential to skin and eyes. However further information is needed to prove if all type of fullerenes are not irritating.

No information has been identified on respiratory irritation.

9.3.4.4 Sensitisation

No effects were seen in a human patch testing to assess the skin irritant potential (Huczko *et al.* 1999). No other information on skin and respiratory tract sensitisation is identified. This endpoint needs more extensive investigation. There are indications that C₆₀ derivatives may act as sensitising agents following intraperitoneal exposure (Erlanger *et al.* 2001). The relevance of these findings to the skin and respiratory tract has to be scrutinised.

9.3.4.5 Repeated dose toxicity

Repeated dose toxicity – oral:

No effects were observed after subacute (50 mg kg⁻¹ daily for 12 days) oral exposure to polyalkylsulfonated (water soluble) C₆₀ (Chen *et al.* 1998b). No information on effects after subchronic or chronic exposure has been identified.

Repeated dose toxicity - inhalation:

In a subacute inhalation study 0.12 mg m⁻³ fullerenes did not induce significant inflammation and tissue injury during the inhalation exposure period (28 days). However, some genes associated with the immune system were up-regulated by C₆₀ fullerene particles. It was concluded that fullerenes might not have severe pulmonary toxicity (Fujita *et al.* 2009).

No inflammation was seen after 10 days nasal inhalation of 2.22 mg m⁻³ for 3 hours per day (Baker *et al.* 2008) and 3 months after a single intratracheal instillation of 3 mg kg⁻¹ C₆₀ or C₆₀(OH)₂₄ (Sayes *et al.* 2007). No information after subchronic or chronic exposure has been identified.

Repeated dose toxicity – dermal:

No information after repeated dermal exposure is identified.

9.3.4.6 Biological mechanisms and target organ toxicity of fullerenes

Cardiovascular effects:

In vitro investigations of endothelial cells following acute exposure to C₆₀(OH)₂₄ (1-100 µg ml⁻¹) resulted in internalisation by cells, and a dose dependent decrease in cell viability. Subsequent to a chronic exposure (10 days), fullerenes detrimentally affected cell attachment and slowed cell growth (Yamawaki and Iwai 2006).

Fullerenes have shown to be less effective than other nanoparticles in eliciting the aggregation of platelets and therefore being less thrombogenic (Radomski *et al.* 2005).

Oxidative stress – inflammation – cytotoxicity and formation of granulomas:

In vitro investigations indicate that an inflammatory response may be instrumental to the toxicity of fullerenes, as demonstrated by the enhanced production of pro-inflammatory mediators such as IL-8 and TNFα (see for example Rouse *et al.* 2006). A concentration dependent effect is likely as fullerol has been demonstrated to have an anti-inflammatory effect within the mouse lung at lower doses, but a pro-inflammatory effect at higher concentrations (Roursgaard *et al.* 2008). There is a lack of information regarding *in vivo* inflammatory mediated responses.

Cytotoxicity mediated through enhanced ROS production, lipid peroxidation and membrane damage has been demonstrated for nano-C₆₀ (0.24 – 2400 ppb) in a variety of cell lines (dermal fibroblasts, hepatocytes and astrocytes) (Sayes *et al.* 2005). Similar observations were made for C₆₀, and C₆₀(OH)₁₈ (Kamat *et al.* 2000) eliciting membrane damage under photosensitive conditions, which was accounted for by the appearance of lipid peroxidation within isolated rat liver microsomes. C₆₀(OH)₁₈ showed greater toxicity than C₆₀. No stimulation of ROS production, depletion of GSH or stimulation of HO-1 expression and TNFα production was associated with fullerol within RAW 264.7 macrophages (Xia *et al.* 2006).

A number of studies relating to antioxidant properties of fullerenes suggest that contrary from being toxic, C₆₀ and its derivatives could actually exhibit beneficial health effects through their potential free radical scavenging activity (see for example Xiao *et al.* 2006, Wang *et al.* 1999, Dugan *et al.* 1996, Gharbi *et al.* 2005, Yin *et al.* 2009, Yin *et al.* 2008, Lin *et al.* 2002, Injac *et al.* 2009, Bogdanovic *et al.* 2008). However, it appears that the antioxidant properties exhibited by fullerenes are restricted to particular fullerene forms and depend on water solubility and the concentration administered.

The cell uptake of fullerenes has been demonstrated on numerous occasions, within a variety of cell types. Subsequent to pulmonary exposure fullerenes have been observed within alveolar macrophages (Fujita *et al.* 2009, Xia *et al.* 2006) and subsequent to intraperitoneal administration accumulation within Kupffer cells in the liver has been observed (Gharbi *et al.* 2005). Macrophages therefore appear to be capable of taking up particles, to thereby fulfil their role within host defence. Following uptake of fullerenes oxidative or inflammatory events may be stimulated. In addition, a number of other cell types have been demonstrated to internalise fullerenes, such as keratinocytes (Rouse *et al.* 2006), epithelial cells (Fujita *et al.* 2009) and eye lens cells (Roberts *et al.* 2008) often with oxidative and lethal consequences.

In summary, no sub-chronic or chronic studies with fullerenes for any of the exposure routes have been identified. Information on possible effects after repeated exposure can be received from studies with acute/sub-acute exposure and from in vitro studies with possible target cells. However the results from a single dose administration with toxicity assessed at a number of post exposure time points might be quite different from a repeated dose exposure, which would be a more realistic scenario within occupational or consumer exposure. This should be considered within future studies.

Following acute and subacute oral exposure fullerenes have been shown in a few studies to have a very low toxicity with little absorption through the gutwall. No information has been identified for subchronic and chronic exposure. A NOAEL of 50 mg kg⁻¹ for polyalkylsulfonated

C_{60} can be determined for deriving a DNEL for a risk characterisation exercise from a subacute study (12 days; Chen et al. 1998b) which will be extrapolated for chronic exposure by applying assessment factors (see Derivation of DNELs).

Following pulmonary exposure fullerenes have shown no or low activity to induce inflammation, depending probably on several factors as described above. The effects observed are assumed to have a threshold which allows a quantitative risk characterisation. A LOAEC of 0.12 mg m^{-3} from a subacute inhalation study (Fujita et al. 2009) is suggested to be used for the risk characterisation of repeated inhalation exposure.

Assessment factors for extrapolating for the duration are applied. Longer exposure can lead to more severe effects, however there is also the possibility of recovery. For the risk characterisation progressing effects and no recovery are assumed.

No clear conclusion regarding uptake potential or toxicity can be generated for skin at this time. More information on possible local effects and dermal uptake is necessary.

Following intraperitoneal injection of fullerenes, the liver and spleen have been demonstrated to be a target of fullerene toxicity. Cytotoxic effects to vascular endothelial cells *in vitro* give cause for concern that exposure to fullerenes could be a potential risk for cardiovascular disease initiation or progression. However this requires further investigation.

The main effects associated with fullerene toxicity are inflammatory and oxidative responses as well as anti-inflammatory and antioxidant properties. The results from different *in vitro* and *in vivo* studies show that these effects are very much dependent on the fullerene in question, the concentrations used, the cell type being investigated, and the experimental set up.

A focus of further investigations should be sub-lethal toxicity after subchronic/chronic exposure via relevant exposure routes at relevant concentrations (e.g. work place). The relevance of the findings following intraperitoneal and intravenous exposure as well as effects seen *in vitro* need to be seen in relation to the absorption rates via the different primary routes, which still have to be investigated.

9.3.4.7 Mutagenicity

In vitro data:

Fullerenes have shown to induce DNA damage within human lymphocytes in a Comet assay when exposed at concentrations ranging from 0.42 to $2100 \mu\text{g L}^{-1}$, for up to 6 hours (Dhawan et al. 2006). Mutagenic effects were seen at concentrations up to $30 \mu\text{g}$ per plate, for 48 hours on *Salmonella typhimurium*, in light conditions using the Ames test (Sera et al. 1996) whereas no mutagenic responses were evident, if exposure occurred within the dark, suggesting that photoactivity is able to promote mutagenic responses.

No mutagenic responses of fullerenes were evident within a variety of *Salmonella typhimurium* and *Escherichia Coli* strains, using the Ames test (C_{60}/C_{70} mixture; up to $5000 \mu\text{g}$ per plate) and no aberrations within the structure or number of chromosomes were apparent in a chromosomal aberration test (in CHL/IU hamster lung cells) (Mori et al. 2006). C_{60} (0 - $200 \mu\text{g ml}^{-1}$, 24 or 576 hours) was not capable of eliciting strand breaks, and no alterations in mutation frequency were observed when using the Comet assay (Jacobsen et al. 2008).

In vivo data:

No information identified

In summary, to date, only in vitro mutagenicity tests with fullerenes are available which have reported contradicting results which are likely to be influenced by the dose, dispersion, model and endpoint measured. An important component of the genotoxic response is anticipated to be their photoactivity which is able to promote such a response. Appropriate in vivo studies should confirm the presence or absence of mutagenic effects.

9.3.4.8 Carcinogenicity

No information on carcinogenic effects of fullerenes has been identified.

Some studies have reported anti-tumour effects of fullerenes *in vivo* and *in vitro*, depending on derivatisation, dispersion and light irradiation (Chen *et al.* 2005, Tabata *et al.* 1997, Zhu *et al.* 2008). It would appear that fullerenes can accumulate in tumours due to hyperpermeability of tumour vasculature with very low toxicity to other organs. Light irradiation seems to be essential for tumour destructive effect to manifest.

Gd@C₈₂(OH)₂₂ following intraperitoneal administration has been demonstrated to inhibit the growth of malignant tumours within mice, and that this was due to their ROS scavenging activity (Yin *et al.* 2008).

In summary, no information on carcinogenic effects of fullerenes was identified and there are indications that fullerenes may have anti-tumorigenic effects which could be an indication that carcinogenicity is not an endpoint of concern for fullerenes. However the evidence is not sufficient to draw firm conclusions and more information is needed. A testing strategy for carcinogenicity could be set after information from toxicokinetics, sub-chronic and mutagenicity studies becomes available.

9.3.4.9 Toxicity for reproduction

Effects on fertility:

No information on the male reproductive system has been identified. The only identified information on the female reproductive system is a cytotoxicity test of fullerene C₆₀ particles (10 mg, dissolved in 250 ml tetrahydrofuran) in Chinese hamster ovary mammalian cell line (CHO) which showed a dose and time dependent potential toxicity with an LD₅₀, within 24 hours determined at 33 mg l⁻¹ (Han and Karim, 2009). The use of THF as a solvent in this study renders the evidence of little use for risk assessment purposes.

Developmental toxicity:

Only one *in vivo* mammalian study on the effects of fullerenes on the developing embryo has been identified. Following intraperitoneal administration of polyvinylpyrrolidone solubilised C₆₀ (up to 137 mg kg⁻¹) to pregnant mice, effects like abnormal enlargement of the head, tail abnormalities and dead embryos at the higher doses were seen as well as shrunken membrane and narrow blood vessels of the yolk sack (Tsuchiya *et al.* 1996). The NOAEL was 16.7 mg kg⁻¹. The relevance of the results of this study for risk assessment has to be questioned due to the limitations of the study (low number of animals per exposure group) and the unusual route of administration, using a relatively high exposure dose and covering only a small part of the pregnancy period.

Two studies assessed effects on embryonic development using the zebra fish model. Fullerol (C₆₀(OH)₁₆₋₁₈) had no adverse effects on newly fertilised eggs whereas a C₆₀ suspension had a conspicuous adverse effect on all parameters (survival, hatching rate, heart beat rate and pericardial oedema) that was lessened by the addition of GSH. This suggests that the adverse effects of C₆₀ were due, at least partly, to free radical-induced mechanism or another form of oxidative stress.

Reduced light (and therefore reduced photo catalytic activity) and the co-exposure to the glutathione precursor, N-acetylcysteine (NAC), reduced the effects of C₆₀ on embryos such as mortality and pericardial edema. Fin malformations were only reduced with reduced light (Usenko *et al.* 2008).

In summary, the identified information shows that fullerenes can have effects on ovary cells and on developing embryos (mice and zebra fish). However the mammalian developmental toxicity study has limitations and used an unusual route of exposure. No studies have been identified that focused on other organs or cell types in the female and male reproductive system. More information is needed to show if the identified results are relevant for reproductive organs and embryos via primary routes of exposure and for humans at relevant concentrations.

9.3.5 Derivation of DNEL(s)

Based on the identified information from inhalation and oral toxicity studies, it can be assumed that the effects such as inflammation and oxidative responses have a threshold. It has even been suggested that at lower concentrations, fullerenes may have an anti-inflammatory and anti-oxidative effect. There is not sufficient information identified to conclude on non-threshold genotoxicity or other possible effects without a threshold. Therefore the following attempt for deriving a DNEL is made on the assumption of threshold effects.

No guideline studies have been identified that would be the preferred studies for a regulatory risk assessment. From the identified information, some key studies were selected for deriving DNELs. These studies are described in the effects assessment part and some more information on their quality is given below. The results of these studies are used for the purpose of this risk characterisation exercise only, to get a perception if there could be a risk for workers at the available measured workplace concentrations.

DNELs are derived from the dose descriptors of these key studies by applying assessment factors as described in the REACH guidance on information requirements and chemical safety assessment.

The following results should however not be used for any conclusions or decisions on a risk of fullerenes and have therefore no regulatory relevance as there are too many uncertainties as regards the quality and the representativeness of the data.

Inhalation of fullerenes

Short term exposure:

For acute exposure a NOAEC of 2.22 mg m^{-3} in rats (3 hours per day for 10 consecutive days) is proposed to be used, as no inflammatory potential was seen at this concentration (Baker *et al.* 2008). The Baker *et al.* (2008) study is not a guideline study and was not performed for the purpose of a risk assessment. Therefore it would normally not be sufficient to be used as a key study for deriving a DNEL and for performing a risk assessment. Though, considering the amount and quality of information the study provides, the reliability of the study would correspond to a Klimisch code 2 (Klimisch *et al.* 1997). The results of the study are not in contradiction to results from other similar studies showing low pulmonary toxicity. Therefore the results of this study can be considered to be useful for this risk characterisation.

1) Modification of the starting point (correction of differences between experimental and human exposure conditions)

Worker: 8 hours exposure (light activity): $\text{NOAEC} \times 3 \text{ hours} / 8 \text{ hours} \times 6.7 \text{ m}^3$ (8 hour standard) / 10 m^3 (8 hours light activity)

→ NAEC worker (8 hours): 0.55 mg m^{-3}

2) Interspecies variation:

Allometric scaling: not applicable as DNEL based on an inhalation study
Other interspecies factors: 2.5

3) Intraspecies variation: 5 (worker)

Overall assessment factor:

Short term exposure (worker): $2.5 \times 5 = 12.5$

→ $\text{DNEL}_{\text{inhalation, acute}}$

- for short term inhalation (worker): $0.044 \text{ mg m}^{-3} = 44.4 \text{ } \mu\text{g m}^{-3}$

Chronic exposure:

For chronic exposure a LOAEC of 0.12 mg m^{-3} (6 hours per day, 5 days per week for 28 days) based on weak inflammation is proposed (Fujita *et al.* 2009). The Fujita *et al.* (2009) study is not a guideline study and was not performed for the purpose of a risk assessment. Therefore it would normally not be sufficient to be used as a key study for deriving a DNEL and for performing a risk assessment. However the results of this study are considered to be used for this risk characterisation exercise (with no regulatory relevance) for the following reasons:

No subchronic or chronic study has been identified on fullerenes and no other sub-acute study is available. Considering the amount and quality of information the Fujita *et al.* (2009) study provides the reliability of the study would correspond to a Klimisch code 2 (Klimisch *et al.* 1997). The results of the study are not in contradiction to results from other similar studies showing low pulmonary toxicity. There was only one concentration tested and the study authors suggest that the NOAEL for lung inflammation might be even higher than this dose. Taking into account the up-regulation of a few genes involved in the inflammatory response, oxidative stress, apoptosis and metalloendopeptidase activity as well as in the immune system process (MHC mediated immunity) the concentration of 0.12 mg m^{-3} will be used as a LOAEC. (The expression of these genes was significantly higher in the positive control of ultrafine NiO, leading to acute inflammation).

1) Modification of the starting point (correction of differences between experimental and human exposure conditions)

Worker: 8 hours exposure (light activity): $\text{LOAEC} \times 6 \text{ hours} / 8 \text{ hours} \times 6.7 \text{ m}^3$ (8 hour standard) / 10 m^3 (8 hours light activity)

→ LAEC worker (8 hours): 0.06 mg m^{-3}

2) Extrapolation from a LAEC to a NAEC (extrapolation factor of 3):

→ $\text{NAEC}_{\text{worker}}: 0.02 \text{ mg m}^{-3}$

3) Interspecies variation:

Allometric scaling: not applicable as DNEL based on an inhalation animal study

Other interspecies factors: 2.5

4) Intraspecies variation: 5 (worker)

5) Duration: extrapolation from sub-acute to chronic: 6

Overall assessment factor:

Long term exposure (worker): $2.5 \times 5 \times 6 = 75$

→ $\text{DNEL}_{\text{inhalation, chronic}}$

- chronic inhalation (worker): $0.0003 \text{ mg m}^{-3} = 0.27 \text{ } \mu\text{g m}^{-3}$

Acute oral exposure to fullerite (mixture of C₆₀ and C₇₀)

A NOAEL of 2000 mg kg^{-1} bodyweight for fullerite is derived from an acute oral toxicity study where no signs of toxicity were observed in rats (Mori *et al.* 2006). This study is not a guideline study and was not performed for the purpose of a risk assessment. However considering the amount and quality of information this study provides a reliability corresponding to a Klimisch code 2 (Klimisch *et al.* 1997).

The oral route is not considered an important route of exposure for workers or consumers via consumer products. However after having more knowledge about absorption from different routes, the identified data could be used for assessing the risk via the dermal route following

route-to-route extrapolation. As no exposure data for the oral route (e.g. indirectly via the environment) are available, no risk characterisation exercise will be performed.

1) Interspecies variation:

Allometric scaling: 4 (might not be necessary, depending on type of acute effects)

Other interspecies factors: 2.5

2) Intraspecies variation: 5 (worker) 10 (general public)

Overall assessment factor:

Acute exposure:	2.5 x 4 x 5	= 50 (worker)
	2.5 x 4 x 10	= 100 (general public)

→ DNEL_{oral}

- for acute exposure: **40 (worker) and 20 mg kg⁻¹ bw (general public)**

Chronic exposure to polyalkylsulfonated (water soluble) C₆₀

A NOAEL of 50 mg kg⁻¹ bodyweight for polyalkylsulfonated C₆₀ derived from a sub-acute oral toxicity study in rats (12 days) where no signs of toxicity were observed (Chen *et al.* 1998b) will be used for deriving a chronic DNEL. This study is not a guideline study and was not performed for the purpose of a risk assessment. However considering the amount and quality of information this study provides a reliability corresponding to a Klimisch code 2 (Klimisch *et al.* 1997) could probably be assigned.

1) Interspecies variation:

Allometric scaling: 4 (rat)

Other interspecies factors: 2.5

3) Intraspecies variation: 5 (worker), 10 (general public)

4) Duration: extrapolation from sub-acute (although even shorter) to chronic: 6

Overall assessment factor:

Acute exposure:	2.5 x 4 x 5 x 6	= 300 (worker)
	2.5 x 4 x 10 x 6	= 600 (general public)

→ DNEL_{oral}

- for chronic exposure: **0.17 (worker) and 0.083 mg kg⁻¹ bw (general public)**

9.3.6 Risk characterisation

Exposure to fullerenes will mainly occur via inhalation and the dermal route at the workplace and possibly in addition via the dermal and/or oral route through consumer products and/or indirect exposure via the environment.

Fullerenes are assumed not to be effectively absorbed and to remain at the deposition site (mainly lung and gut). A small proportion has been suggested to be absorbed through the gut wall. No information is available for possible dermal absorption.

More information is needed on the ADME profile of different fullerene types, in order to interpret the effects seen following intraperitoneal and intravenous injection and those *in vitro*.

Fullerenes seem to have a very low toxicity after oral exposure and an acute NOAEL of 2000 mg kg⁻¹ bw for fullerenes and a sub-acute NOAEL of 50 mg kg⁻¹ bw for polyalkylsulfonated C₆₀ from a subacute toxicity study (12 days) are suggested.

Following exposure via the pulmonary route fullerenes were able to induce pro- or anti-inflammatory responses with the factors driving these effects still unknown. An acute NOAEC of

2.22 mg m⁻³ for acute inhalation is suggested as no inflammatory potential was seen at this concentration. In addition a LOAEC of 0.12 mg m⁻³ from a 28 day whole body inhalation study where weak inflammation was observed (Fujita *et al.* 2009) is suggested for deriving a DNEL for chronic exposure.

No clear conclusion regarding uptake potential or toxicity can be generated for skin. Identified studies suggest that fullerenes may not have an irritating potential to skin and eyes. No conclusions can be made on the sensitising properties of fullerenes. Further clarification concerning an irritating and sensitising potential of fullerenes is needed.

No sub-chronic or chronic studies with fullerenes for any of the exposure routes have been identified. The main effects associated with fullerene toxicity as shown by *in vitro* and acute toxicity studies are inflammatory and oxidative responses as well as anti-inflammatory and anti-oxidant properties. Further studies should focus on the sublethal effects following subchronic/chronic exposure via relevant exposure routes at, for humans, relevant concentrations which have to be determined based on a better operational exposure database. These studies should investigate the chronic effects of the observed inflammation. These effects could become more severe with prolonged period, there could however also be recovery of the organism. Prolonged inflammation could also lead to the release of factors which could induce systemic effects –however there is no indication of that yet.

Identified *in vitro* mutagenicity tests have reported contradicting results. Appropriate *in vivo* studies should confirm the presence or absence of a mutagenic effect.

No information on carcinogenic effects of fullerenes has been identified and there are indications that fullerenes may have anti-tumorigenic effects which could be an indication that carcinogenicity is not an endpoint of concern for fullerenes. However the identified information is not sufficient to reach this conclusion. If results from subchronic toxicity and *in vivo* mutagenicity studies become available this endpoint should be re-evaluated.

Effects on female reproductive organs and embryos have been shown under quite artificial conditions. This endpoint should be re-evaluated once more information on the absorption and subchronic/chronic effects become available.

Despite limited information and a lot of uncertainties, the following section attempts to conduct a quantitative risk characterisation for the purpose of this exercise.

9.3.6.1 Risk characterisation for workers

Based on the identified data a threshold for fullerene induced effects is assumed and a risk characterisation exercise is performed. NOAEL(C)s and DNELs for fullerenes have been determined based on results of some key studies.

For acute oral exposure a NOAEL of 2000 mg kg⁻¹ bw for fullerenes is suggested from which DNELs of 40 (worker) and 20 mg kg⁻¹ bw (general public) for acute exposure were derived. For prolonged oral exposure a NOAEL of 50 mg kg⁻¹ bw for polyalkylsulfonated C₆₀ from a subacute toxicity study (12 days) is suggested and DNELs of 0.17 (worker) and 0.083 mg kg⁻¹ bw (general public) for chronic exposure have been derived. As there is no data on oral exposure, no risk characterisation for the oral route can be performed.

For acute inhalation exposure the concentration of 2.22 mg m⁻³ fullerenes is suggested as a NOAEC and a DNEL of 0.044 mg m⁻³ (44.4 µg m⁻³) for short term inhalation was derived.

From a LOAEC of 0.12 mg m⁻³ (28 day whole body inhalation study), where weak inflammation was observed, a chronic DNEL of 0.003 mg m⁻³ (0.27 µg m⁻³) for inhalation is derived.

These DNELs are compared with measured exposure values at the workplace of 50 – 125 µg m⁻³ (PM2.5). The acute DNEL for inhalation is slightly below the lower range of exposure and under such conditions the risk might be controlled. However the lower range of exposure is

higher than the chronic DNEL and the higher range of exposure is higher than the acute and chronic DNEL. Under such conditions the risk is not considered to be controlled.

There are several uncertainties in this risk characterisation exercise from the exposure site as well as from the effects part. Only information on inhalation exposure of fullerenes in factories has been identified and is of limited accuracy. It is not known whether all PM_{2.5} measured were fullerenes, as also carbon nanotubes were produced in the same facility and there is no information about the representativeness of the exposure data.

The chronic DNEL was derived from a LOAEC of a subacute inhalation study with only one dose tested. It is not known from the identified data if effects would increase and become more severe with progressing time and dose applied or if the organism would recover from the effects. A factor of 6 was used for extrapolating from the subacute to the chronic duration. This factor could change when more information on the progression of the effects with dose and time becomes available. However, altogether the available data suggests that there might be a risk for workers, in particular following long-term exposure.

9.3.6.2 Risk characterisation for consumers

Consumers can be exposed to fullerenes via skin creams. Consequently the skin is a potential exposure route for consumers and chronic exposure might be expected. Little information is available investigating irritating or sensitising effects and other local or systemic effects of fullerenes to the skin. Priority should be given to get more conclusive information on possible absorption and thus systemic effects but also on topical toxicity. No quantitative data on exposure of consumers has been identified. However if more information becomes available that other exposure routes of consumers might be relevant, more information would be needed also for inhalation and/or the dermal routes.

9.3.6.3 Risk characterisation for humans exposed via the environment

Based on currently identified information exposure to fullerenes via the environment is unlikely to be at detectable levels. Consequently no risk characterisation can be performed.

9.3.7 Summary

This risk characterisation exercise has shown that based on currently available data risk seems not to be controlled for fullerenes at the workplace. However this risk characterisation exercise is based on the little identified information and includes many uncertainties with respect to the quality and representativeness of the data for toxicity as well as for exposure. The derived DNELs cover only the endpoints acute and chronic inhalation and oral exposure. No suitable data to perform a risk characterisation were identified for dermal exposure and for endpoints like irritation, sensitisation, genotoxicity, carcinogenicity or reproductive toxicity. The risk characterisation would normally have to be conducted on the leading effect or the lowest DNEL for a given exposure pattern, which however it not known, based on the little identified data available. Therefore the results of this risk characterisation exercise should be taken with caution and no definite conclusion should be drawn on a general risk of fullerenes.

It is clear that more information is needed and the main recommendations for further testing or information requirements are listed below.

- Reliable exposure measurements of fullerenes in mass/volume, identification of fullerenes/fullerene type and distinction from background at workplace, but also in or released from consumer products and possibly in the environment;
- Characterisation of fullerenes - reference materials;
- Toxicokinetics (with careful consideration of detection methods and their influence on the results):
 - Absorption of fullerenes via the different exposure routes (inhalation, dermal and oral if it is a relevant exposure route);

- Metabolism/elimination via alveolar macrophages, Kupffer cells or other pathways;
- Repeated dose toxicity studies via inhalation at concentrations relevant for the workplace to detect local and possible systemic effects; Discussion is required to decide whether a 28 day or 90 day study would be more appropriate to see early/prolonged effects, but also not to be affected by recovery; dependent on the results from these studies (e.g. low toxicity confirmed) no guideline acute inhalation study might be necessary;
- Dermal studies:
 - dermal uptake with a skin model;
 - Local/systemic dermal effects (depending on results on dermal uptake);
- Irritation (skin/eye);
- Sensitisation (skin, respiratory tract);
- Genotoxicity: further *in vitro* and *in vivo* investigations to decide on primary and/or secondary genotoxic effects;
- Carcinogenicity: depending on results from genotoxicity tests and subacute/subchronic studies a testing strategy for carcinogenicity might be developed;
- Reproductive toxicity: depending on results from absorption studies (systemic availability) and indications of effects on reproductive organs/hormons from a repeat dose toxicity study.

The information requirements as listed above are based on endpoints that are normally required for testing of chemicals. The requirements for fullerenes or nanoparticles in general might differ from those for chemicals and test systems more appropriate for nanomaterials would be needed to ensure that endpoints of potential particular concern are properly addressed.

Fullerenes exist in a variety of forms (carbon atom number, surface modifications, aggregations states, etc) and it is difficult to make generalisation about their toxic behaviour. At the same time it makes it difficult to set up a testing strategy which could be applied to all fullerene types.

One important issue to be solved is the question regarding what drives the pro- and anti-oxidative and inflammatory properties of fullerenes, to estimate the hazard of different fullerene types and maybe allow for a grouping of them with regard to their toxicity.

So far it has been shown that one of the main factors for differences in fullerene toxicity seems to be the water solubility with fullerenes of greater water solubility being less toxic. In addition, residual solvents (or their derivatives) used within the preparation of fullerene samples and the modification of fullerenes are able to contribute to the observed toxicity. Some of these properties (e.g. functionalisation) have been shown to effect *in vitro* toxicity, however this may not necessarily translate to *in vivo* effects and more information is necessary.

Full physicochemical characterisation is essential to allow comparisons between fullerene toxicity and a risk assessment should focus on the fullerene type and concentrations to which humans are exposed.

9.3.8 Environmental risk assessment for fullerenes

In this basic risk assessment, only data from non-functionalised C₆₀ were considered, apart from one study concerning fullerol (hydroxylated fullerene, C₆₀(OH)₂₂). Therefore, the results of this assessment are valid only for nC₆₀ (i.e. stable dispersed C₆₀ aggregates forming when C₆₀ is suspended in water), and cannot be generalised to all fullerenes including modified forms.

9.3.8.1 Environmental fate properties

Degradation

No studies on the degradation of fullerenes have been identified in the literature.

Environmental distribution

Although studies on the environmental distribution of fullerenes are still lacking there are some reports on their distribution in water and soil. With respect to water, the reported studies concern the influence of natural water composition (i.e. natural substances dissolved in water) on C₆₀ aggregates formation and stability. Normally, fullerenes in water tend to aggregate, forming agglomerates and aggregates, which will settle to a certain extent. However, in some conditions, due to fullerenes' physico-chemical properties (e.g. zeta potential) and environmental characteristics (e.g. pH), fullerenes are stable and stay as primary molecules or very small agglomerates.

Fortner *et al.* (2007) and Xie *et al.* (2008) identified ozone and natural organic matter (NOM) as two environmental factors than can enhance the C₆₀ aggregate dissolution via oxidation and adsorption, respectively.

Studies were also carried out to simulate the transport of fullerenes in soil and sediments, by using synthetic porous media such as quartz sand or glass beads. The main factors influencing the C₆₀ transport were ionic strength, organic content of the soil, and C₆₀ functionalisation (i.e. hydrophilic groups enhance water solubility and thus transport in porous media). The velocity of the water flow seems not to be an important factor, especially for non-functionalised C₆₀, for which affinity between solid phase, fullerene, and fullerene aggregates explained the obtained results. Lecoanet and Wiesner (2004) observed that C₆₀ reached a 55% plateau of removal from porous media (silica glass beads), while 99% of fullerol characterised by a higher hydrophilicity was transported outside the porous media.

Boxall *et al.* (2007) developed a framework of simple models and algorithms to estimate nanomaterial concentrations in water, soil and air of the UK from different routes of exposure (e.g. direct application, air emissions, run-off). For fullerenes, they estimated 0.31 µg l⁻¹ C₆₀ in water and 13.2 µg kg⁻¹ C₆₀ in soil. Some limitations of these exposure estimates are discussed in Hansen (2009).

Bioaccumulation

Only one study on bioaccumulation was carried out. No BCF were reported in the literature. However, Oberdoster *et al.* (2006) reported that uptake can be observed in *Daphnia magna* after exposure to 30 ppm C₆₀ for 5 days. It is not clear whether the uptake would result in a bioaccumulation.

In summary, the available data on environmental fate properties of fullerenes are scarce. Data from carbon nanotubes suggest that there is the possibility of biodegradation of functional groups that can modify the physico-chemical properties of fullerenes in water (Roberts et al. 2007). Transport of fullerenes in water and soil as small aggregates/agglomerates or as primary molecules is the result of the combination of different factors, such as chemical composition of the water and fullerene properties. Uptake of fullerenes by organisms can be expected, but no information is available concerning biomagnification through the food web. Taking into account existing data, it is likely that fullerenes can be transported in water systems for some distance from the source point given the presence of dissolved (or natural) organic material widespread in aquatic systems, and eventually settling when environmental conditions changes (e.g. increased ionic strength e.g. in marine water).

9.3.8.2 Environmental hazard assessment

Aquatic compartment (including sediment)

Fish:

Data available for fish toxicity are often obtained by suspending fullerenes with the support of solvents, and the solvent most used in published studies was THF, which is toxic to fish. There is much debate regarding the use of THF in hazard assessment. Although THF is evaporated after suspensions with nanoparticles, it has been suggested that some THF may be trapped within the fullerene molecule or by-products of THF degradation (Henry *et al.* 2007) may

enhance toxicity. However, other authors have indicated that THF is not instrumental in increasing the toxicity of fullerenes (e.g. Lyon and Alvarez, 2008). At present this issue is still unresolved. Therefore, all data obtained by using the THF dispersion method were not considered in this hazard assessment due to their low reliability.

Table 9.1: Short-term effects of fullerenes on fish

Method	Results	Remarks	Reference
<i>Pimephales promelas</i> Adults male (freshwater)	No observed effects (48h): 0.5 ppm	Water stirred C ₆₀	Zhu <i>et al.</i> (2006a)
<i>Danio rerio</i> Larvae (freshwater)	No observed effects (72h): 6.25 mg l ⁻¹ Significant change in 10 gene expression (72h): 6.25 mg l ⁻¹	Stirred/sonicated C ₆₀	Henry <i>et al.</i> (2007)
<i>Fundulus heteroclitus</i> Larvae and adults (salt water)	No significant mortality, no development and malformation effects; increasing GSH levels (96h): 10 mg l ⁻¹	Stirred C ₆₀ suspension	Blickley and McClelland-Green (2008)
<i>Danio rerio</i> Embryo (freshwater)	Fin malformation and pericardial oedema EC ₅₀ (96h): 0.11 ppm Mortality: LC ₅₀ (96h): 0.19 ppm Mortality LC ₁₀₀ (24h): 0.5 ppm	Exposure under light conditions. DMSO dispersed C ₆₀ No link to light exposure	Usenko <i>et al.</i> (2008)

DMSO = Dimethyl sulphoxide

The endpoints considered in the short term studies for fish included mortality, as well as sublethal effects such as development, malformation, oxidative stress, and gene expression (Table 9.1). Often, no toxicological endpoint was established. If the paper stated e.g. 'no significant mortality', this was not considered as NOEC, since the NOEC is a statistically derived value. In all these cases, the narrative result was kept as reported in the paper, and statistical endpoints such as NOEC or EC₅₀ were only reported if explicitly mentioned by the author of the paper.

The general trend is that exposure to sonicated and stirred C₆₀ (no dispersant) does not lead to mortality or malformation in fish at any life stage, while oxidative stress can be measured via enzymatic (Blickley and McClelland-Green, 2008) and genetic (Henry *et al.* 2007) activity, especially after exposure under light conditions. Mortality (LC₅₀), as well as development abnormalities, were observed by Usenko *et al.* (2008) for *Danio rerio* embryos at 0.19 ppm and 0.11 ppm C₆₀, respectively, but the role of DMSO in the final toxicity results is not clear (may lead to increased fullerene bioavailability), and the relevance of experimental conditions for the environmental exposure conditions cannot be evaluated. Taking into account the available studies, a NOEC_{fish} (short-term toxicity) can be established as 6.25 mg l⁻¹, while the LC50_{fish} (short-term) is over 10 mg l⁻¹.

Table 9.2: Long-term effects of fullerenes on fish

Method	Results	Remarks	Reference
<i>Fundulus heteroclitus</i> Embryo (saltwater)	No mortality, development delays, or malformations (12 days): 10 mg l ⁻¹	Aqueous C ₆₀ suspension	Blickley and McClelland-Green (2008)
<i>Carassius auratus</i> Juvenile (freshwater)	No unusual behaviour and no mortality (32 d): 1.0 mg l ⁻¹ Lower mean total length NOEC (32 d): 0.04 mg l ⁻¹ ; LOEC (32d): 0.2 mg l ⁻¹ Body weight LOEC (32d): 1.0 mg l ⁻¹ Increase of oxidative stress (32d): 1.0 mg l ⁻¹	Stirred aqueous C ₆₀ suspension	Zhu <i>et al.</i> (2008b)

The two studies carried out for 12 and 32 days exposure on embryos and juveniles are not comparable, due to the different experimental conditions, such as different species, stages, and habitat (Table 9.2). However, in both studies no mortality or developmental delays and abnormalities were observed up to 10 mg l⁻¹ and 1 mg l⁻¹ C₆₀, respectively. The longer study on *C. auratus* juveniles highlighted the possibility of reduced growth (body weight) and increase of oxidative stress at 1 mg l⁻¹ C₆₀ after 32 days of exposure. The lowest long-term NOEC_{fish} (length) reported is 0.04 mg l⁻¹ C₆₀ (Zhu *et al.* 2008b).

Aquatic invertebrates:

Data available on invertebrate toxicity are often obtained by suspending fullerenes with the support of solvents with the most used solvent being THF. As described above, results from such experiments may be controversial. Therefore, all the data obtained by using the THF dispersion protocol were not considered in this hazard assessment.

Table 9.3: Short-term effects on aquatic invertebrates

Method	Results	Remarks	Reference
<i>Daphnia magna</i> (freshwater)	Mortality LC ₅₀ (48h): 7.9 mg l ⁻¹ ; LOEC (48h): 0.5 mg l ⁻¹ ; NOEC (48h): 0.2 mg l ⁻¹	Sonicated C ₆₀ suspension	Lovern and Klaper (2006)
<i>Daphnia magna</i> (freshwater)	LC ₅₀ (48h): 35 mg l ⁻¹	Exposure to hydroxylated fullerenes (fullerol); sonicated C ₆₀	Zhu <i>et al.</i> (2006a)
Harpacticoid copepod (species not specified)	No mortality (96h): 22.5 mg l ⁻¹	Stirred C ₆₀	Oberdoster <i>et al.</i> (2006)

In the short-term studies with crustaceans, as summarised in Table 9.3, lethal concentrations were 7.9 mg l⁻¹ (LC₅₀) for *D. magna* exposed to sonicated C₆₀ and over 22.5 mg l⁻¹ for copepod species exposed to stirred C₆₀. Moreover, functionalised C₆₀ (hydroxylated) was less toxic than nC₆₀, with a LC₅₀ over 35 mg l⁻¹. According to these data, *D. magna* is the most sensitive

species, and an $LC_{50\ Daphnia}$ (short-term) of $7.9\ mg\ l^{-1}\ C_{60}$ can be established. In addition, an acute no effect concentration $NOEC_{Daphnia}$ (short-term) of $0.2\ mg\ l^{-1}$ is provided.

Table 9.4: Long-term effects of fullerenes on aquatic invertebrates

Method	Results	Remarks	Reference
<i>Daphnia magna</i>	Significant reduction of number of offspring (21d): $2.5\ mg\ l^{-1}$ Delay of moulting of carapace (21d): $2.5\ mg\ l^{-1}$ 40% organisms dead (21d): $2.5\ mg\ l^{-1}$	Stirred C_{60} Effect from day 5. Could be the cause of reduction of offspring (comment from the authors)	Oberdoster <i>et al.</i> (2006)

Long-term exposure of *Daphnia magna* to $2.5\ mg\ l^{-1}\ C_{60}$ revealed in a delay of moulting and a significant reduction in offspring (Table 9.4). However, the authors comment that the effect on reproduction could have been caused by mortality which occurred from day 5 onwards. A $NOEC_{Daphnia}$ (long-term) was not reported, but should be $< 2.5\ mg\ l^{-1}\ C_{60}$.

Sediment organisms:

Table 9.5: Effects of fullerenes on sediment organisms

Method	Results	Remarks	Reference
<i>Hyalella azteca</i>	No mortality observed (96h): $7\ mg\ l^{-1}$	Stirred C_{60}	Oberdoster <i>et al.</i> (2006)

Hyalella azteca, a benthic amphipod living in freshwater sedimentary habitats, exposed to up to $7\ mg\ l^{-1}$ stirred C_{60} , showed no sign of mortality (Table 9.5). In addition, no sub-lethal endpoints were addressed.

Terrestrial compartment

Terrestrial studies carried out for fullerenes included two with microbial communities and one with invertebrate taxa. Microbial communities are relevant both for natural environments and for wastewater treatment plants, where they are instrumental in the degradation of nutrients and a range of other chemicals.

Toxicity to soil macro organisms:

Table 9.6: Effects of fullerenes on soil macro organisms

Method	Results	Remarks	Reference
<i>Eisenia veneta</i> (earthworm)	No significant mortality observed (28d): $1\ g\ kg^{-1}$ dry food No significant reduction in growth and cocoon production and hatchability (28d): $1\ g\ kg^{-1}$ dry food No significant hatchability reduction: $1\ g\ kg^{-1}$ dry food	Single dose exposure	Scott Fordsmand <i>et al.</i> (2008)

No lethal or sub-lethal effects were measured on earth worms exposed up to $1\ g\ kg^{-1}\ C_{60}$ dry food (Table 9.6). A $NOEC_{earthworm}$ (long-term) was not reported by the author, but this study suggest that it is over than $1\ g\ kg^{-1}$ food.

Toxicity to soil micro-organisms:

Short term exposure (3 hours) of microbial communities to 50 µg C₆₀ per g soil inhibited colony forming, while long-term exposure (23 days) to the same concentration showed no significant effects (Table 9.7). It should be noted that fullerenes in soil are present as aggregates, rather than primary particles, and the size of the nanomaterial can affect the toxicity. The no effect concentration identified in these studies range from 1 to 50 mg kg⁻¹ soil. Tong *et al.* (2007) added nutrients to the soil, this resulted in no observed effects up to the maximum exposure concentration (1 g kg⁻¹ soil).

Table 9.7: Effects of fullerenes on soil micro organisms

Method	Results	Remarks	Reference
Bacterial community	No change of basal respiration in soil and bacterial activity after adding nutrients (30 days): 1000 µg g ⁻¹ soil No change of basal respiration in soil (30d): 1 µg g ⁻¹ soil	Granular C ₆₀ Granular C ₆₀	Tong <i>et al.</i> (2007)
Bacterial community	60% inhibition of CFU (3 hours): 50 µg g ⁻¹ soil No significant effects on CFU (23 days): 50 µg g ⁻¹ soil No significant effect on biomass (3 hours): 50 µg g ⁻¹	C ₆₀ aggregates	Johansen <i>et al.</i> (2008)
Protozoan species	No significant organisms number reduction (3 hours): 50 µg g ⁻¹	C ₆₀ aggregates	Johansen <i>et al.</i> (2008)

CFU = Colony Forming Unit

9.3.9 Risk characterisation and gap analysis

Quantitative risk assessment:

The regional PEC (0.31 µg l⁻¹ C₆₀) estimated by Boxall *et al.* (2007) for UK is highly uncertain due to lack of data about source, use, fate, and transport. Furthermore, with respect to the PNEC for the aquatic environment, short-term data are only available for *Daphnia magna*, while no data for fish and algae were identified. Long-term studies are available for fish and *Daphnia magna*. For fish the most sensitive endpoint was length with a NOEC_{fish} of 0.04 mg l⁻¹, while for *D. magna* no NOEC was established. Therefore, given the poor ecotoxicity database, and taking into account that for one trophic level (primary producers) no single study was identified, a reliable PNEC for fullerenes cannot be calculated. Thus, a quantitative risk assessment cannot be performed due to the lack of data. Therefore, only a qualitative assessment is made.

Qualitative risk assessment:

The information available so far leads to the conclusion that non-functionalised C₆₀ is toxic for aquatic organisms. A study with fish observed sub-lethal effects on growth at 0.04 mg l⁻¹. For *Daphnia magna* effects were observed both in short-term and long-term studies at concentrations of 7.9 and 2.5 mg l⁻¹, respectively. In addition, there is evidence that *D. magna* can uptake C₆₀ from water. However, no lethal toxicity was observed in other species (both aquatic and terrestrial). Given the very limited exposure information available, it is not possible to evaluate the relevance of the toxicity concentrations for the environment. The C₆₀ dispersion stabilisation/disaggregation due to natural organic matter should also be considered, since it can modify the distribution of C₆₀ in different environmental compartments (e.g. along a river, even at a large distance from the source).

Concerning functionalised fullerenes, the study on fullerol (Zhu et al. 2006a) suggests that hydroxylised fullerenes are less toxic to *Daphnia magna* than C₆₀. However, this cannot be generalised to other functionalised fullerenes and to other species.

In order to estimate the risk of C₆₀ for the aquatic environment, it would be necessary to measure/estimate fullerenes concentrations in water, and to study any toxic effect under realistic exposure conditions, considering the use of well characterised natural waters. Moreover, the effect of different functionalisations in relation to both biotic and abiotic factors should be studied case by case.

Additional gaps identified are:

1. Lack of information on production volumes of C₆₀, in its many forms;
2. Lack of information on the number of C₆₀ products, market penetration, and amounts of the nanoparticles in these products;
3. Lack of information on behaviour of C₆₀ during wastewater treatment;
4. Lack of exposure and monitoring data on C₆₀ in environmental compartments;
5. Very few information about environmental fate, limited to aggregation in natural water;
6. General lack of toxicity data, but especially for algae, sediment organisms and terrestrial organisms;
7. Lack of toxicity long-term studies for aquatic compartment, i.e. long-term studies on fish and *Daphnia*;
8. Lack of information concerning interaction between organisms and C₆₀ (adsorption, uptake, bioaccumulation, etc.);
9. Interaction of fullerenes with chemicals should also be considered in a full risk assessment, since interaction with fullerenes can increase the bioavailability and accumulation of organic and inorganic chemicals, increasing also their toxicity.

9.4 CARBON NANOTUBES

9.4.1 Identity

Carbon nanotubes (CNT) are carbon allotropes with cylindrical graphite structure. Nanotubes are categorised in two families: single walled nanotubes (SWCNT) and multi walled nanotubes (MWCNT).

SWCNT can be described as a strip of graphene rolled up to form a cylinder. They have a typical diameter of ca. 1 nm (ca. 10 atoms around the cylinder), with a tube length that can vary over 6 orders of magnitude from 1 nm to 1 mm. Double walled carbon nanotubes (DWCNT) consist of two SWCNT arranged in a co-axial form.

MWCNT consist of multiple rolled layers (concentric tubes) of graphite and have either the form of a coaxial assembly of SWCNT similar to coaxial cable or as graphene sheet rolled into the shape of a scroll. The diameters of MWCNT are typically in the range of 5 nm to 50 nm. MWCNT can vary between samples with respect to the number of walls and therefore diameter, resulting in variation of their properties between samples.

Beside their wall number carbon nanotubes can differ from each other in their length, surface modification, presence of contaminants and their propensity to form agglomerates and aggregates. DWCNT are an important group because they can be functionalised without disrupting the CNT peculiar mechanical properties. Examples of CNT functionalisation are fluorination, aryl diazonium salt addition, organic acid groups addition and hydroxylation. CNT can also be coated with proteins (i.e. phosphatidyl choline), polymers, or metals in order to be better dispersed in other materials or to obtain specific functions.

All these factors impact the physico-chemical properties of CNT and consequently different types of CNT should be seen as different substances having their own chemical identity. These different physico-chemical properties may influence their biological activity, which therefore renders it difficult to make generalisations with regard to toxicological effects of CNT and consequently for a risk assessment.

9.4.2 Manufacturing and use

CNT are usually produced in closed systems, thus exposure and emissions can be expected only when the system is opened because of e.g. extraction or cleaning of the system.

CNT are mainly used in electronics and polymer industry additives/inclusions to enhance or modify the properties of other materials e.g. polymers, semiconductors, fibres, batteries and hydrogen containers, etc. All these uses require incorporation of the CNT into a composite material, make the probability of direct release into the environment during the service life less likely.

However, the market request of CNT is high, with a global production capacity over 2.5 T per day (mainly MWCNT). Therefore the potential for widespread uses and distribution of CNT is high. SWCNT are presently only produced on a small scale.

9.4.3 Human exposure assessment

Occupational exposure

Occupational exposure to CNT may occur during production, in the laboratory and during downstream applications. In the occupational setting the following activities have been identified: synthesis, recovery/bagging, cleaning, handling/processing, deliberate agitation and sawing composite.

Inhalation exposure:

Measurements of carbon nanotubes at workplace/laboratories were reported in 5 publications of which 4 give concentrations for inhalation exposure. The measurements were for handling unrefined material within a range of $0.7 \mu\text{g m}^{-3}$ (ablation facility) and $53 \mu\text{g m}^{-3}$ (HIPCP process) (Maynard *et al.* 2004) and between 64 and $93 \mu\text{g m}^{-3}$ for weighing and mixing of CNT and up to $1094 \mu\text{g m}^{-3}$ for operations with the wet saw (Methner *et al.* 2008).

Han *et al.* (2008) measured concentrations in a carbon nanotube research laboratory of up to $430 \mu\text{g m}^{-3}$ during blending and before any exposure control was introduced. After introduction of control measures the maximum measured concentration was $40 \mu\text{g m}^{-3}$. In the blending scenario quite high fibre concentrations of between 193.6 and $172.9 \text{ fibre ml}^{-1}$ were found, however strictly applying the WHO rules (WHO 1997) where the minimum size of a fibre is 5000 nm the fibre count would be zero.

In a study by Bello *et al.* 2009, airborne concentrations of respirable fibres ($5\text{--}20 \text{ nm}$ in diameter) were measured to be 1.6 (CNT alumina, Base alumina) and $3.8 \text{ fibres cm}^{-3}$ (Base carbon) measured at source during dry cutting, while this was reduced to $0.2 \text{ fibres cm}^{-3}$ at the breathing zone (Bello *et al.* 2009).

From the identified information (dry) cutting and blending seem to be exposure situations which result in high exposure to CNT in the workplace. Exposure control might reduce exposure by 90% according to some studies (Han *et al.* 2008).

It is however difficult to derive absolute exposure values of CNT that could be used for risk assessment. Measurements for different worker scenarios were made, for which only a few measurements are available, therefore making it difficult to judge on their representativeness.

Often only the particle concentration is measured which makes it difficult to distinguish the measurements of particles from background and to know the fraction of the airborne concentration which can be attributed to CNT. It would also be important to have more information on different types and sizes of CNT in order to know the fraction of respirable CNT or of "fibre".

Different measurement techniques also give concentrations in units of particles/volume and mass/volume, which again makes it difficult to compare studies with each other.

From the described exposure data the following exposure situations and mass concentrations of CNT could be used as an example for a risk assessment:

1. Production of SWCNT (HIPCO process): $53 \mu\text{g m}^{-3}$ (Maynard *et al.* 2004)
- 2a. Blending before implementation of exposure control: $430 \mu\text{g m}^{-3}$
- 2b. Maximum measured concentration following implementation of exposure control (hood and ventilation): $40 \mu\text{g m}^{-3}$ (both from a carbon nanotubes research laboratory; Han *et al.* 2008)
- 3a. Mixing of CNT: $93 \mu\text{g m}^{-3}$
- 3b. Operations with the wet saw: $1094 \mu\text{g m}^{-3}$ (both measured during polymer composite laboratory operations; Methner *et al.* 2006)

The following measurements of respirable CNT fibres (5–20 nm in diameter) could also be used as an example for a risk assessment:

- 4a Dry cutting at source: CNT alumina: $1.6 \text{ fibres cm}^{-3}$
- 4b Dry cutting at breathing zone: CNT alumina: $0.2 \text{ fibres cm}^{-3}$ (both measured during machining of hybrid advanced composites containing carbon nanotubes; Bello *et al.* 2009)

Dermal exposure:

Information on occupational dermal exposure to CNT has been identified in one publication only (Maynard *et al.* 2004). Using the highest value from this study as an example for risk assessment would lead to a dermal exposure value of 6020 μg per person or a concentration of $14.3 \mu\text{g cm}^{-2} \text{ day}^{-1}$ (assuming a surface of 420 cm^2 , both hands, fingers and palms). The use of gloves could reduce the exposure by 90%.

Consumer exposure

Relatively little information has been identified on how consumers are exposed to carbon nanotubes. Mueller and Nowack (2008) indicated that possible applications of CNT which could result in exposures to humans included plastics, sporting equipment and electronic equipment. The use of CNT in textiles has also been reported. In addition there is potential for significant use of CNT in building materials. The main exposures would result from abrasion from these products and from disposal/recycling. Exposure via medical devices is not within the scope of this risk assessment.

Exposure of humans via the environment

Mueller and Nowack (2008) used a life-cycle perspective to model the quantities of engineered nanoparticles (CNT, nanoparticle silver and nanoparticle TiO_2) released into the environment. The quantification was based on a substance flow analysis from products to air, soil, and water in Switzerland. The model inputs used were: estimated worldwide production volume, allocation of the production volume to product categories, particle release from products, and flow coefficients within the environmental compartments. The calculated Predicted Environmental Concentrations (PEC) for CNT are shown below in Table 9.8.

Table 9.8 Calculated PEC values for CNT

Environmental compartment	Realistic exposure	High exposure
air ($\mu\text{g m}^{-3}$)	0.0015	0.0023
water ($\mu\text{g l}^{-1}$)	0.0005	0.0008
soil ($\mu\text{g kg}^{-1}$)	0.01	0.02

The authors note that the lack of information concerning nanoparticle usage in consumer products is a limitation to their work. In order to address the uncertainty of data in this work, two scenarios were modelled; a realistic exposure scenario based on the most reliable data and a

high exposure scenario including the worst-case assumptions. It is likely that the production volumes of CNT and other nanoparticles will increase significantly in the coming years.

9.4.4 Human health effects assessment of carbon nanotubes

The potential toxicology relating to carbon nanotubes exposure focuses on respiratory and dermal effects, due to their propensity to be exposed during their manufacture or utilisation. Ingestion is considered not a relevant route of exposure at the workplace. However some exposure via the GIT can occur as a consequence of hand-to-mouth contact and due to mucociliary clearance. Oral exposure could be relevant for certain consumer products and for exposure via the environment (i.e. drinking water, food).

The toxicity of CNT in biomedical applications (e.g. drug delivery vehicles) and their risk are not part of this review.

Based on their different physico-chemical properties different carbon nanotubes samples may have different biological activities, which makes it therefore difficult to make generalisations regarding their toxicity.

9.4.4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

Absorption

Inhalation:

Subsequent to pulmonary exposure, it is anticipated that a considerable fraction of CNT remain within the lung, suggesting that they are biopersistent (Deng *et al.* 2007, Muller *et al.* 2005), which is likely to derive from their composition, and dimensions. The removal of deposited MWCNT, from the lung, was illustrated by Deng *et al.* (2007) who demonstrated that subsequent to intratracheal administration by day 28 only 20% of the administered dose of the MWCNT remained within the lung.

Thus far, no studies have addressed whether CNT can translocate into the blood from the lungs, skin or GIT. One *in vitro* study with lung airway Calu-3 epithelial cells (Rotoli *et al.* 2008) has suggested that MWCNT (110-170 nm diameter, 5-9 µm long) may increase lung paracellular permeability, which may therefore allow for the greater translocation of CNT from the lung into blood or the pleura, but this finding requires further confirmation *in vivo* and it was not demonstrated with SWCNT (0.7-1.2 nm diameter, 2-20 µm long) or ultrafine carbon black exposure.

Oral:

In one study, after oral administration, the majority of MWCNT were evident within the faeces and also remained within the stomach, small and large intestines, with no detectable transport into the blood. The MWCNT remained unchanged suggesting that they could be biopersistent if not excreted (Deng *et al.* 2007).

Dermal uptake:

No information identified.

Distribution

The distribution of CNT to various organs has been reported following intravenous exposure (Yang *et al.* 2008; Cherukuri *et al.* 2006; Yang *et al.* 2007; Wang *et al.* 2004; Deng *et al.* 2007), with predominant localisation within the liver, lungs and spleen. Following intraperitoneal administration, SWCNT have also been illustrated to accumulate within a number of body compartments (with predominant localisation within bone, stomach and kidneys), with their elimination within urine also apparent (Wang *et al.* 2004).

It is conceivable that subsequent to exposure via other routes (i.e. pulmonary, dermal or oral administration), a similar distribution pattern would be expected to that observed following intravenous or intraperitoneal injection, but this would be dependent on confirmation of the fact

that CNT can actually access the blood from the lungs, skin and GIT, which at this time is unknown.

Translocation from the lung to the pleura is important to consider if CNT are to behave like pathogenic respirable fibres. To date there is no peer reviewed published evidence for or against such transport, although there are web reports that this might be possible.

There is evidence that CNT can elicit toxicity at sites of accumulation following intravenous injection. In one study it was evident that serum transaminases were increased, thus indicating damage to the liver that may be associated with accumulation of SWCNT (Yang *et al.* 2007). In another study, MWCNT were phagocytosed by Kupffer cells within the liver with no apparent toxicity, as indicated by serum indicators and histopathological analysis (Deng *et al.* 2007).

Metabolism

No information regarding the metabolism of CNT has been identified, but it is unlikely that CNT are degraded, due to their biopersistent nature as shown by Deng *et al.* (2007) and Muller *et al.* (2005). Modification of physicochemical characteristics can impact upon biopersistence, for example shorter fibres have shown to be more easily cleared by macrophages (Muller *et al.* 2005).

Elimination

Limited evidence is available that describes the elimination of CNT. In a study conducted by Singh *et al.* (2006), mice were injected (up to 400 µg per mouse) with radiolabelled and ammonium functionalised SWCNT to make them more water soluble, and their biodistribution and subsequent clearance was followed. A rapid excretion via the kidneys was evident, so that SWCNT were demonstrated to have a blood half-life of 3 hours.

It is important to note that the CNT used were relatively short (1 nm diameter, 300-1000 nm long) and functionalised in such a way to ensure that their removal from the body was promoted, and so the applicability of the findings to other CNT requires assessment. Wang *et al.* (2004) have also demonstrated similar results, whereby hydroxylated SWCNT were excreted into urine.

*In summary, subsequent to pulmonary exposure, it is likely that a considerable fraction of CNT remain within the lung, which would suggest that they are biopersistent in nature, which is likely to derive from their composition, and dimensions. Deposition of CNT within the lung depends upon their aerodynamic properties; it is expected that long, thin fibres will penetrate deeper into the airways than aggregates (Warheit *et al.* 2004). Deposition in the deeper airways such as the alveoli will require removal via phagocytic cells, which will be influenced by fibre length. After oral exposure, the majority of CNT are eliminated within the faeces or remain within the GIT, with no detectable transport into the blood. Thus far no studies have specifically addressed whether CNT can translocate into the blood from the lungs, skin or GIT and no studies have identified migration from the lung into the pleura.*

*Epithelial barriers, evident at the exposure site are in place to protect against the transfer of CNT into the circulation, and thereafter prevent their access into other secondary target sites. As a result, it is necessary to determine if this protection is overcome by CNT, to evaluate their systemic uptake and therefore availability to multiple target sites, where they may accumulate and elicit toxicity. It is possible that exposure to either CNT themselves, other toxic substances, or disease could inflict damage to the epithelium and thereby lead to an increase in cellular permeability, as MWCNT have in fact been demonstrated to impair the paracellular permeability *in vitro* (Rotoli *et al.* 2008). However, such penetration has not yet been demonstrated *in vivo*.*

It is anticipated that if CNT are absorbed into the circulation, they would behave in a similar manner to that observed following intravenous exposure, with distribution to various organs expected, and predominant localisation within the liver, lungs and spleen likely. The localisation of CNT within these organs is likely to derive from their accumulation within resident macrophage populations, which could initiate an inflammatory response.

The behaviour of CNT within the human body is likely to be dependent on their properties. Long straight or intact unground MWCNT have been shown to be biopersistent with the propensity of formation of granulomas (Muller et al. 2005; Poland et al. 2008). The study by Poland et al. 2008 suggests that long straight CNT have the potential to behave like pathogenic fibres when in contact with the mesothelium, the tissue that generate the fatal tumour mesothelioma. It is also relevant that the attachment of different surface moieties, or utilisation of CNT of different dimensions, may alter their behaviour.

Detection of CNT within organs will be influenced by their aggregation status and individual CNT may not be visible by conventional imaging. Radiolabelling of CNT or attaching fluorescent probes to their surface could in turn alter their physicochemical characteristics and hence translocation or toxicity.

No information regarding the metabolism of CNT has been identified, but it is unlikely that CNT are degraded due to their biopersistent nature.

It would appear that CNT can be eliminated in urine, but this is likely to be driven by particular physicochemical characteristics (size, functionalisation).

At present it is not possible to make definite conclusions regarding the adsorption, distribution and longevity of CNT within the body. More information is needed and future studies should mainly focus on investigating if and how carbon nanotubes can be absorbed. This is important to know in order to determine the relevancy of toxicological findings within different target sites following intravenous or intraperitoneal exposure or in vitro, as if CNT are unable to access the systemic circulation, addressing their toxicity at the secondary target sites becomes irrelevant.

All of these investigations should include an analysis of the influence of different compositions of CNT and their physicochemical characteristics in order to decide whether results are applicable to CNT in general or to certain CNT types only. It should also give an indication for further toxicity testing whether a grouping on CNT is possible in relation to certain endpoints and results can be used mutually.

9.4.4.2 Acute toxicity

Most of the studies on CNT after single exposure or exposure for a short period (4 – 24 days) have investigated the pulmonary effects after instillation, aspiration and inhalation.

Acute toxicity: oral

No information identified

Acute toxicity : inhalation

The effects of CNT (SWCNT and MWCNT) after single/acute exposure via intratracheal instillation or inhalation have been investigated in mice and rats. CNT exposure resulted in an acute, neutrophil driven inflammatory and fibrotic response, with granuloma development associated with CNT aggregates following single intratracheal instillation (Lam *et al.* 2004, Warheit *et al.* 2004,) and pharyngeal aspiration (Shvedova *et al.* 2005, Shvedova *et al.* 2007). This response is predominantly derived from studies focused on SWCNT but was also seen in one study after instillation of MWCNT (Muller *et al.* 2005). However some studies (Mitchell *et al.* 2007, Li *et al.* 2007a) showed no evidence of granuloma after exposure to CNT. Mortality has been observed in 2 studies (Lam *et al.* 2004, Warheit *et al.* 2004) following intratracheal instillation of high doses (16.7 mg kg⁻¹ in mouse; 5 mg kg⁻¹ in rat) of SWCNT.

Acute toxicity: dermal

Most information can be derived from a limited number of rodent studies with implanted carbon based nanomaterials which were made to gain an understanding of their biocompatibility for use in medical devices. The duration of such implants was between 1 week and up to 3 months.

One mouse study with dermal exposure to unpurified SWCNT for 5 days showed inflammation and oxidative stress.

9.4.4.3 Irritation/Corrosivity

No information on irritating/corrosive effects of CNT has been identified.

9.4.4.4 Sensitisation

No information on sensitising effects of CNT has been identified.

9.4.4.5 Repeated dose toxicity

Repeated dose toxicity: oral

No information has been identified.

Repeated dose toxicity: inhalation

From the studies investigating the effects after pulmonary exposure to CNT only a few have investigated the effects after repeated inhalation exposure. The periods of repeated exposure of mice were between 4 and 24 days and post exposure observation periods were up to 90 days. The exposure duration was relatively short, however the airborne concentrations used were relatively high and such a cumulative exposure could potentially be expected over the working lifetime of an individual.

Mitchell *et al.* (2007) assessed both the pulmonary and systemic immune response of mice to the inhalation of MWCNT (10-20 nm x 5-15 μm , 0.5% nickel and iron impurities). The whole body exposure occurred for 6 hours per day and for a duration of 7 or 14 days, at concentrations of 0.3, 1 and 5 mg m^{-3} (with agglomeration increasing with concentration). The exposure system used in this inhalation study was designed to simulate a potential exposure to MWCNT in the workplace or by an end user. While the highest exposure concentration mimicked the current occupational exposure guideline for nuisance dusts, it was approximately 100 times the concentration of CNT found in an industrial hygiene report by Maynard *et al.* (2004).

Following inhalation for 14 days it was observed that MWCNT were engulfed by alveolar macrophages and MWCNT were distributed throughout the lung. However, no increases in inflammatory cell infiltration were found, with the lack of inflammation, granuloma formation, fibrosis and tissue injury (confirmed by histopathological analysis) up to the highest dose tested. Based on these results the highest concentration tested of 5 mg m^{-3} could be used as a NOAEC for pulmonary effect. However, despite the lack of local, pulmonary effects the systemic immunity was affected at all concentrations tested. Immune function measurement on spleen-derived cells showed suppressed T-cell-dependent antibody response, decreased proliferation of T-cells following mitogen stimulation, and altered NK cell killing. These results were accompanied by increased NQO1 and IL-10 gene expression (indicators of oxidative stress and altered immune function, respectively) in the spleen, but not in the lung. Based on these results the lowest tested dose of 0.3 mg m^{-3} could be used as a LOAEC for systemic immunity effects.

Subsequent to inhalation (6 hour exposure/day, at a mean concentration of 32.61 mg m^{-3} for 8, 16 or 24 days, Li *et al.* (2007b) observed that MWCNT (50 nm x 10 μm , > 95% purity) agglomerates were observed adhered to, and within the bronchial wall, but no inflammatory cells surrounded them. Smaller MWCNT agglomerates were present within alveoli, and promoted the thickening of the alveolar wall (indicative of a fibrotic response), but their structure remained intact. The pathological lesions that developed within inhalation groups where MWCNT that were better dispersed than those observed within intratracheal instillation groups in the same study, and were suggested to occur due to differences in the size and therefore distribution of CNT within the lung. In the instillation groups 0.05 mg MWCNT per mouse were administered, which is slightly lower than the calculated intralung deposition dose in the 8 day

inhalation group (0.07 mg). However the effects seen following intratracheal instillation were more severe. Similar size clumps of MWCNT were distributed in bronchi and alveoli. The clumps led to inflammation to the lining wall of bronchi and severe destruction to the alveolar structure.

Shvedova *et al.* (2008a) also exposed mice via whole body inhalation, using an aerosol of SWCNT of 5 mg m⁻³, at 5 hours per day, for 4 days and conducted toxicological investigations at 1, 7 and 28 days after exposure. The SWCNT sample contained 17.7% iron and possessed dimensions of 0.8-1.2 nm diameter and 100-1000 nm in length. The pulmonary response to inhalation was compared to that following exposure via pharyngeal aspiration. Overall, it was demonstrated that although the pathology exhibited within both exposure scenarios was similar, inhalation was most effective at producing inflammatory, oxidative, fibrotic and mutagenic responses, as the magnitude of toxicity was consistently lower when mice were exposed via aspiration.

Repeated dose toxicity: dermal

In vivo:

Most information about dermal effects can be retained from rodent studies with subcutaneous implants of CNT tested for medical devices. Both SWCNT and MWCNT were able to induce inflammation and the formation of granulomas that encapsulated CNT aggregates (Koyama *et al.* 2006, Yokoyama *et al.* 2005, Sato *et al.* 2005).

Only one short term dermal study in mice has been identified. Murray *et al.* (2009) exposed mice dermally to unpurified SWCNT, daily for 5 days at doses of 40, 80 or 160 µg per mouse. They demonstrated at 160 µg per mouse a significant increase in skin bi-fold thickness (150%), which is a measure of oedema and inflammation development. Inflammation was confirmed by an increase in cells in the epidermis (25% and 58% at 80 and 160 µg per mouse respectively), mast cells in the dermis (90%; 160 µg per mouse), and neutrophil influx (21%; 160 µg per mouse). A significant increased collagen accumulation (12%) was observed at the highest dose only. Oxidative stress was also associated with exposure, and was indicated by a decrease in GSH (11% at 160 µg per mouse), and oxidation of protein thiols and carbonyls (34 and 41% at 80 and 160 µg per mouse respectively). The highest dose (160 µg per mouse) also induced a significant release of the inflammatory cytokines IL-10 and IL-6 (100% and 80% respectively), whereas no changes were observed in MCP-1, IFN-γ, TNF-α and IL-12. Exposure to the lowest dose tested (40 µg per mouse) did not reveal any statistically significant changes. In the absence of any other qualitatively better data this study will be used for deriving a dermal DNEL and the lowest tested dose of 40 µg per mouse will be used as a NOAEL.

In vitro:

Results from *in vitro* tests to assess the dermal toxicity of CNT suggested that inflammatory and oxidative responses are involved in the response of skin cells (keratinocytes) to SWCNT and MWCNT (Shvedova *et al.* 2003; Monteiro-Riviere *et al.* 2005a; Witzmann and Monteiro-Riviere 2006; Zhang *et al.* 2007). However, the studies were predominantly conducted using one single cell type and a dermal hazard is likely to be reliant on the capability of CNT of penetrating through the stratum corneum barrier *in vivo*, which is unknown at this time (Monteiro-Riviere and Inman 2006).

Unpurified SWCNT were shown to induced inflammation in the skin tissue model, EpiDermFT (Murray *et al.* 2009).

Repeated dose toxicity: other routes

Intraperitoneal injection:

Studies with intraperitoneal exposure have been performed to investigate whether CNT have the capacity to elicit toxicity comparable to other pathogenic fibres such as asbestos through formation of mesotheliomas.

Intravenous exposure/circulation of CNT:

Exposure to CNT via intravenous injection is only relevant for medical application. Results from studies following intravenous exposure are relevant to assess systemic effects of CNT if their translocation into the circulation from other organs (lungs, skin or GIT) is realised.

9.4.4.6 Biological mechanisms and target organ toxicity

Lung toxicity

Several authors have investigated the mechanism of CNT pulmonary toxicity *in vitro* and have shown that CNT were cytotoxic to different lung epithelial cell lines (Manna *et al.* 2005; Magrez *et al.* 2006; Muller *et al.* 2005; Ye *et al.* 2009). The underlying molecular mechanisms for the cytotoxic response, is likely to be driven by enhanced cellular ROS production, and the subsequent induction of an inflammatory response. ROS production can be partially inhibited by metal chelators, indicating that metal components (nickel, iron, yttrium) of CNT are able to contribute to the oxidant response observed (Pacurari *et al.* 2008). CNT can contain quite high concentrations of metals as impurities (e.g. 30%), which can contribute to their toxicity. CNT toxicity also seems to be promoted by aggregation of CNT (Davoren *et al.* 2007; Wick *et al.* 2007).

Cytotoxicity via apoptosis has been shown in macrophages and SWCNT were able to impair phagocytosis more effectively than MWCNT and C₆₀ (Jia *et al.* 2005). The consequences of it could be a reduced immune defence as macrophages are responsible for the clearance of foreign particles from different tissues in the body. The extent of these effects are likely to be driven by the morphology and dimensions of CNT. The assessment of the cytotoxic potential of CNT was demonstrated to be dependent on the assay used to assess cell viability, which may be due to an interference with the CNT in the assays used to assess their toxicity. This finding that has also been observed by Casey *et al.* (2007a).

Cardiovascular effects

In vivo and *in vitro* studies suggest that exposure to CNT via the cardiovascular system could be associated with vascular damage, pro-thrombic responses (Radomski *et al.* 2005), inflammatory and/or oxidative responses at sites distal to the exposure site (Yang *et al.* 2008, Li *et al.* 2007b) and a potential increased risk of cardiovascular diseases by affecting normal cardiac electrophysiology (Helfenstein *et al.* 2008).

Extrapulmonary responses subsequent to pulmonary exposure raise the question whether CNT might be able to stimulate the release of factors into the circulation that mediate toxicity at sites distal to the exposure site (Li *et al.* 2007b).

Immunological effects

The immune system is anticipated to play a key role in the removal of CNT from the body and in the induction of inflammatory and fibrogenic responses (Murr *et al.* 2005; Shvedova *et al.* 2005; Muller *et al.* (2005).

The effectiveness of CNT uptake by immune cells has been shown to be influenced by morphology, dimensions and solubility of CNT and cannot be regarded as universally applicable to all CNT forms. Ground MWCNT, chrysotile asbestos and ultrafine carbon black were all shown to be capable of inducing production of pro-inflammatory cytokines and stimulating cytotoxicity with MWCNT being more pathogenic than ultrafine carbon black, and either equivalent or less toxic than asbestos (Muller *et al.* 2005).

Oxidative stress – inflammation – cytotoxicity and formation of granulomas

CNT have been found to induce an increase in ROS production and/or antioxidant depletion in the lung and heart (Shvedova *et al.* 2005, Li *et al.* 2007b) *in vivo*, while *in vitro* oxidative stress has been observed in models of the skin (Shvedova *et al.* 2003) and lung (Manna *et al.* 2005).

Inflammatory responses have been identified in a number of *in vivo* investigations into CNT, including the pulmonary exposure of mice (Shvedova *et al.* 2005) and rats (Muller *et al.* 2005), as well as intraperitoneal exposure of mice (Poland *et al.* 2008). *In vitro*, a variety of CNT exposed cell types, including keratinocytes (Monteiro-Riviere *et al.* 2005a), and macrophages (Brown *et al.* 2007), have also been shown to enhance production of pro-inflammatory mediators such as IL-8 and TNF α suggesting that inflammation might not be limited to the lungs.

*In summary, subsequent to pulmonary exposure CNT have shown inflammatory and fibrotic responses. Following intratracheal instillation, significant inflammation, oxidative stress, the formation of granulomas and tissue injury at doses of 1-5 mg kg⁻¹ SWCNT (Lam *et al.* 2004, Shvedova *et al.* 2005, Warheit *et al.* 2004) and up to 10 mg kg⁻¹ MWCNT (Muller *et al.* 2005) have been reported. Mortality has been observed following intratracheal instillation of high doses of SWCNT (16.7 mg kg⁻¹ and 5 mg kg⁻¹) which was most probably due to congestion of airways, leading to death via asphyxiation, rather than a specific toxic effect. Death via asphyxiation due to CNT exposure of humans is highly unlikely due to the difficulty in generating sufficient concentrations of airborne respirable aggregates.*

*Following inhalation exposure only weak or no pulmonary effects were seen even at relatively high concentrations which is in contrast to many of the findings following intratracheal instillation. The reason why intratracheal instillation showed in general more severe effects may be explained by the fact that via instillation higher concentrations of particles reached the lung. Particles that would normally not be respirable are deposited in the deeper lung from where they cannot be removed. Instillation and to a lesser extent aspiration, resulted in the agglomeration of material. This suspension is placed either directly into the lung or on the back of the tongue and may lead to granuloma formation because large concentrations of material collect in specific foci which result in a "foreign body" type response (Mitchel *et al.* 2007). Therefore the findings from studies assessing pulmonary effects using instillation or aspiration can be used to identify possible effects of CNT but have to be carefully examined when used for a quantitative risk assessment and preference should be given to inhalation studies.*

*There are no guideline inhalation studies identified which could be used as key studies to derive dose descriptors for a regulatory risk assessment. The effects seen in the different studies like inflammatory and fibrotic response, with granuloma development are assumed to have a threshold. Based on the identified information an attempt is made for a quantitative risk assessment based on results in the inhalation study in mice by Mitchel *et al.* 2007.*

*No inflammation and other lung effects were seen at concentrations up to 5 mg m⁻³ MWCNT 14 days post exposure. This value could be used as a NOAEC for MWCNT for pulmonary effects as it is also supported by the findings in the Li *et al.* 2007b study which showed no inflammation in mice after 24 days exposure to MWCNT at the more than 6 times higher concentration of 32.61 mg m⁻³, but there were indications of a fibrotic response. It has to be borne in mind that the effects in both studies were only analysed for quite a short time (14 days and 24 days respectively) and that the responses to inhaled CNT may manifest over several months or years.*

*Despite the absence of pulmonary effects in the Mitchel *et al.* (2007) study systemic immune effects were seen at all concentrations tested and these are difficult to interpret. It is intriguing that these immune suppressing effects were seen in the spleen but not in the lung, which suggests that MWCNT are potentially bypassing pulmonary defence mechanisms and reaching the circulation. It could however also be that due to the whole body exposure MWCNT could become systemically available via other routes of exposure (orally or even dermally).*

*Such observations have not been reported in the other studies assessing the effects following pulmonary exposure and assessing systemic effects following intraperitoneal and intravenous exposure, however it might be that they were not looked at. It has been shown in *in vitro* studies that CNT can have detrimental effects on immune phagocytic cells (Shvedova *et al.* 2005, Muller *et al.* 2005, Murr *et al.* 2005). In addition impaired immune function following inhalation has been reported for other carbonaceous particles (Harrod *et al.* 2003, 2005; Shay *et al.* 1999, Fujita *et al.* 2009) and therefore these effects should not be neglected and a second*

scenario for the risk assessment exercise will be set taking the lowest tested dose of 0.3 mg m^{-3} as a LOAEC for MWCNT.

In the Shvedova et al (2008a) study the concentration of 5 mg m^{-3} of SWCNT containing 17.7% iron caused inflammation, oxidative, fibrotic and mutagenic responses being most evident at day 28. The higher toxicity shown in this study could be due to the longer observation period compared to Mitchel et al. (2007) but also due to the CNT type, with SWCNT being potentially more potent in inducing inflammation. The iron content of SWCNT in this study might have an impact on the toxicity too.

Therefore the risk characterisation exercise will focus on MWCNT (without surface modification and low content of impurities, i.e. $< 0.5\%$ metals) as have been studied by Mitchel et al. (2007) and Li et al. (2007b).

It is important to note that the available database does not allow the generation of general conclusions regarding the effects and a risk assessment for carbon nanotubes.

The different test results obtained from different studies which do not allow the identification of a CNT induced toxicological or pathological effect is probably due in part to physico-chemical characteristics of the CNT used in the different tests. In addition, the concentrations tested, the exposure time and the experimental set up (method of delivery and for *in vitro* studies the cells investigated) can influence the test results.

Further studies are needed which use multiple techniques, doses and CNT manipulated systematically to vary in specific characteristics in order to determine the factors that might drive pathogenicity and possibly allow the determination of doses with and without effects.

The limited number of identified studies on dermal exposure suggest that there is a hazard associated with the exposure of skin to CNT, with the response being primarily inflammatory in nature but also showing the appearance of granulomas. A dermal hazard is likely to be reliant on the capability of CNT to penetrate through the stratum corneum barrier *in vivo*, which is not proved yet. Future studies need to consider the systemic availability of CNT following dermal exposure, for example, using *in vitro* skin models. For the risk characterisation exercise the lowest dose tested of $40 \mu\text{g}$ per mouse (exposure 5 days; Murray et al. 2009) is suggested as a LOAEL for deriving a DNEL.

No information on effects after oral exposure was identified.

9.4.4.7 Mutagenicity

In vitro:

MWCNT have been demonstrated to induce mutation within lung cells as shown in a study by Muller et al. (2008) using the micronucleus assay *in vitro* models ($10\text{-}150 \mu\text{g ml}^{-1}$, following a 6 hour exposure).

A number of genotoxic effects, including DNA damage (using the Comet assay) and H2AX phosphorylation (indicative of DNA damage) were identified following SWCNT exposure in mesothelial cells, at concentrations up to $500 \mu\text{g ml}^{-1}$, for a period of up to 24 hours (Pacurari et al. 2008). It was postulated that DNA damage was a consequence of the direct interaction of SWCNT with DNA, and due to SWCNT stimulated ROS production.

Zhu et al. (2007) demonstrated the ability of MWCNT to elicit DNA damage within mouse embryonic stem cells, at concentrations of 5 or $100 \mu\text{g ml}^{-1}$, for up to 24 hours. There was evidence of damage to the DNA double strand, an increased expression of DNA repair enzymes and of phosphorylation of the p53 protein to arrest cells in the cell cycle in order to repair the DNA.

Kisin et al. (2007) determined the ability of SWCNT to induce genotoxicity, within three separate *in vitro* tests. SWCNT mediated DNA damage was found within V79 fibroblasts, using the Comet assay. There was also a trend for an increase in micronucleus formation within cells.

However, no evidence of genotoxicity was observed within *S. Typhimurium* using the Ames test. A decrease in cell viability was also associated with the response, which interferes with the accurate detection of genotoxic responses.

No cytotoxicity and no mutations were associated with MWCNT exposure (110-170 nm diameter, 5-9 μm , length; up to 3.5 $\mu\text{g ml}^{-1}$) of *S. Typhimurium* and *E.Coli* strains (Di *et al.* 2009).

In vivo:

Increased micronucleated cells were observed in a micronucleus assay in the rat lung subsequent to MWCNT exposure (0.5-2 mg kg^{-1} , 3 days post exposure), which however may be related to a marked inflammatory response in the lung (Muller *et al.* 2008).

K-ras mutations within the lungs of mice, subsequent to pulmonary exposure to SWCNT were seen with greater frequency via inhalation (and increased with time), than via aspiration showing that the exposure method has the ability to impact on the evaluation of CNT toxicity (Shvedova *et al.* 2008a).

In summary, several genotoxic effects with SWCNT and MWCNT were seen in micronucleus assays (Muller et al. 2008, Kisin et al. 2007), comet assays (Pacurari et al. 2008, Kisin et al. 2007) and a test to determine DNA damage in mouse embryonic stem cells (Zhu et al. 2007) at concentrations between 5 and 500 $\mu\text{g ml}^{-1}$. No evidence of genotoxicity was observed in Ames tests (Kisin et al. 2007, Di et al. 2009).

The genotoxic potential of CNT is uncertain at this time, and is likely to be dictated by the experimental set up; including the model, exposure route, CNT type, concentration administered and endpoint assessed. There was no indication whether fibre like CNT would be more genotoxic. Genotoxic events may transpire as a secondary consequence of CNT mediated inflammatory and oxidative responses, or alternatively act primarily due to their direct interaction with DNA following cell internalisation. Consequently it is at date not possible to decide whether CNT induced effects have a threshold or not. More definite conclusions regarding the genotoxicity of CNT would require more extensive in vitro and in vivo investigations.

9.4.4.8 Carcinogenicity

No carcinogenicity studies have been identified with CNT.

Studies with intraperitoneal exposure have been performed to investigate whether CNT have the capacity to elicit toxicity comparable to other pathogenic fibres such as asbestos through formation of mesotheliomas. Intraperitoneal exposure of the mesothelial lining of abdominal cavity to CNT has been used as a surrogate for the mesothelial lining of the pleural cavity surrounding the lungs. These studies have aimed to investigate whether CNT conform to the pathogenic fibre paradigm, which dictates that long (>approximately 15 μm), relatively straight and biopersistent fibres are more pathogenic than short fibres. As for pathogenic fibres such as asbestos, not all mesotheliomas are limited to the pleural cavity, but in fact many have also been observed in the peritoneum of humans (Selikoff *et al.* 1990; Mossman *et al.* 1996) the findings following intraperitoneal injection might be of considerable relevance.

Poland *et al.* (2008), investigated the toxicity of a variety of MWCNT with varied morphology; specifically long (>20 μm rigid and straight) or short and entangled (<20 μm length) forms, and compared the response to that of asbestos 'controls' (long and short fibre amosite), subsequent to their intra-peritoneal injection within mice.

Following injection of albumin dispersed fibres at a dose of 50 μg per mouse analysis were made 24 hours or 7 days post-exposure. It was suggested that the ability of MWCNT to possess asbestos-like properties is dependent upon their length and morphology with long straight MWCNT being able to induce an inflammatory response and form granuloma pathology on the peritoneal surface of the diaphragm and foreign body giant cells comparable to long fibre amosite asbestos. The short or entangled MWCNT and short asbestos were incapable of eliciting such a response.

In a study by Takagi *et al.* (2008) MWCNT, crocidolite asbestos or fullerenes (3mg/animal) were administered via intraperitoneal injection in p53 (+/-) mice to investigate their carcinogenicity. It was concluded that MWCNT (average width 100 nm, 27.5% of particles longer than 5 µm) and asbestos had the greatest carcinogenic potential, which was based on the visualisation of mesotheliomas, with no tumours evident within control and fullerene treated mice.

The animals were monitored until 100% mortality (week 25) and excessively high concentrations were used. Beside mesotheliomas also peritoneal adhesion (and fibrous thickening) was considered to contribute to the thickening.

In summary, the most important organ to be affected by inflammation and consequent carcinogenic responses would be the lung, as this is the organ where the highest concentration of CNT following inhalation would be expected. However, if CNT translocate to other locations either via the cardiovascular system, or into the pleura, there might be additional target organs for carcinogenicity.

*Depending on their length and morphology, MWCNT can behave in a similar manner to asbestos (no such information has been reported on SWCNT). Specifically, long, straight MWCNT have been shown to have the potential to induce mesotheliomas following intraperitoneal exposure (Poland *et al.* 2008). In this study mice exposed to long MWCNT intraperitoneally developed inflammatory and granulomatous changes at concentrations of 50 µg/mouse 24 hours or 7 days post-exposure. However it is not known whether these effects seen would go on to develop mesotheliomas.*

*In a different study mesotheliomas were induced with short latency in p53 deficient mice following intraperitoneal injection of MWCNT at concentrations of 3 mg per mouse (Takagi *et al.* 2008).*

These mice are very sensitive to oxidative stress-mediated carcinogenesis as a massive number of cells are mutated. In p53-proficient animals a protracted inflammatory reaction is needed to stimulate the proliferation of the few mutated cells and their progression to carcinogenesis. Prediction of the potential of MWCNT to induce mesotheliomas in humans cannot be conducted using this p53 +/- model study.

*A high rate of mesotheliomas was observed in rats following single intrascrotal administration of 1 mg kg⁻¹ bw⁻¹ MWCNT (same type as in Takagi *et al.* 2008) (Sakamoto *et al.* 2009). No carcinogenic effects were seen in a 2-year bioassay in rats following single intraperitoneal injection of 2 or 20 mg per rat MWCNT with and without structural defects (Muller *et al.* 2009). MWCNT in that assay had a diameter of 11.3 ± 3.9 nm and a length of about 0.7 µm and did not contain a sufficient high number of fibres >5 µm. The intraperitoneal bioassay may not be sufficiently sensitive to such short CNT <5 µm.*

None of the studies described above addressed whether CNT would be able to reach the mesothelium in sufficient numbers to cause mesothelioma following inhalation exposure. It is also important to clarify whether CNT conform to the fibre paradigm, and if they are biopersistent for which to date little evidence has been provided.

To decide whether carcinogenicity is an endpoint of concern more information is needed especially for physiologic routes of exposure (especially inhalation). Future studies should also investigate the sub-lethal effects after a sub-chronic (or chronic) timeframe at relevant exposure concentrations and give more conclusive information about possible genotoxic effects of CNT in order to decide whether possible carcinogenic effects should be regarded as having a threshold or not.

9.4.4.9 Toxicity for reproduction

Effects on fertility

No information identified

Developmental toxicity

The only information on reproductive effects of CNT have been identified in zebra fish, where lower survival rates in the second generation (Cheng *et al.* 2009) and a significant hatching delay in zebra fish embryos, were observed. It was also shown that the embryo chorion is an effective protective barrier to SWCNT when exposed as micro-scaled or larger agglomerates (up to 360 mg l⁻¹) (Cheng *et al.* 2007).

The limited literature examining the effects of CNT during pregnancy highlight effects on the developing foetus but are limited to the zebra fish model with no published studies looking at effects in mammals. No specific in vitro or in vivo studies were found examining CNT effects on male and female reproductive systems.

9.4.5 Derivation of DNEL(s)

Based on the identified information from the pulmonary and dermal exposure studies it can be assumed that the effects seen like inflammation, oxidative and fibrotic responses have a threshold. There is uncertainty with regard to genotoxic effects which could be secondary due to an inflammatory or oxidative response and then would have a threshold but could also be primarily due to an intrinsic property of the particles themselves. The latter case would not allow determination of a threshold for genotoxic effects of CNT. Thus the DNELs derived in this section would not apply if such direct genotoxicity is shown.

A possible risk of mesotheliomas by certain MWCNT would probably have a threshold as it depends on certain number of fibres reaching the lung and being translocated from the lung into the pleura. However it is important to keep in mind that with asbestos there is a long latency in the effects apparent and this might also be the case for CNT. The DNELs derived in this section are however not intended to cover the behaviour and effects of fibre-like CNT.

No guideline studies have been identified that would be the preferred studies for a regulatory risk assessment. From the identified data on inhalation and dermal exposure some key studies were selected for deriving a Derived No Effect Levels (DNELs) assuming a threshold for CNT induced effects. These studies are described in the effects assessment part and some more information on their quality is given below.

DNELs are derived from the dose descriptors of these key studies by applying assessment factors as described in the REACH guidance on information requirements and chemical safety assessment.

The results of the studies are used for the purpose of this risk characterisation exercise only, to get a perception of whether there could be a risk for workers at the measured workplace concentrations or for the general public at modelled predicted environmental concentrations in the air. The following calculations should however not be used for any conclusions or decisions regarding a risk assessment of carbon nanotubes and have therefore no regulatory relevance as there are too many uncertainties as regards the quality and the representativeness of the data.

Inhalation

For acute toxicity the only studies that give effects at certain concentrations levels (e.g. mortality at 5 mg kg⁻¹ and 16.7 mg kg⁻¹) were using intratracheal instillation, which seems inappropriate to be used for a human health effects assessment as the mortality seen was not due to a toxic effect but rather due to congestion of airways, leading to death via asphyxiation. Death via asphyxiation due to CNT exposure of humans is highly unlikely due to the difficulty in generating sufficient concentrations of airborne respirable aggregates.

Dose descriptors from inhalation studies are suggested for deriving a short term and a chronic DNEL.

Engineered Nanoparticles: Review of Health and Environmental Safety

No inflammation and other lung effects were seen in mice at concentrations up to 5 mg m^{-3} MWCNT (10-20 nm x 5-15 μm , 0.5% nickel and iron impurities) 14 days post exposure (Mitchel *et al.* 2007) and it is suggested to use this value as a NOAEC for pulmonary effects in a first scenario.

The study by Mitchel *et al.* (2007) also reported systemic immune effects at all dose levels tested, therefore a second scenario using the lowest tested dose of 0.3 mg m^{-3} as LOAEC will be performed.

The Mitchel *et al.* (2007) study is not a guideline study and was not performed for the purpose of a risk assessment. Therefore it would normally not be sufficient to be used as a key study for deriving a DNEL and for performing a risk assessment. Though, considering the amount and quality of information, the reliability of the study would correspond to a Klimisch code 2 (Klimisch *et al.* 1997). The results of the study are supported by results from other similar studies showing low pulmonary toxicity. Therefore it is suggested to use the results of this study for this risk characterisation exercise.

Scenario 1: NOAEC for pulmonary effects: 5 mg m^{-3} (6 hours)

1) Modification of the starting point (correction of differences between experimental and human exposure conditions)

Worker: 8 hours exposure (light activity): $\text{NOAEC} \times 6 \text{ hours} / 8 \text{ hours} \times 6.7 \text{ m}^3$ (8 hour standard) / 10 m^3 (8 hours light activity)

General public: 24 hours exposure (standard conditions): $\text{NOAEC} \times 6\text{h}/24\text{h}$

→ NAEC worker (8 hours): 2.5 mg m^{-3}
→ NAEC general public (24 hours): 1.25 mg m^{-3}

2) Interspecies variation:

Allometric scaling: not applicable as DNEL based on an inhalation study

Other interspecies factors: 2.5

3) Intraspecies variation: 5 (worker), 10 (general public)

4) Duration: extrapolation from sub-acute (although even shorter) to chronic: 6 (not needed for short term DNEL)

Overall assessment factor:

Short term exposure (worker):	2.5×5	= 12.5
Long term exposure (worker):	$2.5 \times 5 \times 6$	= 75
Long term exposure (general public):	$2.5 \times 10 \times 6$	= 150

→ $\text{DNEL}_{\text{inhalation}; \text{scenario1}}$

- short term inhalation (worker):	0.2 mg m^{-3}	=	$201 \mu\text{g m}^{-3}$
- chronic inhalation (worker):	0.034 mg m^{-3}	=	$33.5 \mu\text{g m}^{-3}$
- chronic inhalation (general public):	0.008 mg m^{-3}	=	$8.3 \mu\text{g m}^{-3}$

Scenario 2: LOAEC for systemic immune effects: 0.3 mg m^{-3} (6 hours)

1) Modification of the starting point (correction of differences between experimental and human exposure conditions)

Worker: 8 hours exposure (light activity): $\text{NOAEC} \times 6 \text{ hours} / 8 \text{ hours} \times 6.7 \text{ m}^3$ (8 hour standard) / 10 m^3 (8 hour light activity)

Engineered Nanoparticles: Review of Health and Environmental Safety

General public: 24 hours exposure (standard conditions): NOAEC x 6 hours / 24 hours

- LAEC worker (8 hours): 0.15 mg m⁻³
- LAEC general public (24 hours): 0.075 mg m⁻³

2) Extrapolation from a LOAEC to a NAEC (extrapolation factor of 3):

- NAEC_{worker}: 0.05 mg m⁻³
- NAEC_{genpub}: 0.025 mg m⁻³

3) Interspecies variation:

Allometric scaling: not applicable as DNEL based on an inhalation study

Other interspecies factors: 2.5

3) Intraspecies variation: 5 (worker), 10 (general public)

4) Duration: extrapolation from sub-acute (although even shorter) to chronic: 6
(not needed for short term DNEL)

Overall assessment factor:

Short term exposure:	2.5 x 5	= 12.5
Long term exposure (worker):	2.5 x 5 x 6	= 75
Long term exposure (general public):	2.5 x 10 x 6	= 150

→ DNEL_{inhalation, scenario 2}

- short term inhalation (worker): 0.004 mg m⁻³ = **4 µg m⁻³**
- chronic inhalation (worker): 0.0007 mg m⁻³ = **0.67 µg m⁻³**
- chronic inhalation (general public): 0.00017 mg m⁻³ = **0.17 µg m⁻³**

Dermal

From the only available dermal study (Murray *et al.* 2009) the lowest tested dose of 40 µg/mouse unpurified SWCNT which did not induce inflammation (as seen at the higher tested doses 80 and 160 µg per mouse) is suggested to be used as a short term NOAEL. Assuming a weight of 30 g for a mouse, a NOAEL of 1333 µg kg⁻¹ or 1.3 mg kg⁻¹ bodyweight is calculated.

The Murray *et al.* (2009) study is not a guideline study and was not performed for the purpose of a risk assessment. Therefore it would normally not be sufficient to be used as a key study for deriving a DNEL and for performing a risk assessment. Though, considering the amount and quality of information the study provides, the reliability of the study would correspond to a Klimisch code 2 (Klimisch *et al.* 1997). As there are no other similar studies identified that could support the results, a second scenario is performed, using an assessment factor of 3 for the quality of the whole database. Under these conditions it is suggested to use the results of this study for this risk characterisation exercise.

Assessment factors

1) Interspecies variation:

Allometric scaling: not needed as the observed effects do not involve metabolism (local effects)

Other interspecies factors: 2.5

2) Intraspecies variation: 5 (worker)

(no risk characterisation is performed for the general public, due to lack of exposure data)

3) Duration: extrapolation from sub-acute (although even shorter) to chronic: 6
(not needed for short term DNEL)

Only for Scenario 2:

4) Quality of the whole data base
only one short term non guideline study identified 3

Overall assessment factor:
Short term exposure: 2.5×5 (x3) = 12.5 (37.5)
Long term exposure: $2.5 \times 5 \times 6$ (x3) = 75 (225)

→ DNEL_{dermal worker}

Scenario 1

- for short term exposure: $0.0011 \text{ mg kg}^{-1} \text{ bw} = 106 \text{ } \mu\text{g kg}^{-1} \text{ bw} = \mathbf{7448 \text{ } \mu\text{g per person}^1}$
- for chronic exposure: $0.0018 \text{ mg kg}^{-1} \text{ bw} = 17.7 \text{ } \mu\text{g kg}^{-1} \text{ bw} = \mathbf{1241 \text{ } \mu\text{g per person}^1}$

Scenario 2 (Assessment factor 3 for quality of the whole database)

- for short term exposure: $0.0035 \text{ mg kg}^{-1} \text{ bw} = 35.5 \text{ } \mu\text{g kg}^{-1} \text{ bw} = \mathbf{2483 \text{ } \mu\text{g per person}^1}$
- for chronic exposure: $0.0059 \text{ mg kg}^{-1} \text{ bw} = 5.9 \text{ } \mu\text{g kg}^{-1} \text{ bw} = \mathbf{414 \text{ } \mu\text{g per person}^1}$

9.4.6 Risk characterisation

Exposure to carbon nanotubes will mainly occur via inhalation and the dermal route at the workplace and possibly in addition via the dermal and/or oral route through consumer products and/or indirect exposure via the environment.

Subsequent to pulmonary exposure, it is likely that a considerable fraction of CNT remain within the lung, depending on their biopersistence. After oral exposure, the majority of CNT are eliminated within the faeces or remain within the GIT, with no detectable transport into the blood.

Thus far no studies have specifically addressed whether CNT can translocate into the blood from the lungs, skin or GIT. Epithelial barriers, evident at the exposure site are in place to protect against the transfer of CNT into the circulation, however if this protection is overcome, CNT might be distributed to various organs like liver, and spleen, as has been shown following intravenous or intraperitoneal injections.

No information regarding the metabolism of CNT has been identified, but it is unlikely that CNT are degraded due to their probably biopersistent nature. It would appear that CNT can be eliminated within urine, but this is likely to be driven by particular physicochemical characteristics (size, functionalisation).

At present it is not possible to make definite conclusions regarding the absorption, distribution and longevity of CNT within the body. However this information is important and further studies should clarify the toxicokinetic behaviour in order to determine the relevancy of toxicological findings within different target sites that have been shown following intravenous, intraperitoneal or *in vitro* exposures. If CNT are unable to access the systemic circulation, addressing their toxicity at secondary target sites becomes irrelevant.

The behaviour of CNT within the human body is likely to be dependent on their properties. Long straight or intact unground MWCNT have been shown to be more biopersistent than their ground counterparts; also the attachment of different surface moieties, or utilisation of CNT of different dimensions, may alter their behaviour (in particular their water solubility). This has to be taken into account when planning the testing of CNT and when interpreting the data.

The most important target organ following inhalation is the lung, where CNT can accumulate and induce inflammation and fibrotic responses with the development of granulomas.

¹ Assuming a bodyweight of 70 kg for humans

Despite differences in terms of their wall number, source, metal contamination and particle dimensions it has been demonstrated by a number of investigators that most types of CNT are capable of eliciting similar underlying toxicity, like oxidative responses (enhanced production of cellular ROS), inflammation and cytotoxicity. This may ultimately lead to a threshold driven secondary genotoxicity. Metal components of CNT may contribute to the oxidative response and enhance the toxicity.

However there will be differences between CNT depending upon their aerodynamic properties, with long, thin fibres expected to penetrate deeper into the airways where they have to be removed by phagocytic cells, and if this clearance mechanism fails, it is anticipated that CNT will more easily translocate from the lung to reach the circulation. Certain CNT may show higher toxicity at the site of accumulation depending on their size, solubility and impurities (metals).

No information on CNT with regard to irritation and sensitisation has been identified.

No conclusion can be drawn on a genotoxic potential of CNT as there was no consistency in the results from different *in vitro* and *in vivo* studies. Genotoxic events could act primarily due to their direct interaction with DNA or secondary as a consequence of inflammatory and oxidative responses. More definite conclusions regarding the genotoxicity of CNT would require more extensive *in vitro* and *in vivo* investigations.

Subsequent to intraperitoneal exposure (at high concentrations) long, straight MWCNT have been shown to have asbestos-like properties, exemplified by their ability to induce mesothelial pathology. This hazard has been identified under human irrelevant conditions and whether this poses a risk for humans would depend on the possibility for natural migration of nanotubes to the pleural mesothelium from the airspace. It also has to be clarified whether there is inhalation exposure to these specific types of CNT. Studies under more realistic conditions or models which are able to show the migration of CNT are needed to realistically estimate a possible risk of carbon nanotubes to induce mesotheliomas in humans.

No specific studies were found examining CNT effects on male and female reproductive systems. The only identified information on developmental effects is from zebra fish; the relevance of the findings has to be seen in relation to the systemic availability of CNT.

Based on the identified studies it is, to date, not possible to decide whether CNT induced effects have a threshold or not and more information is necessary. From the available information it seems that there might be substantial differences in the potential of different CNT in inducing toxic effects or even tumors, depending on the form and properties of the carbon nanotubes. Therefore evaluations will have to be made on a case by case basis. In any case more information is needed and further suggested information requirements are listed at the end of this section.

Despite these difficulties, it is attempted in the following section to conduct a quantitative risk characterisation. The assessment would not be relevant should further studies suggest that (some types of) CNT possess non-threshold characteristics.

9.4.6.1 Risk characterisation for workers

For this risk characterisation exercise a threshold for CNT induced effects is assumed and NOEL(C)s and DNELs for MWCNT have been determined based on results of some key studies.

Inhalation

Inhalation seems to be the main exposure route of concern for occupational exposure. From a short term repeated dose inhalation study a NOAEC of 5 mg m⁻³ for pulmonary effects (Scenario 1) and a LOAEC of 0.3 mg m⁻³ for systemic immune effects (Scenario 2) for MWCNT were determined. Usually the risk assessment would be based on the leading effect or the lowest dose descriptor and DNEL respectively. However due to the uncertainties in the

relevance/origin of the systemic immune effects, two scenarios with 2 different DNELs considering both possible cases were selected.

Scenario 1 (pulmonary effects):

DNEL worker:

- for short term inhalation: **201 $\mu\text{g m}^{-3}$**
- for chronic inhalation: **33.5 $\mu\text{g m}^{-3}$**

DNEL: General public/chronic inhalation: **8.3 $\mu\text{g m}^{-3}$**

Scenario 2 (systemic immune effects):

DNEL worker:

- for short term inhalation: **4.02 $\mu\text{g m}^{-3}$**
- for chronic inhalation: **0.67 $\mu\text{g m}^{-3}$**

DNEL: General public/chronic inhalation: **0.17 $\mu\text{g m}^{-3}$**

Several measurements have been reported from different occupational activities. For a risk characterisation exercise two measured values were selected, 53 $\mu\text{g m}^{-3}$ which could represent a typical exposure of CNT production and 1094 $\mu\text{g m}^{-3}$ for operations with the wet saw, an activity with high exposure.

A risk characterisation exercise based on the pulmonary effects and a short term DNEL of 201 $\mu\text{g m}^{-3}$ would show that for the lower exposure measured during production the risk seems to be controlled, but for the higher exposure during operations with the wet saw the risk would not be controlled. For chronic inhalation and applying a DNEL of 33.5 $\mu\text{g m}^{-3}$ the risk for neither of these two occupational exposure situations would be controlled.

Performing the risk characterisation exercise based on the systemic immune effects and the lower DNELs of 4.02 $\mu\text{g m}^{-3}$ and 0.67 $\mu\text{g m}^{-3}$ for short term and chronic inhalation respectively, there seems to be no exposure situation, where the risk would be controlled.

Dermal

Dermal exposure may be another important exposure route for workers. Following dermal exposure, inflammation and granulomas have been seen. From the only available dermal study the following DNELs for SWCNT were derived:

Scenario 1:

- for short term exposure: **7448 $\mu\text{g per person}$**
- for chronic exposure: **1241 $\mu\text{g per person}$**

Scenario 2 (additional factor 3 for quality of the whole database):

- for short term exposure: **2483 $\mu\text{g per person}$**
- for chronic exposure: **414 $\mu\text{g per person}$**

For a risk characterisation exercise the only identified measured dermal value is 6020 $\mu\text{g per person}$ which is assumed to be reduced by 90% to 602 $\mu\text{g per person}$ when gloves are worn. These exposure values are compared to DNELs derived from a short term dermal study. Based on these data, the risk for dermal exposure during short term exposure to SWCNT might be controlled as the exposure value and DNEL are close to similar, and for chronic exposure it would not be controlled without wearing gloves. As only one study on dermal effects is available a second scenario with a lower DNEL including an additional assessment factor for quality of the whole database is performed. In this second scenario the risk would not be controlled for chronic exposure and without wearing gloves. Assuming 90% efficiency of gloves, risk would be controlled for acute exposure

9.4.6.2 Risk characterisation for consumers

No exposure values are reported on CNT in consumer products and therefore no risk assessment can be performed. The main exposure would be expected to result from abrasion from consumer products and from disposal/recycling and risks are thus expected to be much

lower than for the workplace, even considering cumulative exposures from several consumer products.

9.4.6.3 Risk characterisation for humans exposed via the environment

Based on modelled environmental concentration in the air (0.0015 – 0.0023 $\mu\text{g m}^{-3}$) and the DNELs derived for chronic inhalation of MWCNT for the general public (8.3 $\mu\text{g m}^{-3}$ for pulmonary effects and 0.17 $\mu\text{g m}^{-3}$ for systemic immune effects), it seems that there is probably no concern that humans could be at risk due to environmental exposure to carbon nanotubes. However it has to be considered that there is high uncertainty in the data used and that the environmental exposure can increase in future due to the more wide manufacturing and use of carbon nanotubes.

No statement can be made with regard to risk via drinking water or residues in food as no information has been identified that would allow this study to make a risk assessment.

9.4.7 Summary

This risk characterisation exercise has shown that based on currently available data there are several exposure situations where risk seems not to be controlled for carbon nanotubes at the workplace. However this risk characterisation exercise is based on the little identified information and includes many uncertainties with respect to the quality and representativeness of the data for toxicity as well as for exposure. The little identified information and the uncertainties in the quality of the toxicity data made it difficult to determine reliable dose descriptors and DNELs. The derived DNELs cover only the endpoints acute and chronic inhalation and dermal exposure. No suitable data to perform a risk characterisation were identified for oral exposure and for endpoints like irritation, sensitisation, genotoxicity, carcinogenicity or reproductive toxicity. The risk characterisation would normally have to be conducted on the leading effect or the lowest DNEL for a given exposure pattern, which however is not known, based on the little identified data. Therefore the results of this risk characterisation exercise should be taken with caution and no definite conclusion should be drawn on a general risk of carbon nanotubes.

This risk characterisation exercise also shows that the outcome of the risk characterisation depends a lot on the data used and how assessment factors are applied. If carbon nanotubes, or at least certain carbon nanotubes show effects only at very high airborne concentrations it can be assumed that many of the exposure situations, at least where exposure is controlled would be without risk. However if it is shown that effects following inhalation can start already at much lower concentrations as has been suggested for the immune effects in Mitchell *et al.* (2007) study or as can be expected for any carcinogenic effects, then the risk is probably not controlled in any of the exposure situations.

From the exposure site it is not clear if the measured concentrations were always CNT or other particles and which fraction of it would be inhalable and which of it are fibres. The little identified information and the uncertainties in the quality of the toxicity data made it difficult to determine reliable dose descriptors and DNELs.

Finally, it should be stressed again that the presented risk characterisation exercise should not be used as a regulatory risk assessment or as basis for any conclusions on risks of carbon nanotubes. No statement can be made whether this risk characterisation exercise represents a realistic or a worst case scenario. This assessment is based solely on shorter term studies (up to 14 days) with the use of assessment factors. However nothing is known about how the effects would progress after a longer exposure period. Relatively high airborne concentrations were used to compensate for the short term exposure. The validity of such a model for accumulative exposure over a long time, such as the working lifetime of an individual has to be questioned. Sub-chronic (or chronic) studies at representative exposure levels at the workplace would be more relevant for investigating subchronic/chronic effects that could occur in humans after a lifetime exposure at work.

Engineered Nanoparticles: Review of Health and Environmental Safety

This risk characterisation exercise focused on available data from specific carbon nanotube types (i.e. MWCNT for inhalation, SWCNT for dermal exposure, see description in the effects part). It is not possible to extrapolate the conclusions of this risk characterisation exercise to all carbon nanotubes. Based on the current knowledge a risk assessment for different carbon nanotubes has to be performed on a case by case basis.

More information is needed for a proper risk assessment of CNT and the main recommendations for further work are:

- Reliable exposure measurements and identification of CNT (distinction from background) particularly at the workplace, but also in, or released from consumer products and possibly in the environment;
- Characterisation of CNT/reference materials;
- Toxicokinetics: (with careful consideration of detection methods and their influence on the results):
 - Absorption of CNT via the different exposure routes (inhalation and dermal; oral if oral exposure is proved to be relevant);
 - investigate dermal uptake with a skin model;
- If absorbed, assessment of the distribution of CNT to, and their longevity in multiple target sites is required, with a particular focus on translocation from the lung to the pleural cavity following inhalation;
- acute and repeated dose toxicity studies via inhalation at concentrations relevant for workplace (bases on more and better exposure information)
- dermal exposure:
 - studies on local/systemic dermal effects (duration of the study has to be carefully considered);
 - oral exposure (duration of the study has to be carefully considered);
- irritation (skin + eye irritation);
- sensitisation;
- Systemic effects:
 - Interference of CNT with immune cell function;
 - Ability of CNT to stimulate the release of factors into circulation to mediate toxicity at distal sites;
- genotoxicity: further *in vitro* and *in vivo* investigations to come to a definite conclusion;
- carcinogenicity: investigate carcinogenic potential, depending on the results of a possible translocation from the lung to the pleural cavity and depending also on results from genotoxicity and from a sub-acute/sub-chronic inhalation studies;
- reproductive toxicity: depending on results from absorption studies (systemic availability) and indications of effects in a sub-chronic toxicity study.

All of these investigations should include an analysis of the influence of different compositions of CNT and their physico-chemical characteristics in order to decide whether results are applicable to CNT in general or to certain CNT types only. It should also give an indication for further toxicity testing whether a grouping on CNT is possible in relation to certain endpoints and thus whether results can be used mutually.

The information requirements as listed above are based on endpoints that are normally required for testing of chemicals. The requirements for carbon nanotubes or nanoparticles in general might differ from those for chemicals and test systems more appropriate for nanomaterials would be needed to ensure that endpoints of potential particular concern are properly addressed.

9.4.8 Environmental Risk Assessment for carbon nanotubes

9.4.8.1 Environmental fate properties

Degradation

No studies explicitly addressing degradation of CNT have been identified in the literature. A study from Roberts *et al.* (2008) investigated the biological modification of ingested CNT. In detail, the author reported that *Daphnia magna* ingested water soluble SWCNT coated with lysophosphatidylcholine, and then modified them by digesting the coating, and thus reducing the SWCNT solubility by 50%. The biological modification can be relevant to assess the environmental fate in water of modified CNT after emission.

Environmental distribution

The fate and transport of MWCNT in water was investigated by several authors. The factors affecting the stability in natural water are similar to those already identified for C₆₀, i.e. concentration of Natural Organic Matter (NOM), pH, ionic strength, and functionalisation. In particular, NOM (e.g. humic acids) in water can stabilize MWCNT dispersions for over 1 month, but the NOM adsorption efficiency is dependent on aromatic hydrocarbon content (as reported by Hyung and Kim 2008). Environmental parameters can also alter the NOM-MWCNT adsorption efficiency, which decreases as pH decreases and ionic strength increases. Kennedy *et al.* (2008) observed the role of functional groups and coating for the dispersion stability, even if the functionalisation type remains a key factor. Finally, Han *et al.* (2008) observed an interaction between surfactants-facilitated MWCNT suspensions and clay (kaolinite and montmorillonite), leading to MWCNT deposition. This result can suggest that clay may act as an aggregation factor also in natural waters.

In the review only one study concerning transport in soil was dealing with carboxyl-modified SWCNT in quartz sand (Jaisi *et al.* 2008). This paper reported that an increasing ionic strength or the addition of calcium ions increased the SWCNT deposition. Moreover, shape and aspect ratio of the SWCNT are important to filter SWCNT out from flow. Finally, adding a low ionic strength solution (i.e. KCl) can mobilize deposited SWCNT.

Mueller and Nowack (2008) used a life-cycle perspective to model the quantities of engineered nanoparticles released into the environment including CNT. The quantification was based on a substance flow analysis from products to air, soil, and water in Switzerland. They calculated PECs between 0.0005 and 0.0008 $\mu\text{g l}^{-1}$ CNT in water, and between 0.01-0.02 $\mu\text{g kg}^{-1}$ CNT in soil. However, given the lack of information, numerous assumptions and estimates had to be made.

Bioaccumulation

The bioaccumulation of CNT was investigated in invertebrates, both aquatic and terrestrial, sometimes considering CNT as carriers of other compounds such as xenobiotic organic chemicals (e.g. PAHs), with results outlined in Table 9.9 and 9.10.

Table 9.9: Aquatic bioaccumulation of carbon nanotubes

Method	Results	Remarks	Reference
<i>Amphiascus tenuiremis</i> copepod marine sediments	No detectable SWCNT uptake into tissue (14d): 5 mg g ⁻¹		Ferguson <i>et al.</i> (2008)
<i>Streblospio benedicti</i> polychaete worm marine sediments	No detectable SWCNT uptake into tissue (14d): 5 mg g ⁻¹	Evident ingestion of SWCNT	Ferguson <i>et al.</i> (2008)
<i>Lumbriculus variegatus</i> freshwater sediments	SWCNT BSAF (28d): 0.28 MWCNT BSAF (28d): 0.40	CNT dispersed in water by sonication. High depuration rate (CNT concentration < LOD after 2 days)	Petersen <i>et al.</i> (2008b)

BSAF = Biota/Sediment Accumulation Factor
LOD = Limit of detection

Table 9.10: Terrestrial bioaccumulation of carbon nanotubes

Method	Results	Remarks	Reference
<i>Eisena foetida</i> (earthworm) soil	MWCNT BSAF (14d): 0.016 to 0.023 SWCNT BSAF (14d): 0.0061 to 0.022	Exposure to 0.03 to 0.3 mg g ⁻¹ MWCNT Exposure to 0.03 to 0.1 mg g ⁻¹ SWCNT	Petersen <i>et al.</i> (2008a)

In summary, no data regarding CNT persistence are available. However, functionalisation groups can be biologically degraded resulting in modification of the CNT properties. In fact, functional groups and coating of CNT are two of the main factors affecting dispersion and transport in water (e.g. Roberts *et al.* 2008). Other factors concern the chemical composition of the water, such as NOM, ionic strength, pH, and clay content. The influence of ionic strength, shape and aggregation status on the transport of carboxyl-modified SWCNT were studied in quartz sand. An interesting result was that the addition of a low ionic strength solution to the flow in soil can mobilise deposited SWCNT.

In conclusion, according to the available experimental results, the bioaccumulation of CNT into organisms is low, BSAF < 1 (for comparison, literature BSAF values in *Lumbriculus variegatus* for PCBs are in the range of 0.44 to 4.64, with a majority of values over 1 (Trimble *et al.* 2008)). Results reported in the literature indicate that CNT do not translocate from gut into tissues, furthermore a high depuration rate was observed in sediment polychaetes (Petersen *et al.* 2008a).

9.4.8.2 Environmental hazard assessment

Aquatic compartment (including sediment)

Fish:

Table 9.11: Short-term effects of carbon nanotubes on fish

Method	Results	Remarks	Reference
<i>Danio rerio</i> Embryo (freshwater)	Delayed hatching LOEC (96h): SWCNT 120 mg l ⁻¹ ; DWCNT 240 mg l ⁻¹	Differences in LOEC may be due to differences in impurities (authors' remark)	Cheng <i>et al.</i> (2007)

The endpoints considered in the short-term studies for fish included only sub-lethal effects. LOEC values for delayed hatching caused by exposure to SWCNT and DWCNT were estimated by Cheng *et al.* (2007) at 120 and 240 mg l⁻¹, respectively (Table 9.11). However, the difference between the two nanomaterials can be mainly due to impurities, according to the author. From these data, no LC50_{fish} (short-term) can be determined.

Table 9.12: Long-term effects of carbon nanotubes on fish

Method	Results	Remarks	Reference
<i>Oncorhynchus mykiss</i> Juvenile (freshwater)	Increase in ventilation rate, gill pathologies, mucus secretion (10d): 0.1 mg l ⁻¹ Damages on brain surface (swelling) (10d): 0.25 mg l ⁻¹ Oxidative stress in liver and brain (10d): 0.25 mg l ⁻¹ Aggressive behaviour (10d): 0.1 mg l ⁻¹ Liver cells pathological effects (10d): 0.25 mg l ⁻¹	Exposure from 0.1 to 0.5 mg l ⁻¹ SWCNT, dispersed by using SDS and stirring. Solvent control does not highlight toxic effects Suggesting subtle neurotoxic/cardiovascular effects Suggesting carcinogenic potential of SWCNT after long term exposure	Smith <i>et al.</i> (2007)

SDS = sodium dodecylsulfate

The Smith *et al.* (2007) study was considered a long-term study because it concerned sub-lethal endpoints for juveniles, even if 10 days is not really a chronic exposure (Table 9.12). SWCNT can exert significant adverse effects to early life stages of fish at concentrations as low as 0.1 mg l⁻¹. Effects on juveniles can be negative at population level, while effects on nervous system and carcinogenic potential in liver could become relevant for long term exposure. Moreover, Smith *et al.* (2007) speculate that exposure to low levels of SWCNT for more than 2 weeks can lead to high risk of mortality due to low cardiovascular fitness in trout. A NOEC_{fish} (long-term) < 0.1 mg l⁻¹ can be established.

Aquatic invertebrates:

Table 9.13: Short-term effects of carbon nanotubes on aquatic invertebrates

Method	Results	Remarks	Reference
<i>Ceriodaphnia dubia</i>	MWCNT LC50 (48h): 50.9 mg l ⁻¹ No effect on survival of MWCNT-OH (48h): 120 mg l ⁻¹ ; MWCNT-COOH (48h): 88.9 mg l ⁻¹	Stirred MWCNT + 100 mg l ⁻¹ NOM	Kennedy <i>et al.</i> (2008)
<i>Daphnia magna</i>	No effect on survival (96h): 0.5 mg l ⁻¹ 20% mortality (96h): 10 mg l ⁻¹ 100% mortality (96h): 20 mg l ⁻¹	Lysophosphatidylcholine-coated SWCNT	Roberts <i>et al.</i> (2008)

NOM = Natural Organic Matter

CNT may cause mortality of daphnids. However, the data highlight the importance of the functionalisation or coating of the CNT on mortality. While -OH and -COOH modified MWCNT

did not affect survival at concentrations up to 120 mg l⁻¹, lysophosphatidylcholine-coated SWCNT caused 100% mortality at 20 mg l⁻¹ (Table 9.13).

Sediment organisms:

The available data highlighted two aspects, one related to the species sensitivity, and one related to the purity of the CNT (Table 9.14). Kennedy et al (2008) reported two different LC₅₀ for two amphipods, with a difference of around an order of magnitude at similar exposure conditions. It is important to note that the salinity requirements of these species are different. The different species sensitivity highlights the need to have more data (i.e. more species) in order to address properly the interspecies variability in a PNEC estimation.

The second aspect is that impurities of CNT can lead to significant toxicity, in this case for reproduction, in that there was a 64% reduction in fertilisation rate for non-purified SWCNT, where as no effects were observed for the same concentration (10 mg l⁻¹) of purified SWCNT. Due to the difference in CNT tested, and lack of data on the different types, species and conditions, no general conclusion can be made about effects on sediment invertebrates. Care should be taken to address impurities of CNT in the interpretation the results of toxicity tests.

Table 9.14: Effects of carbon nanotubes on sediment organisms

Method	Results	Remarks	Reference
<i>Leptocheirus plumulosus</i> marine burrowing amphipod	MWCNT LC50 (10d): 68 g kg ⁻¹ sediment		Kennedy <i>et al.</i> (2008)
<i>Hyalella azteca</i> freshwater sedimentary amphipod	MWCNT LC50 (10d): >264 g kg ⁻¹ sediment		Kennedy <i>et al.</i> (2008)
<i>Amphiascus tenuiremis</i> marine harpacticoid copepod	34% reduction development success from nauplius to adult (35d): not purified SWCNT 10 mg l ⁻¹ 64% reduction fertilisation rate (35d): not purified SWCNT 10 mg l ⁻¹ Significant effects on development: fluorescent by- product fraction of nanocarbon 0.58 mg l ⁻¹ No significant effects: purified SWCNT 10 mg l ⁻¹	Effects seems caused by impurities	Templeton <i>et al.</i> (2006)
<i>Lumbriculus</i> <i>ariegates</i> freshwater sediment oligochate	No increase in mortality (28d): 0.03 mg l ⁻¹ SWCNT; 0.37 mg l ⁻¹ MWCNT	CNT dispersed by sonication	Petersen <i>et al.</i> (2008b)

Other organisms:

Table 9.15 includes a mixture of data on different organisms not included in the other categories. The tests were carried out either on SWCNT, MWCNT, or mixtures of CNT, functionalised or non-functionalised. Therefore, it is not possible to formulate a general

conclusion about a potential NOEC using these data. However, mortality was observed only for MWCNT concentrations $\geq 10 \text{ mg l}^{-1}$ over an exposure period of 5 days, while sublethal effects (membrane damage) were recorded for lower concentrations (i.e. 1.0 mg l^{-1}) in protozoa (Zhu *et al.* 2006b). Mortality was observed for bacteria exposed to functionalised and unbundled MWCNT after 60 minutes of exposure (Kang *et al.* 2008).

Table 9.15: Effects of carbon nanotubes on other organisms

Method	Results	Remarks	Reference
<i>Stylonychia mytilus</i> protozoa (Freshwater, sediment, soil)	43% survival (5d): 50 mg l^{-1} 53% survival (5d): 10 mg l^{-1} Damage to macronucleus and external membrane (5d): 1.0 mg l^{-1}	MWCNT from 0.1 to 200 mg l^{-1}	Zhu <i>et al.</i> (2006b)
<i>Tetrahymena thermophila</i> protozoan (Freshwater)	No cell viability loss (72h): 6.8 mg l^{-1} Blocked bacteria ingestion (72h): 7.3 mg l^{-1}	Suspended SWCNT	Ghafari <i>et al.</i> (2008)
<i>Xenopus laevis</i> larvae, frog (amphibian)	85% mortality and dose dependent size reduction (12d): 500 mg l^{-1} (no air bubbling) Abnormal behaviour (12d): $10\text{-}500 \text{ mg l}^{-1}$ No genotoxicity (12d): 500 mg l^{-1}	CNT mixture: 80% DWCNT, 15% SWCNT, 5% TWCNT	Mouchet <i>et al.</i> (2008)
<i>Ambystoma mexicanum</i> Salamander (amphibian)	No genotoxicity: 1000 mg l^{-1}	CNT mixture: 80% DWCNT, 15% SWCNT, 5% TWCNT	Mouchet <i>et al.</i> (2007)
<i>Escherichia coli</i> K12 Bacteria	41.6% inactivated cells (60 min): 20 mg l^{-1}	Functionalised (hydroxyl and acidic groups) short and unbundled MWCNT	Kang <i>et al.</i> (2008)

Terrestrial compartment

Toxicity to soil macro organisms:

The exposure of earthworms to DWCNT resulted in sub-lethal toxic effects at long-term exposure, with reproduction as most sensitive endpoint. A $\text{NOEC}_{\text{earthworm}}$ (long-term) was not reported by the authors, but an EC_{10} of 37 mg kg^{-1} food for CNT was determined (Table 9.16).

Table 9.16: Effects of carbon nanotubes on soil macro organisms

Method	Results	Remarks	Reference
<i>Eisenia veneta</i> Earthworm	Reproduction EC ₁₀ (28d): 37 mg kg ⁻¹ food for DWCNT Reproduction EC ₅₀ (28d): 176 mg kg ⁻¹ food for DWCNT Growth reduction EC ₁₀ (28d): 94 mg kg ⁻¹ food for DWCNT Growth reduction EC ₅₀ (28d): >500 mg kg ⁻¹ food for DWCNT 60% cocoon production reduction (28d): 495 mg kg ⁻¹ food for DWCNT		Scott Fordsmand <i>et al.</i> (2008)

Toxicity to terrestrial plants:

Concerning the effects of CNT on plants, no general conclusions can be made, because different CNT and different species were investigated (Table 9.17). Available data showed no effects on germination and root growth or positive effects on root growth for most of the tested species. For two species very high concentrations of SWCNT reduced the root growth.

Table 9.17: Effects of carbon nanotubes on terrestrial plants

Method	Results	Remarks	Reference
Higher plants (6 species)	No significant effects on germination and root elongation: 2000 mg l ⁻¹ MWCNT	Seeds soaked in MWCNT suspension	Lin and Xing (2007)
6 crop species	Reduced tomato and lettuce root growth (48 hours): 1750 mg l ⁻¹ Increased onion and cucumber root elongation (48 hours): 1750 mg l ⁻¹	Exposure to functionalised and non-functionalised SWCNT. Contrasting results among species, with mainly no effect or positive effects	Cañas <i>et al.</i> (2008)

9.4.9 Risk characterisation and gap analysis

The risk assessment for CNT is focused on non-functionalised nanotubes. Information on functionalised CNT were reported when data were available. However, it should be stressed that also the non-functionalised CNT include different types of nanotubes with different properties. Therefore, the results of the assessment can only be considered as preliminary findings for non-functionalised CNT and should be refined for specific CNT types (e.g. MWCNT vs. SWCNT).

Quantitative risk assessment:

Mueller and Nowack (2008) estimated very low CNT concentrations in water (0.0005 - 0.0008 µg l⁻¹ CNT) and soil (0.01-0.02 µg kg⁻¹ CNT) which are 3 to 7 orders of magnitude lower than the reported toxicity values. However, since a PNEC for the aquatic compartment was not calculated as data for algae are missing (and no long-term NOECs are available), only qualitative considerations were made.

Qualitative risk assessment:

The use of CNT in solid materials (polymers, microchips, etc.) reduces the possibility of direct releases to the environment during the product service life, but currently no data exist on potential releases during production, disposal and/or recycling. Transport into water and soil is very much a case-by-case issue, depending on CNT functionalisation and water/soil chemistry. In any case, since natural freshwaters contain NOM, transport over some distance may be expected, as well as deposition of CNT on external (gills, skin) and internal (internal gut lumen) organisms surfaces, which may lead to physical obstruction effects. Taking into account the differences between non-functionalised SWCNT and MWCNT, sub-lethal effects on fish can be expected at concentrations of 0.1 mg l^{-1} , while mortality on aquatic crustacean is expected at concentrations around 50 mg l^{-1} . Effects on microbial communities and protozoan can also occur in the same concentration range. Effects on sediment organisms caused by CNT (and not by impurities) were observed only at high concentrations (68 g kg^{-1}). Finally, terrestrial toxicity was observed for an earth worm (176 mg kg^{-1} food for DWCNT), while for plants effects can be expected only at high pore water concentrations (1750 mg l^{-1} SWCNT) for some species.

Testing of some functionalised CNT suggested that MWCNT with oxidised groups (i.e. -OH and -COOH) are less toxic to aquatic invertebrates than non-functionalised MWCNT, showing no toxicity at concentrations of 120 and 89 mg l^{-1} to *Ceriodaphnia dubia*, respectively (Kennedy *et al.* 2008). For plants, Canas *et al.* (2008) observed that SWCNT functionalised with sulfonic acid showed lower toxicity on root length than non-functionalised SWCNT. However, according to Kang *et al.* (2008), functionalised (i.e. -OH and -COOH), short and unbundled MWCNT are more toxic to *Escherichia coli*, and they suggested that the effect is related to the higher dispersion state enhancing the contact opportunities between CNT and cell membrane. Lysophosphatidylcholine-coated SWCNT (Roberts *et al.* 2008) caused 100% mortality at 20 mg l^{-1} in *Daphnia*, a concentration lower but comparable with that observed for non-functionalised MWCNT. These results highlighted that the functionalisation affects the toxicity of CNT. It seems that in general a higher hydrophilicity reduces the toxicity.

The following gaps can be highlighted:

1. Lack of information on production volumes of CNT, in its many forms;
2. Lack of information on the number of CNT products, market penetration, and amounts of the nanoparticles in these products;
3. Lack of information on behaviour of CNT during waste treatment and recycling;
4. Lack of information about environmental fate. This aspect is especially important because CNT interact with different natural components, which may result in different size distributions (aggregation/agglomeration), and thus in different transport potential. CNT can be functionalised in many different ways, and the available data suggest completely different environmental behaviour of different nanomaterials;
5. Lack of monitoring field data on CNT levels in environmental compartments;
6. Lack of toxicity data on several species categories for aquatic compartment, as for example on algae and long-term studies on *Daphnia*; no PNEC can be derived for CNT as a group;
7. Limited toxicity data, with little and inconclusive information, for the terrestrial compartment;
8. Limited information concerning interaction between organisms and CNT (adsorption, uptake, bioaccumulation, etc.).

9.5 METALS NANOPARTICLES

9.5.1 Identity

Metal nanoparticles should not be considered as one group in terms of risk assessment and no general conclusion on the general toxicity/ecotoxicity of metal nanoparticles can - or should - be made. Just like "bulk forms" of metals there are large differences in speciation, behaviour, fate and effects. Also functionalisation of metal nanoparticles is an issue of high relevance. The major part of the scientific literature published deals with effects of silver nanoparticles. Therefore, silver was chosen to carry out a case study for a basic risk assessment for a metal nanoparticle.

9.5.2 Manufacturing and use

Silver (all forms, including particulate) is used in a wide range of products, and has been over centuries. In recent years silver nanoparticles have been used in many consumer products (at least 235) as anti-microbial, such as in wound dressing, toothpaste, textiles, etc. All these products are used daily, allowing direct exposure to humans and potential release into the environment mainly via water discharge and waste release (e.g. textiles washing), both as nanosilver and as ionic Ag^+ . It has been estimated that nanosilver is produced worldwide at a level of 500 tonnes per annum (Mueller and Nowack 2008).

9.5.3 Human exposure assessment for silver

Occupational exposure

Occupational exposure to nano-silver can occur during manufacturing, including handling and packaging, during formulation into various preparations, during use of these preparations and/or incorporation of these into articles/solid goods. The main exposure route for occupational settings are expected to be inhalation and dermal contact. However, oral intake should not be completely discarded due to swallowing of particles that following inhalation can be cleared via the mucociliary escalator and due hand-to-mouth behaviour. The latter however should in general be avoided via good hygiene. Unfortunately very few data are available to assess exposure to silver nanoparticles, and the authors are not comfortable with applying existing modelling tools for nanomaterials.

Inhalation exposure:

Tsai *et al.* (2008) assessed airborne nanoparticle exposures associated with the manual handling of nano-alumina and nano-silver in fume hoods in a laboratory scale facility. The silver nanoparticles appeared to have a primary size of less than 100 nm but were highly aggregated into particles of several μm . For handling 15 g of silver, a peak size on 100-200 nm and a peak count of 7000 particles cm^{-3} was reported. For the purpose of a risk characterisation exercise as carried out in this review (see end of this chapter), this is converted into a surface area estimate. By assuming a uniform size of 150 nm this gives $0.5 \times 10^9 \text{ nm}^2 \text{ cm}^{-3}$. This number should of course be used with all possible care in the subsequent risk assessments.

Demou *et al.* (2008) reported a study which was carried out at a pilot scale "nanostructured particle" gas phase production facility. The facility produced metal-based nanoparticles embedded in a larger porous oxide matrix. Given the few nano-silver exposure data otherwise available, the data from this study are for the purpose of the exercise assumed to represent nano-silver manufacturing, although of course it is highly recommended to establish "real data" for a "real assessment".

Condensation Particle Counters, a DustTrak and Scanning Mobility Particle Sizer were used to quantify real-time size, mass and number, over a 25 day period. Temporal and spatial analysis of particle concentrations and sizes was performed during production, maintenance and handling. Number-based particle retention of breathing mask filters used under real-time production and exposure conditions in the workplace was quantified.

The results demonstrate elevated number concentrations during production, which can be an order of magnitude higher than background levels. Elevated number concentrations were also observed during reactor cleaning although not as great as during the production.

Average concentrations during production were 59100 cm^{-3} and 0.188 mg m^{-3} for sub-micron particles (particle size not further identified). There were no indications of particle size distribution and therefore it does not seem possible to calculate a surface area. The study demonstrates real-time worker exposure during gas-phase nanoparticle manufacturing and indicates clear differences between periods where the reactor was operational and other periods. Assessment by particle number was more sensitive than mass concentration measurements.

As these data are from a pilot production and read-across from a "nano-metal manufacturing" facility, respectively, these data can at most be used as a rough indication of exposure levels in a well-controlled manufacturing facility.

No other data on occupational exposure have been identified for manufacturing or downstream professional/industrial use of nano-silver.

Dermal exposure:

No information on dermal exposure is identified, although it must be assumed that dermal exposure will take place, not least, during handling and bagging and possible downstream handling of silver nanoparticles.

Consumer exposure

Silver nanoparticles are used in at least 235 consumer products (PEN, accessed at http://www.nanotechproject.org/inventories/consumer/analysis_draft/, 16th October 2009). Many of these are applications within pharmaceuticals (like wound dressings) and cosmetics (like toothpaste). These applications are outside the scope of this risk assessment. Use as food supplements has also been reported (PEN, accessed at: http://www.nanotechproject.org/inventories/consumer/analysis_draft/, 16th October 2009), but is also considered outside the scope of this assessment. The main consumer uses within the scope of this assessment appears to be within clothing (for coating and for preventing odour), anti-microbial applications for leisure equipment, metal products plastics, sprays and paints. No quantitative data on consumer exposure have been identified.

It must be assumed that dermal, ingestion and inhalation exposures are the main consumer exposure routes – dermal in particular for the clothes and paints applications, ingestion due inclusion in health remedies and inhalation for the spray applications. The magnitude of the exposure will depend on the extent to which nano-silver will be release from consumer products, between others the concentration in paints and sprays and how strongly it is bound in the 'solid'/matrix applications (e.g. in clothes and plastics). No data to investigate this has been identified.

9.5.4 Human health effects assessment

Nano-silver can occur in many different sizes, shapes and as agglomerates or combined with particles of other materials.

As a consequence identifying distinct hazards related to nano-silver exposure and making generalisations about the biological activity of nano-silver is difficult.

9.5.4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

Absorption

Inhalation:

Takenaka *et al.* (2001) demonstrated that a fraction of inhaled nano-silver (4-10 nm) was deposited within the lungs after exposure to 3×10^6 particles cm^{-3} equivalent to $133 \mu\text{g m}^{-3}$ for 6 hours. The burden was decreased to 4% after one week. Deposition was also seen in the nasal cavity and accumulation in the lung-associated lymph nodes was seen. It could not be seen whether the burden accumulated in macrophages in the lung.

In the same study Takenaka *et al.* (2001) measured silver in the blood and low concentrations of silver were found in the liver, kidney, spleen, brain and heart. It was not clear whether the absorbed silver included silver nanoparticles, free silver ions (Ag^+), a silver complex or a combination.

Sung *et al.* (2008, 2009) conducted a 90 day whole body inhalation study (6 hours per day, 5 days a week) to silver nanoparticles (18-19 nm), at low ($49 \mu\text{g m}^{-3}$, equivalent to 0.6×10^6 particles cm^{-3}), medium ($133 \mu\text{g m}^{-3}$, equivalent to 1.4×10^6 particles cm^{-3}) and high ($515 \mu\text{g m}^{-3}$, equivalent to 3.0×10^6 particles cm^{-3}) doses. The study showed deposition of silver in the lung, as well as silver in the blood and secondary organs such as liver, olfactory bulb, brain and kidneys. Also this study did not clarify whether the absorbed silver was nano-silver or free ions. Ji *et al.* (2007) found similar distribution patterns in a 28 day inhalation study.

In an intratracheal instillation study Semmler-Behnke *et al.* (2008) investigated the tissue distribution of radiolabelled gold nanoparticles (1.4 nm and 18 nm) within rats, in order to assess the translocation of nanoparticles from the respiratory tract to the blood. They showed that 24 hours following administration, the majority of 18 nm nanoparticles remained within the lung (99.8%), whereas for the 1.4 nm nanoparticles 91.5% remained within the lung, and 8.5% found within secondary targets (blood and liver). It was thus demonstrated that (small) gold nanoparticles can be absorbed following inhalation. It is not clear to which extent these findings can be used in a read-across approach for nano-silver.

Oral:

In a 28-days repeated dose oral study, Kim *et al.* (2008) exposed rats to silver nanoparticles (60 nm), at low ($30 \text{ mg kg}^{-1} \text{ day}^{-1}$), medium ($300 \text{ mg kg}^{-1} \text{ day}^{-1}$) or high ($1000 \text{ mg kg}^{-1} \text{ day}^{-1}$) concentrations. The silver content of a number of organs increased, which indicates transfer of silver into blood. Specifically, silver deposition within the brain, liver, kidneys, lungs and testes was observed. Note that the doses used within this study were exceptionally high in relation to what would be seen in an occupational or consumer situation.

Wadhwa and Fung (2005) found argyria (blue-grey colouring of the skin) in other organs following oral exposure, indicating oral silver uptake and distribution to the skin.

As for uptake via the lung, none of the above studies clarify whether the uptake is as nano-silver, silver ions, silver complexes or a combination of the two.

Silver uptake via the gastrointestinal tract is not surprising since a number of drugs (using non-nano silver) are administered this way, where it is absorbed probably as ions. Luoma *et al.* (2008) suggests that the low pH of the stomach converts silver to its ionic form. However, it should also be further considered to which extent silver reacts with Cl^- to form AgCl and to which extent it forms complexes.

Nonetheless, it must be anticipated that at least part of the silver uptake following GIT exposure to nano-silver takes place as silver ions. This assumption is – at least partly – supported by the fact that some of the same toxic effects as seen following non-nano silver exposure are seen following nano-silver exposure (in particular argyria). It must also be assumed that the larger surface area and the larger solubility (regarding increased solubility of nanoforms, see e.g. Torchilin 2006) from nanoforms of silver increases the uptake of silver ions, although this needs further investigation.

Engineered Nanoparticles: Review of Health and Environmental Safety

Savanone *et al.* (2008a) showed that 15 nm gold particles (but not larger particles) can penetrate isolated rat intestine. As for uptake via the lung it is however not clear whether read-across to silver nanoparticles is appropriate.

Further investigations are needed to determine whether the silver uptake observed following GIT exposure is likely to be caused primarily by nano-silver, by ions, as complexes or in combination and whether the particle uptake results seen for gold nanoparticles in an *in vitro* model are relevant to silver *in vivo*.

Dermal:

Silver nanoparticles are used in wound dressings and some studies have been conducted to investigate the efficiency of these dressings within the treatment of burns. Some of these have also (see e.g. Vlachou *et al.* 2007 and Trop *et al.* 2006) looked into systemic uptake and toxicity following the topical exposure (human studies). Vlachou *et al.* 2007 showed that serum levels increased with increasing exposure time peaking after 9 days and returning to normal 6 months after exposure. Trop *et al.* (2006) (silver particles 15 nm) found increased silver levels in the serum (and urine) 7 days post exposure and toxicity in secondary organs. Thus in these models it is shown that silver is absorbed through the skin. In the described studies, the wound dressings were applied to damaged skin every 2-4 days.

An *in vitro* study (Larese *et al.* 2009) investigated the penetration of polyvinylpyrrolidone coated silver nanoparticles (25 nm) within intact and damaged (abraded) human skin. Silver nanoparticles (70 $\mu\text{g cm}^{-2}$, for 24 hours) were able to pass through the skin preparation, and this was 5 times greater within damaged skin.

The relevance of these *in vitro* findings needs to be investigated *in vivo*.

As for uptake via lung and GIT it is not clear whether the uptake takes place as silver nanoparticles, as silver ions, as complexes or as a combination, and again this needs further investigation.

Savanone *et al.* (2008a) investigated the permeation of gold nanoparticles (15, 102 and 198 nm) through isolated rat skin. It was shown that the smaller particles (15 nm) could penetrate the deeper layers, whereas the larger particles did not. As for lung and GIT uptake it is not clear whether these results can be used in a read-across approach.

Distribution

Following absorption, the different studies described above have measured silver in the blood and secondary organs and/or shown toxicity in organs secondary to the exposure site.

In dermal studies, a peak blood concentration was reached after 7 days (Trop *et al.* 2006) and 9 days (Vlachou *et al.* 2007) with dressings changed every 2-4 days, suggesting that a steady state may be reached following dermal uptake. These studies also showed a gradual decrease in the blood levels following termination of treatment, where Trop *et al.* (2006) showed continued elevated levels 7 weeks after treatment and Vlachou *et al.* 2007 showed that blood levels were back to normal after 6 months. This indicates some retention within the blood and a gradual clearance with distribution to other organs and/or excretion. Takenaka *et al.* (2001) (inhalation) also showed a clearance from blood with time.

Silver was seen in the following secondary organs: liver, kidney, spleen, heart, olfactory bulb, brain, testes and skin (discoloration of the skin), with liver being the likely major secondary organ site of accumulation.

As noted above there is no clear indication whether the absorbed and distributed silver occurs as silver nanoparticles, silver ions, silver complexes or a combination.

Distribution of gold nanoparticles (following intravenous injection) has been investigated in a number of studies.

Semmler-Behnke *et al.* (2008) observed that 24 hours following the intravenous administration of 18 nm gold particles, they were completely removed from the blood, and were preferentially located within the liver and spleen. For 1.4 nm gold nanoparticles a proportion (3.7%) remained within the blood at 24 hours with a lower amount located in the spleen and liver as compared to the larger sized particles. Sonavese *et al.* (2008b), de Jong *et al.* (2008) and Cho *et al.* (2009) all showed that following intravenous injection, gold nanoparticles primarily accumulate in liver and a smaller fraction in other organs (like spleen, kidney and lung). In particular smaller particles distribute wider (even to the brain (Sonovane *et al.* 2008b)) and accumulate to a larger extent than larger particles (de Jong *et al.* 2008; Sonavane *et al.* 2008b). Consequently, size influences the behaviour of gold nanoparticles within the body.

As before it is not clear to which extent these gold NP results are relevant for silver nanoparticles. They are in any case not relevant for any silver content appearing as silver ions.

Metabolism

As described several times there is no clear indication whether it is silver nanoparticles, silver ions, silver complexes or a combination that is absorbed. There is thus also no clear data suggesting whether and how the silver is further transformed once in the body.

It should however be noted that one of the biological/toxic effects seen in some studies (argyria - being a blue-grey dis-colouring of the skin) may, at least partly, involve a transformation/reduction of silver ions to metallic silver following exposure to UV-radiation/sunlight (for mechanism see Chang *et al.* 2006; Luoma, 2008). This could indirectly suggest that some of the silver in circulation occurs as silver ions.

Elimination

Trop *et al.* (2006) showed elevated silver levels in the urine following exposure. No other data for nano-silver elimination has been identified.

Semmler-Behnke *et al.* (2008) observed that 24 hours following the intravenous administration of 18 nm gold particles, a small fraction of the administered dose (0.5%) was eliminated via hepatobiliary clearance into the faeces, and <0.1% eliminated within urine and 1.4 nm nanoparticles were excreted within urine (8.6%) and faeces at 24 hours. Consequently, it is apparent that particle size influences the behaviour of gold nanoparticles within the body.

As before it is not clear to which extent these gold nanoparticles results are relevant for nano-silver. They are in any case not relevant for any silver content appearing as silver ions.

In summary, several authors have shown that nano-silver exposure leads to silver uptake after inhalation, oral as well as dermal exposure. It is however not clear whether this uptake occurs as silver nanoparticles, as silver ions (Ag⁺), as silver complexes and/or in combination. There are reasons to believe that part of the uptake via the GIT occurs as silver ions.

Experiments with (the more inert) nano-gold have shown that gold nanoparticles can result in uptake via the relevant exposure routes. The relevance of these findings for silver nanoparticles does indeed depend on: i) whether the silver uptake occurs as particles, and if so; ii) whether the more reactive silver uses the same uptake mechanisms as the more inert gold. However, it would appear that the same target organs are relevant. In addition, the absorption and distribution of particles is likely to be size dependent, so that smaller particles are more readily able to pass through biological barriers, and have a wider tissue distribution.

Following absorption, silver transport within the blood and accumulation within secondary organs has been demonstrated, in addition to evidence of silver dependant toxicity in organs distant to the exposure sites. The main target organ for the absorbed silver (and gold), seems to be the liver and skin (evidenced by the discoloration of the skin), but other targets include the kidneys, spleen, heart, olfactory bulb, brain and testes.

Not knowing in what form silver is absorbed, it is difficult to judge whether and if so how metabolism takes place. However, it should be noted that one of the biological/toxic effects seen in some studies (argyria - being a blue-grey dis-colouring of the skin) may, at least partly, involve a transformation/reduction of silver ions to metallic silver following exposure to uv-radiation/sunlight. This could indirectly suggest that some of the silver in circulation occurs as silver ions.

Some data sources indicate that silver can be eliminated via urine, but more studies are needed to further investigate this.

In conclusion, the main focus in future studies should be on investigating how silver is absorbed and in particular in which form. Having an improved understanding on this will improve understanding of the toxico-kinetics, ease the interpretation of existing studies and improve the design of future toxicity studies.

9.5.4.2 Acute toxicity

Acute toxicity: Oral

Single dose exposure:

Cha *et al.* (2008) directly delivered nano- (15 nm) and micro-particles (2 - 3.5µm) silver, at a dose of 2.5 mg, to the stomach, as a model of ingestion, within mice. Histopathological analysis of the liver tissue, 3 days post-exposure, demonstrated evidence of inflammation in the form of lymphocyte influx for both particle sizes (greater response for the nanoform). This was further supported by changes in the gene expression of 4 genes involved in inflammation. This dose seems to be relatively high in relation to what would be expected in terms of occupational and consumer exposure. The relevance of these findings is therefore dubious for the purpose of this risk assessment.

28 day:

In a 28 day study, Kim *et al.* (2008) investigated the toxicity of silver nanoparticles (60 nm), following the repeated oral exposure of rats, at low (30 mg kg⁻¹ day⁻¹), medium (300 mg kg⁻¹ day⁻¹) or high (1000 mg kg⁻¹ day⁻¹) concentrations. A dose-dependant toxicity was evident within the liver (histopathology changes and inflammation), insinuating that this organ is a target site for nanoparticle toxicity. Again, however, it should be noted that the doses used within this study were relatively high, which should be addressed if used for a risk assessment. However, due to limited histopathological information, no oral No-Observed Adverse Effect Level (NOAEL) is established based on these data.

Human information:

Wadhwa and Fung (2005) and Chang *et al.* (2006) both report skin discolouration (argyria) following pharmaceutical ingestion of colloidal silver (a nano-/micro-particulate silver suspension in an aqueous solution (Luoma, 2008)) most pronounced in sun exposed areas. For the Wadhwa and Fung (2005) study ingestion took place 3 times a day for 10 month (dose unknown). It must be anticipated that the doses seen are considerably higher than what would be expected in occupational settings and following consumer exposure (drugs treatment is outside the scope of this study).

Acute toxicity: Inhalation

In vivo:

No acute toxicity studies have been identified. A number of 28 and 90 days studies are summarised under "Repeated dose toxicity".

In vitro:

A number of studies are described in the section on repeated dose toxicity.

Acute toxicity: Dermal

In vivo:

Most studies focus on the efficiency/beneficial effects of nano-silver in wound dressings (described in the toxicokinetics section) and are considered in the section on "Human information".

Human information:

Trop *et al.* (2006) (evaluating treatment of a burn with wound dressing containing 15 nm silver nanoparticles) found elevated liver enzyme levels, insinuating that liver injury had occurred as a consequence of treatment, and skin discolouration (argyria) was seen.

In vitro:

Arora *et al.* (2008) exposed both HT-1080 (human fibrosarcoma) and A431 (human skin/carcinoma) cells to silver nanoparticles (7-20 nm) and found induction of oxidative stress, as indicated by depletion of glutathione (GSH) and increased lipid peroxidation in both cell types. They also found that the concentrations required to illicit apoptosis to be much lower than the concentrations required for necrosis.

Acute toxicity: Other routes

No studies were identified studying toxicity via other routes of exposure.

In summary, as shown in the toxicokinetics section and as toxicity was observed in secondary organs, silver can be absorbed through the GIT following oral exposure. However, it is not clear whether the silver uptake occurs as nano-silver particles, as silver ions or a combination. The toxicity associated with the ingestion of silver particles, has been demonstrated to include inflammatory responses, liver toxicity and argyria (blue-grey discolouration of the skin). The latter is a typical toxic effect following intake of larger amounts of 'normal'/bulk silver. This may be an indication that some of the silver uptake occurs as silver ions, but this requires confirmation with further investigation, as nanoparticles could also be responsible for this phenomenon. It should be noted that the majority of the data identified relates to the application of silver within pharmaceuticals (or doses as high as expected for pharmaceuticals), that are on occasion self-prescribed (through procurement on the internet). The doses tested do therefore seem to be far above what would be expected in occupational and consumer settings (apart from accidents or deliberate intake). Based on the limited data and the doses tested, it is not possible to give any firm conclusions on acute oral toxicity. All in all, however, the data shows uptake but do not indicate a significant acute toxicity. More detailed investigations involving further (histopathological) investigations are recommended. Further testing should also focus on identifying whether the silver uptake via the GIT involves uptake of nano-silver particles, ions, complexes or a combination. If it is shown that the form and mechanism of uptake is the same as for bulk silver, toxicity data from bulk silver could be used, and as relevant, adjusted for the effects of the smaller sizes of the silver nanoparticles (i.e. possible higher uptake or dissolution of silver nanoparticles).

The available data from 28 and 90 day studies will be discussed under repeated dose toxicity, as well as the in vitro lung studies identified.

No studies investigating the dermal acute toxicity of silver nanoparticles on healthy skin has been identified. However, there are signs of argyria and liver toxicity following treatment of burns with wound dressings. The relevance of this data for healthy skin at lower concentrations and longer exposure time (as expected in occupational and consumer settings) is uncertain. Therefore, further investigations are needed on healthy skin with doses and exposure times relevant for exposures as seen in occupational and consumer settings.

9.5.4.3 Irritation /corrosion

No studies have been identified specifically investigating the irritation or corrosive behaviour of nano-silver to skin, eye and the respiratory tract.

No studies have been identified investigating specifically the irritating behaviour of silver nanoparticles (to the eyes, lungs or skin). Silver nanoparticles are not expected to be irritating to skin based on the human findings of applying it in wound dressings. However, further information would be needed to draw firm conclusions. In relation to inhalation, please refer to sections on acute and repeated toxicity. Based on the above considerations, silver nanoparticles are not assumed to be corrosive.

9.5.4.4 Sensitisation

No studies have been identified specifically investigating sensitisation following dermal or inhalation exposure.

No studies have been identified investigating the sensitising behaviour of silver nanoparticles. No sensitising behaviour has been reported in any of the other studies reported in this assessment. As silver nanoparticle containing wound dressings are routinely applied and none of the studies investigating that application have reported sensitisation, it appears unlikely that nano-silver is a dermal sensitiser. Regarding possible sensitisation following inhalation, further proof/investigation is required.

9.5.4.5 Repeated dose toxicity

Repeated dose toxicity: Oral

A 28-days study was reported under the "Acute toxicity" section.

Repeated dose toxicity: Inhalation

28 days:

Hyun *et al.* (2008) investigated the consequences of repeated exposure to silver nanoparticles (13-15 nm) on the nasal respiratory mucosa of rats. Exposures were conducted via an inhalation chamber for 6 hours per day, 5 times a week for 28 days, at three different doses; low-dose (1.73×10^4 particles cm^{-3} , 1.32×10^7 $\text{nm}^2 \text{cm}^{-3}$, 0.5 mg m^{-3}), middle-dose (1.27×10^5 particles cm^{-3} , 9.68×10^7 $\text{nm}^2 \text{cm}^{-3}$, 3.5 mg m^{-3}) and high-dose (1.32×10^6 particles cm^{-3} , 1.41×10^9 $\text{nm}^2 \text{cm}^{-3}$, 61 mg m^{-3}). The nasal cavity and lungs from the exposed groups were comparable to the control group, although the size and number of goblet cells containing neutral mucins increased significantly in the animals exposed to the middle and high doses of silver nanoparticles. In addition, they identified a slight increase in mucins including sulfomucins, but not sialomucins. The authors concluded that while the silver nanoparticles did influence the neutral mucins in the respiratory mucosa, the results did not suggest any toxicological significance in this model.

Ji *et al.* (2007) exposed rats to silver nanoparticles (<16 nm), at low ($0.48 \text{ } \mu\text{g m}^{-3}$, 1.73×10^4 particles cm^{-3}), medium ($3.48 \text{ } \mu\text{g m}^{-3}$, 1.27×10^5 particles cm^{-3}) or high ($61 \text{ } \mu\text{g m}^{-3}$, 1.32×10^6 particles cm^{-3}) concentrations for 6 hours per day over 5 days a week, for a duration of 28 days. No hematology or biochemical indicator alterations were observed. Some toxicity (cytoplasmic vacuolation and hepatic necrosis) was observed within the liver, but histopathological analysis did not reveal any distinct toxicity within other organs.

90 days:

Sung *et al.* (2008, 2009) conducted a 90 day whole body inhalation study (according to OECD test guideline 413) in rats (6 hours per day, 5 days a week) to silver nanoparticles (18-19 nm), at low ($49 \text{ } \mu\text{g m}^{-3}$, equivalent to 0.6×10^6 particles cm^{-3} and 1.08×10^9 $\text{nm}^2 \text{cm}^{-3}$), medium ($133 \text{ } \mu\text{g m}^{-3}$, equivalent to 1.4×10^6 particles cm^{-3} and 2.39×10^9 $\text{nm}^2 \text{cm}^{-3}$) and high ($515 \text{ } \mu\text{g m}^{-3}$, equivalent to 3.0×10^6 particles cm^{-3} and 6.78×10^9 $\text{nm}^2 \text{cm}^{-3}$) doses. The main targets of particle accumulation and toxicity were observed to be the lungs and liver. Prolonged exposure to silver nanoparticles was demonstrated to elicit an inflammatory response within the lung, and induced alterations in lung function (tidal volume and minute volume), at all particle concentrations (Sung *et al.* 2008) indicating that the lower dose would be a Lowest Observed Adverse Effect Level (LOAEL). However, in the Sung *et al.* (2009) study, which reported on clinical

observations, haematology and histopathological examinations, erythrocyte aggregation and kidney function test, a No-observed Adverse Effect Level (NOAEL) of 100 µg m⁻³ was stated (should rather be termed a NOAEC). For risk assessment it is therefore important to determine whether the lung function effects are considered an adverse effect. For the purpose of this assessment both values will be taken forward to the risk characterisation. In any case the authors conclude that surface area seems to be an important parameter for silver nanoparticle toxicity. It is also noted that the nano-silver particles were shown not to aggregate/agglomerate during the experiment.

Repeated dose toxicity: Dermal

No information identified except the dermal studies reported under acute toxicity.

Repeated dose toxicity: Other routes

No studies identified.

9.5.4.6 Biological mechanisms and target organ toxicity of silver toxicity

Lung toxicity

AshaRani *et al.* (2009) showed toxicity of starch coated silver nanoparticles to normal human lung fibroblast cells (IMR-90) and human glioblastoma cells (U251) *in vitro*. The mechanism of toxicity was considered to involve disruption of the mitochondrial respiratory chain leading to production of ROS and interruption of ATP synthesis, which in turn cause DNA damage.

Soto *et al.* (2007) showed cytotoxicity to lung macrophage and epithelial cell lines with lung epithelial cells being more susceptible than macrophages.

Liver toxicity

As shown in the toxico-kinetics section, liver is expected to be a (or the) major target organ of systemic toxicity.

Hussain *et al.* (2005) showed toxicity to BRL 3A liver epithelial cells. Silver nanoparticles induced a significant increase in cellular reactive oxygen species (ROS) which implies that the silver nanoparticles were able to induce oxidative stress, which again was thought to cause a dose dependent decrease in mitochondrial membrane potential and cell viability.

Cha *et al.* (2008) showed a decrease in the DNA content of the human Huh-7 hepatoma cell line after exposure to silver nanoparticles, which they concluded to be indicative of apoptosis, and they suggested that the nanoparticle form was more potent than the micro particle form of Ag.

Arora *et al.* (2009) exposed primary mouse fibroblasts and liver cells to silver nanoparticles. They showed a protective effect on, or response by, the primary fibroblasts, characterised by an increase in GSH and a decrease in lipid peroxidation. The hepatocytes also demonstrated an increase in the antioxidant enzyme superoxide dismutase (SOD) and GSH, again indicative of a protective response.

Oxidative, inflammatory and genotoxic toxicity

As encountered for other nanoparticles (and as indicated in several studies described above), the toxicity of metal nanoparticles appears to be driven by their oxidative, and inflammatory (Cho *et al.* 2009) nature, which drive genotoxic (AshaRani *et al.* 2009) and cytotoxic (Hussain *et al.* 2005) outcomes. Consequently, it would appear that metal nanoparticles exert their toxicity in a sequential manner, so that increases in ROS production, stimulates oxidant sensitive signaling pathways to eventually culminate in inflammatory, genotoxic and cytotoxic consequences. This pattern of events is exemplified by the findings of a number of

investigators. See also Carlson *et al.* (2008), Hsin *et al.* (2008), Ahamed *et al.* (2008) and Chi *et al.* (2009).

Low levels of oxidative stress are considered to induce the expression of protective mechanisms, as suggested here, while larger doses of oxidative stress result in activation of pro-inflammatory mechanisms, or cell death at extreme levels. Thus, this findings suggest that a threshold mechanism for this type of toxicity.

In summary, as shown in the toxicokinetics section nano-silver can accumulate in the lung tissue and silver (as silver nanoparticles, silver ions and/or silver complexes – to be investigated) can enter the circulation following inhalation. Two 28-day studies (silver nanoparticles <16 nm) reported no or limited toxicity (to neutral mucins and liver), whereas a 90 day study (whole body inhalation) showed an inflammatory response and alterations in the lung function (even at the lowest dose tested (18-19 nm silver nanoparticles, $49 \mu\text{g m}^{-3}$, equivalent to 0.6×10^6 particles cm^{-3} and $1.08 \times 10^9 \text{ nm}^2 \text{ cm}^{-3}$), which could be taken as a LOAEC. However, the authors suggested a NOAEC of $100 \mu\text{g m}^{-3}$. This will be applied for an assessment for other signs of toxicity namely liver toxicity. As this was not an observed value, the middle dose of $133 \mu\text{g m}^{-3}$ (observed dose without liver effects), equivalent to 1.4×10^6 particles cm^{-3} and $2.39 \times 10^9 \text{ nm}^2 \text{ cm}^{-3}$ will be applied as the NOAEC. As can be seen in the DNEL derivation (see below), this value will however be corrected for daily exposure time and thereby will be close to the value suggested by the authors (it is not clear whether the authors have actually also done this to get to the $100 \mu\text{g m}^{-3}$). In any case, the difference in values is within the general uncertainty.

In conclusion, for risk assessment it is important whether the lung function data are considered an adverse effect. For the purpose of this assessment and with the information available, both values (LOAEC of $49 \mu\text{g m}^{-3}$ for lung effects and NOAEC of $133 \mu\text{g m}^{-3}$ for liver effects) will be taken forward to the risk characterisation.

Although studies are not directly comparable (administration, dose, and size of nanoparticles), it appears that dose, size, surface area and exposure time are relevant. It therefore seems relevant to adjust for exposure time when doing a risk assessment and there seems to be a dose-response relationship. Further, as discussed below the mechanism causing the toxicity seems to be threshold driven and it therefore seems appropriate to calculate a Derived No-Effect Level (DNEL).

Quite a few, mainly in vitro, studies have shown that the main mechanism of silver nanoparticle toxicity seems to be mediated by an increase in ROS production, which stimulates oxidant sensitive signaling pathways, and stimulates inflammation and genotoxic events, to eventually culminate in cell death (necrosis and apoptosis).

It is also relevant that the concentration of nanoparticles administered is able to influence the toxicity that is stimulated; specifically, at low levels of oxidative stress a protective response is initiated which progresses to a damaging response with increasing particle concentration, and therefore oxidant levels. Thus it is anticipated that the toxic mechanism is threshold driven.

It should also be noted that it appears that smaller particles exhibit higher toxicity as compared to larger particles; and thus surface area and, if silver is absorbed as particles, the size itself for crossing biological barriers, are relevant. Thus one should be careful in measuring and reporting particle size as part of toxicity studies and exposure measurements. This should consequently be closely considered when doing any risk assessment.

Finally, it should be noted that the relevance of these findings (largely in vitro) needs to be re-evaluated once more information on the likely uptake of silver (as particles, ions, complexes or a combination) and therefore the form in which silver may reach relevant target organs becomes available. It should also be noted that the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) have indicated the difficulties associated with in vitro studies (SCENIHR 2009).

9.5.4.7 Mutagenicity

In vitro:

No mutagenicity or genotoxicity studies classically used in chemical regulatory setting have been identified.

However, as discussed under repeated dose toxicity, silver nanoparticles seem to initiate a ROS driven process, which may ultimately lead to cytotoxicity and genotoxicity.

However, further studies would be needed to test the true direct genotoxic behaviour of silver nanoparticles, which furthermore may be size dependant.

In vivo:

At the end of the 28 days oral study (described under acute toxicity oral exposure) the bone-marrow was collected for a micronucleus test (Kim *et al.* 2008). The result was negative, although it should be noted that there was no positive control.

A micronucleus study was negative. Otherwise, no studies investigating the direct mutagenicity of nano-silver have been identified. However, nano-silver may cause mutagenicity/genotoxicity via an indirect threshold mechanism driven by formation of ROS. Further testing of the possible direct genotoxicity of nano-silver seems justified.

9.5.4.8 Carcinogenicity

No carcinogenicity information has been identified.

*No studies investigating the carcinogenicity of silver nanoparticles have been identified. However, as silver nanoparticles may be genotoxic, carcinogenicity can not be excluded. Further chronic carcinogenicity studies should, however, await confirmation of the possible genotoxicity of silver nanoparticles and further information on the relevance of the conducted mechanistic *in vitro* studies vis-à-vis the uncertainty around the form in which silver is absorbed and may reach various targets.*

9.5.4.9 Toxicity for reproduction

Effects on fertility

Bradyich-Stolle *et al.* (2005) examined the suitability of a mouse spermatogonial stem cell line as a model to assess nanotoxicity, via the LDH and MTT assay, in the male germline, *in vitro*. Silver (15 nm), molybdenum trioxide (MoO₃, 30 nm), and aluminum (Al, 30 nm) nanoparticles were investigated. Control soluble salts were used as well as a positive control, ~1000 nm cadmium oxide, known for its cytotoxic properties. Each particle was added to cells at a concentration of 10 µg ml⁻¹ for 48 hours. Findings demonstrate a concentration-dependent toxicity for all types of particles tested, whereas the corresponding soluble salts had no significant effect. Silver nanoparticles were the most toxic while molybdenum trioxide nanoparticles were the least toxic.

Wiwanitkit *et al.* (2009) highlighted the direct *in vitro* toxicity of gold nanoparticles on mature male germ cells, spermatozoa. As before, the relevance of gold nanoparticle data for read-across to silver nanoparticles is uncertain.

Developmental toxicity

Bar-Ilan *et al.* (2009) showed uptake and embryonic malformation of colloidal silver and gold nanoparticles on transparent zebrafish embryos. The results showed Ag being toxic and Au being more inert. Results also indicated effects to be size dependant for the silver nanoparticles.

Lee *et al.* (2007) conducted a similar study, and reported that silver nanoparticles were transported into and out of embryos through chorion pore canals (CPCs). They further report that the nanoparticles become trapped inside CPCs and the inner mass of the embryos, showing restricted diffusion. The group concludes that toxicity and biocompatibility of silver nanoparticles and the types of abnormalities observed in zebrafish are highly dependent on the dose.

In summary, one in vitro study indicates toxicity (mortality and function) in a mouse spermatogonial stem cell line. It also showed silver to be more toxic than molybdenum trioxide and aluminium particles. Another in vitro study investigating gold nanoparticles showed toxicity towards mature male germ cells, spermatozoa. The relevance of gold nanoparticle data for read-across to silver nanoparticles is however not clear.

Silver nanoparticles may affect male fertility if they reach the male germ cells. Further interpretation requires better understanding of the toxicokinetics and possibly subsequent confirmation in vivo.

No data on female fertility were identified and no conclusion can be drawn.

Two zebra fish embryo studies investigated the developmental toxicity potential of silver nanoparticles and one compared with the effects of gold nanoparticles. It seems that toxicity (malformation and death) is dose dependent and that silver is considerably more toxic than inert gold nanoparticles. As for fertility, further interpretation requires better understanding of the toxicokinetics and possibly subsequent confirmation in vivo.

9.5.5 Derivation of DNEL(s)

As the identified information indicates that toxicity is driven via a threshold mechanic, it seems appropriate to set Derived No-Effect Levels (DNELs). However, it should be noted that there is uncertainty around whether (some) nanomaterials may have nano-specific (possibly non-threshold) mechanisms. Therefore, this exercise should be seen as an attempt to set a DNEL and to do a quantitative risk characterisation for the case in which we actually deal with a threshold mechanism.

With the data available, it seems only possible to estimate a No-effect Level (and thereby set a DNEL) for repeated/chronic inhalation.

A 90 day study (whole body inhalation - rat) showed an inflammatory response and alterations in the lung function at the lowest dose tested (18-19 nm silver nanoparticles, $49 \mu\text{g m}^{-3}$, equivalent to 0.6×10^6 particles cm^{-3} and 1.08×10^9 $\text{nm}^2 \text{cm}^{-3}$). This is for the purpose of the exercise, taken as a lowest observed adverse effect level (LOAEL).

For other effects (mainly liver effects) a NOAEL (NOAEC) of $133 \mu\text{g m}^{-3}$ (equivalent to, 1.4×10^6 particles cm^{-3} and 2.39×10^9 $\text{nm}^2 \text{cm}^{-3}$) will be applied in line with what was suggested by Sung *et al.* (2009).

As noted, this assessment will not, based on the information available, judge whether the reduced lung function is adverse and therefore both values are taken forward in the risk assessment. However, with inclusion of the LOAEL of $49 \mu\text{g m}^{-3}$, this assessment would like to question the NOAEC suggested by Sung *et al.* (2009).

As noted in the introduction, DNEL will be derived based on the Chapter R.8 of the REACH guidance (ECHA, 2008).

Reduced lung function as critical effect

Modification of the starting point (correction for exposure time and worker light activity)

Corrected LOAEC (8 hours, worker light activity) =

Engineered Nanoparticles: Review of Health and Environmental Safety

6 hours / 8 hours x 6.7 m³ / 10 m³ x LOAEC (6 hours, rat) = 25 µg m⁻³, equivalent to 3.0x10⁵ particles cm⁻³ and 0.54x10⁹ nm² cm⁻³.

LOAEC to NAEC:

Extrapolating the LOAEC to a NAEC is difficult, but again for the purpose of the exercise, two NOAECs are estimated using an extrapolating factors of 3 (scenario 1) and 10 (scenario 2), respectively.

This gives:

NAELscenario1 = 8.2 µg m⁻³, equivalent to 0.10x10⁶ particles cm⁻³ and 0.18x10⁹ nm² cm⁻³
NAELscenario2 = 2.5 µg m⁻³, equivalent to 0.03x10⁶ particles cm⁻³ and 0.054x10⁹ nm² cm⁻³

It should be noted that it would clearly be recommended to design a study to estimate a real No Observed Adverse Effect Concentration (NOAEC).

Interspecies extrapolation:

As the local effects seen do not depend on the metabolic rate, no allometric scaling is used and a factor of 2.5 for other variations is applied.

Intraspecies:

The default factor of 5 (workers) is applied.

Exposure duration:

As it has been shown that exposure time is relevant, a default factor of 2 is applied to extrapolate from sub-chronic to chronic conditions. No factor is applied for severity of effect.

Thus the overall assessment factor (OAF) is:

OAF scenario: 2.5 (Inter) x 5 (intra) x 2 (sub-chronic to chronic) = 25.

This gives:

DNEL_{lung scenario 1} = NAEL1 / OAF = **0.33** µg m⁻³, equivalent to 4000 particles cm⁻³ and 7.2x10⁶ nm² cm⁻³

DNEL_{lung scenario 2} = NAEL2 / OAF = **0.098** µg m⁻³, equivalent to 1200 particles cm⁻³ and 2.2x10⁶ nm² cm⁻³

Systemic liver effects as critical effect

NOAEC = 133µg m⁻³ (equivalent to, 1.4x10⁶ particles cm⁻³ and 2.39x10⁹ nm² cm⁻³)

Modification of the starting point (correction for exposure time and worker light activity)

Corrected NOAEC (8 hours, worker light activity) = 6 hours / 8 hours x 6.7 m³ / 10 m³ x NOAEC (6 hours, rat) = 67 µg m⁻³, equivalent to 7.0x10⁵ particles cm⁻³ and 1.2x10⁹ nm² cm⁻³.

Interspecies extrapolation:

Due to lack of other data and including allometric scaling a default factor of 10 is applied.

Intraspecies:

The default factor of 5 (workers) is applied.

Exposure duration:

As it has been shown that exposure time is relevant, a default factor of 2 is applied to extrapolate from sub-chronic to chronic conditions. No factor is applied for severity of effect.

Thus the overall assessment factor (OAF) is:

OAF scenario: 10 (Inter) x 5 (intra) x 2 (sub-chronic to chronic) = 100.

This gives a DNEL_{liver effect} = 0.67 µg m⁻³ (equivalent to, 7000 particles cm⁻³ and 1.2x10⁷ nm² cm⁻³).

9.5.6 Risk characterisation

Silver nanoparticles have been shown to be absorbed into the circulation following all considered routes of exposure (namely the skin, GIT and lungs). It is not clear whether this uptake occurs as silver nanoparticles, free silver ions, silver complexes or in combination, although at least for uptake via the oral route, it is likely that at least some of the uptake occurs as free ions. In line with this, most of the applications of silver nanoparticles are related to the well-known anti-microbial activity of bulk silver. Altogether, it must therefore be anticipated that some of the human uptake of silver from the nanoparticles, occurs as ions.

It should be noted that various studies confirm that gold nanoparticles can be absorbed as particles via all routes of exposure, but the relevance of these findings for assessing silver would need to await the outcome of further toxicokinetic studies of silver (is silver absorbed as particle?) and careful consideration of whether the same uptake mechanisms would be relevant (silver chemistry being considerably more reactive as compared to the more inert gold). But on the other hand, it might be important that the same target organs have been recognised, particularly the involvement of the liver within the uptake, and accumulation of particles, and the size dependence of the tissue distribution, which could indicate that some of the same mechanisms are involved.

Should silver uptake occur solely as ions, the already rich database for silver could be applied to assess systemic silver nanoparticle toxicity. For that exercise, it would need to be considered whether and how the dramatically increased surface area and possibly increased solubility of silver nanoparticles would need to be taken into account.

It is recommended that further clarification of the toxicokinetic behaviour of silver following nanoparticle exposure should be the main focus of future investigation, as this will also heavily influence the interpretation of existing and design of future toxicity studies.

In any case, for the time being, exposure organ as well systemic toxicity is relevant to consider for all routes of exposure to silver nanoparticles.

In terms of mechanisms of toxicity, quite a few, mainly *in vitro* studies have shown that the main mechanism of silver nanoparticle toxicity seems to be mediated by an increase in ROS production, which stimulates oxidant sensitive signalling pathways, and stimulates inflammation and genotoxic events, to eventually culminate in cell death (apoptosis and necrosis). It is also relevant that the concentration of nanoparticles administered is able to influence the toxicity that is stimulated; specifically, at low levels of oxidative stress a protective response is initiated which progresses to a damaging response with increasing particle concentration, and therefore oxidant levels. It is thus relevant to consider that silver nanoparticles may induce their toxicity via a threshold mechanism. It should also be noted that it appears that smaller particles exhibit higher toxicity as compared to larger particles; and thus surface area and if silver is absorbed as particles the size itself for crossing biological barriers, is relevant. A few studies have indicated that silver nanoparticles seem to have the ability to cause reproductive toxicity and be more toxic than some other metals. However, this is based on limited data. Finally, as noted several times the relevance of these findings (largely *in vitro*) needs to be re-evaluated once more information on the likely uptake of silver (as particles, ions or a combination) and therefore the form in which silver may reach relevant target organs becomes available. It is noted that little information is available on possible direct genotoxicity caused by silver nanoparticles. Altogether, the following attempts to do (quantitative) risk characterisations assume that silver nanoparticles act via a threshold mechanism. The assessment should not be applied for any regulatory decision-making, given the large uncertainties associated with toxicity as well as exposure data.

The toxicity data for oral exposure are mainly related to application of high doses of relevance for assessing the risk/safety of silver as used in drug applications. As toxicity has been seen following intake of these drugs (sometimes self-prescribed and bought on the internet) this requires further evaluation and regulation. However, within the scope of this assessment, the toxicity seen in these studies (liver toxicity and argyria) is not necessarily relevant for the doses that would normally be expected to be encountered in the workplace and in the identified

consumer uses. No quantitative exposure data have been identified, but the exposure in the workplace is expected to mainly occur after swallowing silver nanoparticles that have been cleared from the airways via the mucociliary escalator and in the identified consumer uses oral exposure does also not seem to be the most relevant exposure route. In conclusion, the current database does not allow for a quantitative evaluation of the risk for the oral exposure route and more exposure data and toxicity data using lower doses would be needed to appropriately assess the risks. Qualitatively however, risks associated with oral exposure, is expected to be less critical than the risk from other routes of exposure, although intake following hand-to-mouth contact should also be further evaluated. This conclusion does of course not apply to drug applications and deliberate/accidental intake.

Inhalation exposure appears to be the main exposure route of concern for occupational exposure. One study has been identified giving quantitative values from a pilot plant manufacturing activity. Peak concentrations of 7000 particles cm^{-3} (for 100-200 nm particles) was reported. By assuming a uniform size of 150 nm this corresponds to a surface area of $0.5 \times 10^9 \text{ nm}^2 \text{ cm}^{-3}$. Another study reporting on production of manufacturing of metal nanoparticles, not necessarily silver nanoparticles, found average concentrations of 59199 particles cm^{-3} , 0.188 mg m^{-3} (for sub-micron particles). This study is applied for the purpose of this exercise, but the values should be applied with care. It should be stressed that the values represent only two manufacturing facilities (the one a pilot plant) and that they are not necessarily representative. On the other hand, it should be considered whether downstream handling of silver nanoparticles in less controlled settings might cause higher exposures.

Regarding toxicity, in particular a 90 day inhalation study in rats, indicates damage to the lung function and the liver (following systemic uptake). As pointed out in the previous section, for the purpose of the exercise, several DNELs have been identified (particle size 18-19 nm):

DNEL_{lung scenario 1} : 0.33 $\mu\text{g m}^{-3}$, equivalent to 4000 particles cm^{-3} and $7.2 \times 10^6 \text{ nm}^2 \text{ cm}^{-3}$
DNEL_{lung scenario 2} : 0.098 $\mu\text{g m}^{-3}$, equivalent to 1200 particles cm^{-3} and $2.2 \times 10^6 \text{ nm}^2 \text{ cm}^{-3}$
DNEL_{liver effect} : 0.67 $\mu\text{g m}^{-3}$, equivalent to, 7000 particles cm^{-3} and $1.2 \times 10^7 \text{ nm}^2 \text{ cm}^{-3}$.

As several studies have indicated that smaller particles appear to be more toxic than their larger counterparts, a direct comparison of the identified exposure data (for larger particles) and toxicity data (for smaller particles) should be approached with care. However, it is interesting that in terms of particle number the exposure values are in the same order of magnitude (or higher) than the estimated DNELs. The mass concentrations are also higher as is the surface area (to the extent it could be estimated). It should be noted that the toxicity study reported that the particles did not agglomerate during the study. It is therefore interesting to further investigate the state of the silver nanoparticles to which workers are actually exposed. All in all, it seems that concentrations encountered in occupational settings might cause a risk after prolonged inhalation exposure. This is of course even more relevant if exposures are higher in less controlled conditions than those reported in the exposure studies identified in this assessment; for example in less controlled downstream handling.

Further investigations should focus on generating more data for the workplace exposure (manufacturing and downstream uses) and doing a proper characterisation of the particles measured (e.g. are workers only exposed to larger agglomerates?) and likely conduct (sub-) chronic toxicity studies focusing on silver nanoparticle sizes and agglomeration states as encountered in the workplace.

No inhalation data for consumers have been identified and therefore no quantitative risk estimation can be carried out. However, qualitatively, given that silver nanoparticle application in sprays have been reported, this exposure route should be of concern and priority in terms of identifying exposure values. On the other hand, it should be noted that consumer exposure may be less frequent than in the workplace and therefore (given the likely time dependency of effects) possibly of less concern.

No quantitative data estimating occupational or consumer dermal exposure to silver nanoparticles have been identified. However, especially consumers must be assumed to be exposed to silver nanoparticles, not the least from the relatively widespread use in clothes. Also

use in paints may provide a significant dermal consumer exposure. In estimating consumer exposures, it may be relevant to include considerations of cumulative exposures from several consumer products. Some toxicity data (also in humans) have been identified, investigating the use of silver in wound dressings for treatment of burns. These data show some toxicity (in particular argyria and liver toxicity) following repeated treatment with these wound dressings under certain circumstances, but their focus was mainly on determining the efficiency of the wound dressings. The relevance of these data for assessing the risk of occupational and consumer exposure is dubious as the exposure levels encountered in the workplace and consumer uses are expected to be considerably lower and to normally occur on more healthy skin. On the other hand, occupational and consumer exposure is expected to last considerably longer than a short term treatment of a burn. In conclusion, in order to investigate this exposure route further, consumer exposure need to be estimated as well as longer term (repeated dose) toxicity studies using doses/concentrations closer to the expected exposure concentrations conducted. Again for the wound dressing themselves (outside the scope of this assessment), toxicity has been seen even in human studies and further assessment is therefore encouraged for that purpose.

In terms of further work with assessing the risk of silver nanoparticles, the following activities are recommended as main priorities:

- Further investigations of the nano-silver toxicokinetics. In particular, to which extent the silver absorbed via different routes becomes systemically available as ions, as nanoparticles and/or as silver complexes, and whether the size of the silver nanoparticles influences the uptake mechanism(s);
- Generation of exposure data. In particular for occupational inhalation and consumer inhalation and dermal exposure. As noted these should carefully characterise the silver nanoparticles in terms of size and agglomeration states, as well as clearly indicate duration and frequency of exposure;
- Sub-(chronic) inhalation toxicity study/studies with silver nanoparticles as encountered in the workplace (concentrations and agglomeration state) to also identify a No-Observed Effect Concentration (NOAEC) based on an in-depth investigations of all relevant endpoints, including histopathological examinations.

Also relevant seems to be:

- Further testing for the possible direct genotoxicity of silver nanoparticles;
- Oral and dermal toxicity studies at doses and durations relevant for occupational and consumer exposure;
- Further investigation of the possible reproductive behaviour of silver nanoparticles.

Depending on the outcome of these investigations, further activities should be prioritised.

9.5.7 Environmental risk assessment for silver nanoparticles

9.5.7.1 Environmental fate properties

Degradation

Not relevant for metals.

Environmental distribution

The environmental chemistry and fate of silver (not nano) is relatively well investigated and understood (see e.g. Adren and Armstrong 1999; WHO 2002). However, up until now little is known about the fate and behaviour of nanosilver.

The effect of the chemical composition of the water on the particle size distribution and the dispersion stability of nanosilver in natural river water were investigated taking natural organic matter (NOM) and cations concentration into consideration (Gao *et al.* 2009). The results of the study showed the aggregation effect of electrolytes and the stabilisation effect of NOM. An interesting finding was that metal cations (e.g. Ca^{2+} , Mg^{2+}) as a mixture in natural waters act as

aggregating agents even if nanosilver concentration is below the theoretical Critical Coagulation Concentration (CCC) determined by NOM. Natural organic matter can also reduce the leaching of Ag^+ in water from nanosilver by rapidly coating the Ag nanoparticles. This effect is important because Ag^+ ion is toxic to most organisms at low concentrations.

Given the likelihood of particulate silver to dissolve to a certain level, and the fact that different forms of nanosilver may be associated with different dissolution rates, it is very difficult to predict from work published to date realistic environmental concentrations. Mueller and Nowack (2008) used a life-cycle perspective to model the quantities of engineered nanoparticles released into the environment including nanosilver. The quantification was based on a substance flow analysis from products to air, soil, and water in Switzerland. They calculated PECs between 0.03 and 0.08 $\mu\text{g l}^{-1}$ silver nanoparticles in water, and between 0.02 to 0.1 $\mu\text{g kg}^{-1}$ silver nanoparticles in soil. However, given the lack of information, numerous assumptions and estimates had to be made. Some details and limitations of these calculations are discussed in Hansen (2009).

Boxall *et al.* (2007) developed a framework of simple models and algorithms for estimating nanoparticle concentrations in water, soil and air. For silver, they estimated 0.01 $\mu\text{g l}^{-1}$ silver nanoparticles in water and 0.43 $\mu\text{g kg}^{-1}$ in soil. In comparison to the values calculated by Mueller and Nowack (2008), the estimate for water is lower, while that for soil is higher. However, both values are in the same order of magnitude. Hansen (2009) discusses some of the differences between the two approaches.

Bioaccumulation

No data available.

In summary, there are limited data on the environmental fate of silver nanoparticles. The effect of the chemical composition of water on the transport of silver nanoparticles in the environment is similar to those observed for carbon-based nanomaterials, with organic matter as dispersion stabiliser and cations as aggregating agents. Moreover, NOM can act as a coating for nanoparticles, preventing the leaching of Ag^+ ions, while divalent cations mixtures can be effective particle aggregating agents even at lower than the CCC. These data suggest low mobility of silver nanoparticles in environmental matrices, due to the formation of large aggregates, and also the resulting reduced leaching of toxic ions into water.

9.5.7.2 Environmental hazard assessment

The hazard assessment of nanosilver was carried out focusing on the effects of nanoparticles in environmental matrices. However, since the main mechanism of toxicity of silver seems to be related to Ag^+ ion dissolution, the toxicity of Ag nanoparticles and Ag^+ ions (generally from inorganic salts of Ag) was compared in order to identify the main mechanism of action of silver nanoparticles, and to answer the question whether the toxicity related to the ion leaching or not.

Aquatic compartment (including sediment)

Fish:

Toxicity data for fish were found in three short-term studies for *Danio rerio*. No long-term studies were found in literature published before December 2008.

Table 9.18: Short-term effects of nanosilver on fish

Method	Results	Remarks	Reference
<i>Danio rerio</i> Adult	Mortality LC ₅₀ (48h): 7.07 mg l ⁻¹	Ag NP 44.5 nm size. No significant Ag ⁺ leaching. Toxicity due to NP.	Griffitt <i>et al.</i> (2008)
<i>Danio rerio</i> Juvenile	Mortality LC ₅₀ (48h): 7.20 mg l ⁻¹	Ag NP 44.5 nm size. No significant Ag ⁺ leaching. Toxicity due to NP.	Griffitt <i>et al.</i> (2008)
<i>Danio rerio</i> Embryo	Mortality LC ₅₀ (72h): 25-50 mg l ⁻¹ Sub lethal effects (72h): 5 mg l ⁻¹	Varying according to development stage Ag NP coated with BSA/potato starch, size 5-20 nm	Asharani <i>et al.</i> (2008)
<i>Danio rerio</i> Embryo	Morphological defects (72h): 10 ppt (10 ng l ⁻¹)	Ag NP in tap water, size 10-20 nm. Effect of Ag ⁺ .	Yeo and Kang (2008)

BSA = bovine serum albumin

Short-term toxicity in fish was tested on *Danio rerio* at different life stages. Mortality, as well as sub-lethal effects, was observed for adults, juveniles, and embryos (Table 9.18). Since nanoparticles with different size and composition were used, no general conclusions can be established. A study concerning the morphological development of embryos observed effects at 10 ng l⁻¹ silver nanoparticles, which according to the authors was mostly in the ionic form (Yeo and Kang, 2008). However, Griffitt *et al.* (2008) reported that toxicity was mostly due to Ag nanoparticles, because ions dissolution was very low.

The lowest LC_{50 fish} (short-term) identified is 7 mg l⁻¹ silver nanoparticles.

Aquatic invertebrates:

Only short-term toxicity data for aquatic invertebrates were reported in the literature. No long-term studies were found in literature published prior to December 2008.

Table 9.19: Short-term effects of nanosilver on aquatic invertebrates

Method	Results	Remarks	Reference
<i>Ceriodaphnia dubia</i>	Mortality LC ₅₀ (48h): 0.067 mg l ⁻¹	Ag NP diameter: 44.5 nm. No significant Ag ⁺ leaching. Toxicity due to NP.	Griffitt <i>et al.</i> (2008)
<i>Daphnia pulex</i>	Mortality LC ₅₀ (48h): 0.040 mg l ⁻¹	Ag NP diameter: 44.5 nm. No significant Ag ⁺ leaching. Toxicity due to NP.	Griffitt <i>et al.</i> (2008)

Toxicity of nanosilver was found to be much higher for the crustacean taxa tested than for fish species studied, with LC₅₀ two orders of magnitude lower than that measured in the same exposure conditions for *Danio rerio* (Table 9.19). Griffitt *et al.* (2008) reported that toxicity was mostly due to Ag nanoparticles, because ions dissolution was very low.

The lowest LC_{50 aquatic invertebrates} (short-term) reported is 0.04 mg l⁻¹ silver nanoparticles.

Algae and aquatic plants:

Table 9.20: Effects of nanosilver on green algae

Method	Results	Remarks	Reference
<i>Pseudokirchneriella subcapitata</i>	Growth inhibition LC ₅₀ (96h): 0.19 mg l ⁻¹	Ag NP diameter: 44.5 nm. No significant Ag ⁺ leaching. Toxicity due to NP.	Griffitt <i>et al.</i> (2008)
<i>Chlamydomonas reinhardtii</i>	Growth inhibition: EC ₅₀ (1h): 0.355 mg l ⁻¹ EC ₅₀ (3-5h): 0.092 mg l ⁻¹	Carbonate coated Ag NP suspension, diameter: 25 nm. Effect due to Ag NP (low Ag ⁺ dissolution).	Navarro <i>et al.</i> (2008)

Available data on algae are not comparable because Griffitt *et al.* (2008) used non-coated nanoparticles (44.5 nm), while Navarro *et al.* (2008) used carbonate-coated Ag nanoparticles (25 nm). The data presented in Table 9.20 indicates that nanosilver is toxic to freshwater micro algae, with the EC₅₀ ranging between 0.092 and 0.19 mg l⁻¹ silver nanoparticles for exposures between 5 hours and 96 hours. These data are in between those reported for daphnids and for *Danio rerio*.

Both authors reported that the toxicity of Ag nanoparticles was mainly related to the nanoparticle itself, acting as a source of Ag⁺ in the presence of algae (Navarro *et al.* 2008), rather than Ag⁺ in solution at the beginning of the exposure (i.e. < 1% mass was in solution during exposure). Navarro *et al.* (2008) also reported that the toxicity of Ag nanoparticles as a function of Ag⁺ was much higher than those of AgNO₃. Therefore, the toxicity of Ag nanoparticles appears to be mediated by Ag⁺ ions dissolved due to interaction with algae. The lowest LC_{50 algae} value is 0.09 mg/L.

Other aquatic organisms:

Table 9.21: Effects of nanosilver on bacteria

Method	Results	Remarks	Reference
Nitrifying bacteria	IC ₅₀ : 0.14 mg l ⁻¹	NP size relevant if < 5 nm. Ag nanoparticle more toxic than Ag ⁺	Choi and Hu (2008)
Nitrifying bacteria	IC ₅₀ : 0.32 mg l ⁻¹ .	IC ₅₀ extrapolated from paper graph. Nanoparticle size 16 nm. Ag nanoparticle more toxic than Ag ⁺	Choi <i>et al.</i> (2008)
<i>Escherichia coli</i>	IC ₅₀ : 0.43 mg l ⁻¹	Ag nanoparticle less toxic than Ag ⁺	Choi <i>et al.</i> (2008)

IC₅₀: Inhibition concentration 50%

Toxicity towards bacteria is a well known property of nanosilver, since it is used in consumer products as antibacterial. The two studies by Choi and Hu (2008) and Choi *et al.* (2008) refer to two different types of bacteria, one autotrophic and the other heterotrophic. Nitrifying bacteria are important for sewage treatment plant efficiency. In general, the test results confirm the antibacterial activity of silver nanoparticles (Table 9.21). However, it is not possible to define a general conclusion about the role of Ag⁺ ions, since for nitrifying bacteria it is reported that the nanoparticles are more toxic than Ag⁺ ions, while for *Escherichia coli* it is the opposite. The measured IC₅₀ are in the same order of magnitude than that of the EC₅₀ for green algae.

Terrestrial compartment

In the literature, no data concerning the terrestrial compartment were identified.

9.5.8 Risk characterisation and gap analysis

Quantitative risk assessment:

Due to the limited data, a quantitative risk assessment is only carried out for the aquatic compartment, freshwater (without sediment, without food chain).

PEC/PNEC comparison can provide provisional information about the potential for environmental impacts of silver nanoparticles. However, the PEC estimated by Mueller and Nowack (2008) and Boxall *et al.* (2007) are highly uncertain; therefore, calculations for different scenarios were made.

A PNEC for water can be calculated by using short-term LC₅₀ and assessment factors. There is at least one short-term LC₅₀ from each of the three trophic levels of the base-set (fish, *Daphnia* and algae). When only short-term data are available, an assessment factor of 1000 will be applied to the lowest LC₅₀. From the species investigated, *Daphnia pulex* was the most sensitive aquatic organism for nanosilver (LC₅₀ = 0.04 mg l⁻¹ silver nanoparticles).

Table 9.22: Risk Characterisation for nanosilver for the aquatic compartment

Compartment	PEC	PNEC	PEC/PNEC	Remarks
Freshwater	0.01 µg l ⁻¹	0.04 µg l ⁻¹	0.25	PEC from Boxall <i>et al.</i> (2007)
	0.03 µg l ⁻¹	0.04 µg l ⁻¹	0.75	PEC corresponds to lower value from Mueller and Nowack (2008)
	0.08 µg l ⁻¹	0.04 µg l ⁻¹	2	PEC corresponds to higher value from Mueller and Nowack (2008)

The PEC/PNEC ratio for the aquatic compartment is around 1, being comprised between 0.25 and 2 (Table 9.22). Given the high uncertainties in the calculation, the results suggest a concern for environmental exposure to silver nanoparticles, leading to the need of a refined assessment. Taking into account the uncertainties with respect to the PEC, improved exposure information would be useful. Additional exposure information, either through more detailed modelling based on better data (e.g. manufacture and use data) or exposure measurements in natural waters are needed. Furthermore, long-term ecotoxicity data on *Daphnia* would be useful to refine the risk assessment: long-term ecotoxicity data would justify the use of a lower assessment factor to derive a PNEC, and decrease the uncertainty of the assessment.

Qualitative risk assessment:

Silver nanoparticles are used in many consumer products every day, allowing direct exposure to humans and potential release into the environment mainly via water discharge and waste release (e.g. textiles washing) as nanosilver, but mainly as ion Ag⁺ released from solid matrices including nanosilver.

Silver is known as a highly ecotoxic metal. Studies with nano silver on fish, crustacean and algae confirm the toxicity of the nanoparticles. It seems that the mechanism of action of Ag nanoparticles is mostly mediated by Ag⁺ ions released after interaction with organisms (e.g. adsorption to exudates, adsorption to membranes, uptake), as generally the dissolved Ag ion concentration leached from nanoparticles during the exposure (< 1% in mass) is not sufficient to explain the observed effects.

Therefore, the effect of natural water components (e.g. natural organic matter, clay, and ionic strength) as well as the effects of the coating and the embedding matrix (e.g. textile) on the ion leaching of silver nanoparticles should be investigated.

Other gaps that prevent the derivation of a robust and reliable risk assessment are:

1. Lack of information on production volumes of nanosilver, in its many forms;
2. Lack of information on the number of nanosilver products, market penetration, and amounts of the nanoparticles in these products;
3. Lack of information on behaviour of nanosilver during wastewater treatment;
4. Lack on monitoring field data on nanosilver levels in environmental compartments;
5. Lack of toxicity data on several species categories for aquatic compartment, as for example long-term studies on fish and *Daphnia*;
6. Complete absence of data (fate, toxicity) for the terrestrial compartment, as well as for the aquatic sediment;
7. Lack of information concerning interaction between organisms and nanosilver (adsorption, uptake, bioaccumulation, etc.).

9.6 METAL OXIDE NANOPARTICLES

9.6.1 Identity

Metal oxide nanoparticles should not be considered as one group in terms of risk assessment and no general conclusion on the general toxicity/ecotoxicity of metal oxide nanoparticles can, or should, be made. Just like “bulk forms” of metal oxides there are large differences in speciation, behaviour, fate and effects. The major part of the metal oxide scientific literature published deals with effects of titanium dioxide (TiO₂) nanoparticles. Therefore, TiO₂ was used to carry out a case study for a basic risk assessment for a metal oxide nanoparticle addressing human health and the environment. However, the physico-chemical properties of the tested TiO₂ nanoparticles (e.g. size, crystallinity, surface coating) differ between studies and thus, can make comparisons difficult. Besides TiO₂, an environmental risk assessment for zinc oxide (ZnO) has been performed.

9.6.2 Manufacturing and use

Titanium dioxide nanomaterials are used in several consumer and industrial products, e.g. personal care products (in particular in sunscreens), protective coatings, building products, and as white pigment in paints, plastics, paper and other applications. The large production volumes (global: 5000 - 64000 million tonnes per annum, Mueller and Nowack 2008) and widespread use of products containing TiO₂ may allow release of TiO₂ nanoparticles into the environment via wastewater and leaching of surfaces exposed to the external ambient environment.

Zinc oxide nanoparticles are used in several products, for example, ZnO is one of the best broad spectrum UV (Ultra Violet Rays) blockers available. It is approved by the US FDA, but not in the EU, for use as a broad spectrum sunblocker in sunscreen lotions. In addition it is used as a food additive although this use has not been evaluated by EU authorities. Furthermore, zinc oxide exhibits anti-bacterial properties and is used in pharmaceutical applications. Nano zinc oxide powders seems to be used in sunscreens and sunblockers, lipsticks, anti-bacterial lotions, rubber emulsions, UV stabiliser in plastics, as a catalyst in the chemical industry, and as a food additive. Zinc oxide is extremely stable and does not degrade from sun exposure; it is insoluble in water and is well suited for water resistant sunblock.

9.6.3 Human exposure assessment

The risk assessment for metal oxides will focus on titanium dioxide (TiO₂), which is the metal oxide nanoparticle with the richest data-base.

Occupational exposure assessment

Occupational exposure to TiO₂ can occur during manufacturing, including bagging and handling and during subsequent downstream formulation and use of the resulting preparations, which

covers various paints/varnishes, paper, ceramics, rubber and printing inks. It is also used in various coatings.

Occupational exposure is foreseen to mainly occur via inhalation (during manufacturing and handling, bagging and formulation of the TiO₂ powder) and as dermal contact following handling of the powder and the liquid preparations in which it is applied. Oral exposure may also be relevant as part of the inhaled fraction if cleared via the mucociliary escalator and subsequently swallowed, and following hand-to-mouth contact.

Inhalation exposure:

No specific studies have been identified. However, Tsai *et al.* (2009) assessed the airborne exposure following the manual handling of aluminium oxide (powder) nanoparticles in fume hoods. Due to a lack of data for TiO₂, these data will be used in a read-across approach for the purpose of this exercise. However, it is recommended that TiO₂ specific data should be generated.

The particles handled had a size of 27 to 56 nm, forming agglomerates of approximately 200 nm when dried. Particle number concentrations (corrected for background) were assessed following handling of 15 and 100 g, respectively, with the latter giving clearly the highest numbers. The maximum worst case concentration found was 200000 particles cm⁻³. Although uncertain, this value will be used for the purpose of this risk assessment, although of course real data for TiO₂ should be established. Assuming this would be TiO₂ (with a density of about 4 g cm⁻³), this would for 50 nm particles result in a concentration of about 52 µg m⁻³ and for 100 nm particle in a concentration of 420 µg m⁻³.

Dermal exposure:

No studies were identified.

Consumer exposure

Titanium dioxide is used in a variety of consumer products. The most well-known use being in sunscreen, which is expected to be a major source for consumer exposure. It is however outside the scope of this assessment as it is considered a cosmetic use in Europe.

TiO₂ is used in a variety of other products to which consumers can be exposed, including solar cells, catalysts (between others for indoor air treatment due to its ability to degrade different pollutants such as nitrogen oxides (NOx) and volatile organic compounds (VOCs)) and various coatings/surfaces (due to its self-cleaning and sterilizing/anti-fouling/anti-bacterial/anti-fungal properties). It is also used in various paints, varnishes and inks.

This information suggests that a large proportion of consumer use is associated with solid matrices (e.g. coatings). One study has been identified investigating TiO₂ nanoparticle emission due to human contact and weathering of TiO₂ nanoparticle coatings applied to three carrier materials (wood, polymer and tile) (Hsu and Chein, 2007). The experiment was run in a laboratory simulation box with UV-light, a fan and a rubber knife to simulate weathering (wind and sun-light) and human contact conditions. The emission rates were different for the three materials, but in general were very low, with the highest release reported as being 22 particles cm⁻³ (55 nm) from the coated tiles. Overall, this study does not point to significant releases from coatings, although one could doubt the possibilities for analytical identification of such low numbers. This single study cannot be taken to be representative (in terms of materials and applications) and the authors also conclude that nano-coated materials should undergo emission evaluations.

For the applications where the product is used in "liquid" products, such as paints and varnishes, dermal contact is likely to be a major route of exposure, whereas inhalation may be significant if used as sprays.

The only study identified that estimated such exposures relates to sunscreens applied as a spray-on formulation. Boxall *et al.* (2007) found, by using a simple modelling approach assuming recommended use of the spray, a possible concentration of 3.5 mg m⁻³. It should be noted that this value would represent an acute/short-term exposure event. However, given that

other consumer products may be supplied as sprays and used for longer periods (e.g. spray cans with paint), exposure of this (or even higher) magnitude may occur over longer periods.

9.6.4 Human health effects assessment

TiO₂ nanoparticles can occur in many different sizes, shapes and as agglomerates or coated with other material. It also occurs in two main crystal forms, anatase and rutile. As a consequence, identifying distinct hazards related to TiO₂ nanoparticle exposure and making generalisations about the biological activity of TiO₂ nanoparticles is difficult.

9.6.4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

Absorption

Inhalation:

Ferin *et al.* (2008) showed that following a 12 week inhalation study, smaller particles (21 nm) had a longer retention time in the lung than larger particles (250 nm), with 501 days and 174 days, respectively.

Bermudez *et al.* (2004) showed differences between species in their ability to clear TiO₂ nanoparticles from the lung. Hamsters seem to clear TiO₂ nanoparticles relatively quickly (and show thereby less toxicity) as compared to mice and rats having longer (and similar) initial retention time. In general, rats were found to be most sensitive.

No studies were identified investigating systemic absorption of TiO₂ nanoparticles following inhalation. As many studies investigating the toxicity of TiO₂ nanoparticles following inhalation do not report on this issue, it is questionable whether TiO₂ enters the circulation to a great extent via this route. This, however, needs further specific investigation.

Following nasal instillation (mice, 500 µg per mouse, every other day for a total of 30 days), Wang *et al.* (2008a, 2008b) reported accumulation of TiO₂ nanoparticles in the brain, with the hippocampus and the olfactory bulb being the major sites of accumulation. The absorption is thought to occur via neuronal transport, and thereby by-passes the blood-brain barrier.

Oral:

Wang *et al.* (2007) orally exposed mice to 5 g kg⁻¹ of nanoparticulate (25 and 80 nm) and microparticulate (155 nm) forms of TiO₂. TiO₂ particles of all sizes, 2 weeks post exposure, were distributed to the liver, spleen, lungs, kidneys. This indicates systemic uptake of nano- and micro-TiO₂ via the oral route of exposure.

Dermal:

Several authors have reported studies investigating the possible dermal absorption of various formulations of TiO₂ nanoparticles *in vivo* (humans) and *in vitro* (porcine and human skin) (Mavon *et al.* 2007; Schulz *et al.* 2002; Kiss *et al.* 2008; Gamer *et al.* 2006; Pflucker *et al.* 1999; Dussert *et al.* 1997). The studies reported none or negligible passage past the *Stratum Corneum* in healthy skin.

No studies were identified investigating the possible passage through skin with damaged *Stratum Corneum*, as for example could be evident within heavily sunburnt skin.

Distribution

Following oral absorption, Wang *et al.* (2007) showed distribution of TiO₂ nanoparticles to the liver, spleen, lungs and kidneys. Following intravenous injection of TiO₂ (single dose 5 mg kg⁻¹, 20-30 nm), Fabian *et al.* (2008) found distribution to primarily the liver, but also the spleen, lungs and kidneys.

As noted, Wang *et al.* (2008a, 2008b) reported the hippocampus to be the main accumulation target for TiO₂ nanoparticles absorbed (via neuronal transport) following nasal instillation.

Metabolism

No information was found on the possible metabolism of TiO₂ nanoparticles within the body.

Elimination

No information was found on the mechanisms for elimination of TiO₂ nanoparticles from the body.

In summary, in one study, TiO₂ nanoparticles have been demonstrated to be systemically available via oral exposure with the liver, spleen, lungs and kidneys being the target organs. A study applying intravenous injection supports this distribution pattern, suggesting the liver to be the main target organ. Several studies suggest that TiO₂ nanoparticles do not pass the Stratum Corneum of healthy skin, but further investigations are needed on the behaviour in skin where the Stratum Corneum is damaged.

Following inhalation TiO₂ nanoparticles deposit in the lung with smaller particles having a longer retention time. No studies have been identified investigating possible systemic uptake of nano-TiO₂ following inhalation therefore, further investigation is needed. One author reported that following nasal instillation, TiO₂ nanoparticles were found in the brain, possibly as a consequence of neuronal transport by-passing the blood-brain barrier. The main target for distribution was the hippocampus.

No information on metabolism or elimination of TiO₂ nanoparticles has been identified.

9.6.4.2 Acute toxicity

Acute toxicity: Oral

In vivo:

No studies identified.

In vitro:

Zhang *et al.* (2004) show limited cytotoxicity of a high dose of TiO₂ nanoparticles (21nm) to human colon carcinoma Ls-174-t cells.

Acute toxicity: Inhalation

In vivo:

Several authors have shown that TiO₂ nanoparticles (with a size in the range of about 20-30 nm) is considerably more toxic than its micro-TiO₂ (> 100nm) counterpart (see e.g. Ferin *et al.* 1992; Renwick *et al.* 2004; Chen *et al.* 2006; Inoue *et al.* 2008).

Most studies identified used a single dose of particles, administered via intratracheal instillation and toxicity observed included: pulmonary inflammatory response (characterised by neutrophil and macrophage infiltration) (Ferin *et al.* 1992; Chen *et al.* 2006; Warheit *et al.* 2007; Inoue *et al.* 2008; Renwick *et al.* 2004; Grassian *et al.* 2007); epithelial damage, increased permeability of the lung epithelium, and cytotoxicity, which were measured within the bronchoalveolar lavage fluid (BALF) (Renwick *et al.* 2004); and morphological alteration within the lung (Chen *et al.* 2006). Finally, Ahn *et al.* (2005) using a high dose (4 mg kg⁻¹) investigated what processes were responsible for particulate mediated stimulation of excessive mucus secretion within humans. TiO₂ exposure stimulated an increase in goblet cell hyperplasia, which is, in part, attributed to an increase in muc5 gene expression and IL-13 production. Therefore, it could be speculated that particle mediated increases in mucus secretion contributed to the aggravation of chronic airway disease symptoms within humans, and therefore warrants further investigation.

Grassian *et al.* (2007) investigated the toxicity of TiO₂ nanoparticles (5 and 21 nm) within mice, subsequent to inhalation (0.7 or 7 mg m⁻³, for 4 hours) or nasal instillation (up to 150 µg per 50 µl). An elevated macrophage population was associated with the inhalation of particles (4 and

24 hours post exposure), and were observed to internalise particles. An infiltration of neutrophils was associated with the nasal instillation of TiO₂.

Several authors suggested that the response subsequent to TiO₂ exposure was dose driven (e.g. Chen *et al.* 2006; Renwick *et al.* 2004). In the Renwick *et al.* (2004) study, no toxicity was seen at 125 µg per rat (corresponding to 0.5 µg kg⁻¹ assuming a rat weight of 250 g), whereas toxicity was seen at the high dose of 500 µg per rat (particle size 29nm). Chen *et al.* (2006) exposed mice and found toxicity (inflammation and histological changes in the lung) at the lowest dose of 100 µg per mouse (corresponding to 33 µg kg⁻¹ assuming a mouse weight of 30 g) (particle size 19-21 nm). Although the Chen *et al.* (2006) study does not indicate a no effect level, it seems justified (assuming the rat is more sensitive) to estimate, a No Observed Adverse Effect Level (NOAEL) of 125 µg per rat (corresponding to 0.5 µg kg⁻¹).

The crystallinity of TiO₂ nanoparticles is thought to influence the toxicity with the anatase form expected to be more toxic than the rutile form (Warheit *et al.* 2007).

Acute toxicity: Dermal

In vivo:

No studies identified.

In vitro:

Kiss *et al.* (2008) and Jin *et al.* (2008) showed that TiO₂ nanoparticles may affect different cell lines *in vitro*. It is however not expected that these effects would materialise in healthy skin as TiO₂ nanoparticles are not likely to pass the *Stratum Corneum*. However, the results highlight that if TiO₂ nanoparticles are able to pass through damaged skin they might cause dermal toxicity.

Acute toxicity: Other routes

Chen *et al.* (2008) investigated the acute toxicity of TiO₂ nanoparticles (80-100 nm) subsequent to intraperitoneal injection of mice (doses ranged from 324 to 2592 mg kg⁻¹). Mortality was observed as was thrombosis at the highest doses (TiO₂ blocking pulmonary vessels), and pathology was evident within the liver, spleen and kidneys. It should be noted that the doses used were high and given the uncertainty around the absorption (see above) data from i.p. exposure should be treated with care. Altogether, the data assessed are of limited relevance for a quantitative risk assessment, but do not indicate severe acute toxicity.

In summary, no in vivo studies investigating toxicity following acute oral exposure were identified. One in vitro study suggests low toxicity, but given the likelihood of systemic uptake via this route, further investigation is recommended.

One study using intra-peritoneal exposure showed mortality using relatively high exposures and is assessed to be of limited value for a quantitative risk assessment. This study does not indicate severe acute toxicity.

Most studies investigating the acute toxicity of TiO₂ following pulmonary exposure, exposed the animals using intra-tracheal instillation. Several of these compared toxicity of nano-sized TiO₂ with micro-sized TiO₂, and there is clear evidence that nano-sized TiO₂ is considerably more toxic. Toxicity identified includes inflammation and morphological changes/damage to the lungs. Similar effects were seen following acute nasal installation and inhalation.

No or very low toxicity is expected following short term dermal exposure to normal skin as several studies have shown that TiO₂ nanoparticles do not pass to the viable layers of such skin. However, further investigations are needed to show whether TiO₂ may pass through damaged skin as in vitro studies have shown that TiO₂ nanoparticles are capable of damaging various types of skin cell lines.

9.6.4.3 Irritation/Corrosivity

No studies were identified specifically investigating irritation or corrosion.

No studies specifically investigating irritation (dermal, eye and inhalation) have been identified. As TiO₂ nanoparticles are used widely in sun creams, it is however not expected to be irritating to the skin. It is not expected that TiO₂ nanoparticles are irritating following inhalation, apart from those effects described in the sections on acute and repeated dose toxicity. Thus TiO₂ nanoparticles are also not expected to be corrosive given the available data described elsewhere in this assessment.

9.6.4.4 Sensitisation

No studies were identified studying sensitisation following dermal contact or inhalation.

No studies specifically investigating the sensitising behaviour of TiO₂ nanoparticles have been identified. However as for irritation, given the widespread use in sun creams, it is not expected to be a dermal sensitiser. TiO₂ nanoparticles are also not expected to be sensitising following inhalation, given the available data described elsewhere in this assessment.

9.6.4.5 Repeated dose toxicity

Repeated dose toxicity: oral

No studies identified.

Repeated dose toxicity: inhalation

Using high doses (about 23.5 mg m⁻³) in a 12 week inhalation study in rats, Ferin *et al.* (1992) showed that TiO₂ nanoparticles (21 nm) caused a pulmonary inflammatory response (characterised by neutrophil infiltration), which micro-TiO₂ (250 nm) did not. This study thus confirms the potency of TiO₂ nanoparticles as already discussed under acute toxicity.

In a 13 week (6 hours per day, 5 days per week, 21 nm particles) inhalation study, Bermudez *et al.* (2004) showed that rats were more sensitive than mice and much more sensitive than hamsters, which is thought to derive from the ability of hamsters to quickly clear the lung (see toxico-kinetics section). As a consequence for this risk assessment, the rat data will be used, assuming it to be the most sensitive species. The authors noted that the particles aggregated during the experiment, but assumed the overall surface area would be important due to disaggregation into single particles once the particles are deposited in the lungs. Doses used were 0.5, 2, or 10 mg m⁻³ and the pulmonary response was assessed up to 52 weeks post exposure. The toxicity investigated covered lung burden, cytology, total protein and LDH, lung cell proliferation and histopathology. The authors concluded that the rats suffered from pulmonary overload at the highest dose and inflammation characterised by severe epithelial proliferative changes, including mataplastic changes in the centriacinar region (bronchiolisation of alveolar epithelium) associated with particle and particle-laden macrophage accumulation was described. Lesions were also observed in the mid-dose group and considered to be minimal to mild in severity and consisted primarily of particle-laden macrophage accumulation and aggregation in subpleural regions and in centriacinar zones. It seems thus likely to assume a no-effect concentration somewhere in the range of 0.5 to 2.0 mg m⁻³.

In a chronic study, Heinrich *et al.* (1995) exposed rats to TiO₂ nanoparticles via inhalation for 2 years (18 hours per day, 5 days per week), at an average exposure concentration of 10 mg m⁻³, which was followed by exposure to clean air for 6 months. TiO₂ was able to increase mortality to 60% after 24 months (control 40%) and 90% after 130 weeks (control 85%). Lung tumours were observed, as a consequence of the chronic exposure to TiO₂. The relevance of these data for risk assessment purposes are however dubious due to the very high doses used. Also, as indicated by the control data, the mortality after such long periods is dubious.

It should be noted that the US National Institute for Occupational Safety and Health (NIOSH), based on these data and a review of the literature, have concluded that TiO₂ is carcinogenic to rats and that it cannot be ruled out that TiO₂ nanoparticles are also carcinogenic to humans (NIOSH, 2005). It is assumed that the mechanism for carcinogenicity would not be chemical specific, but caused by chronic inflammation following pulmonary overload, and thus caused by

the particle nature of TiO₂. As already discussed, several authors have concluded that TiO₂ nanoparticles are more potent compared to micro-sized TiO₂ (with effects often related to surface area). This is also supported by NIOSH (2005) (and discussed in Bermudez *et al.* (2004)) by comparing carcinogenicity data and other toxicity data between nano- and micro-TiO₂. Based on this, NIOSH has published a recommended exposure level, which should protect workers from a possible cancer risk, at least until better data are available.

As already described in the toxicokinetics sections, Wang *et al.* (2008a, 2008b) showed that rutile (80 nm) and anatase (155 nm) TiO₂ particles following nasal instillation exposure (500 µg per mouse, every other day for a total of 30 days) were able to access the brain, with accumulation within the cerebral cortex, thalamus and hippocampus (main target) evident, and was postulated to occur via the olfactory bulb. Accumulation of TiO₂ resulted in morphological alterations and loss of neurones in the hippocampus. In addition it was suggested that TiO₂ elicited oxidative stress within the brain due to the elevation of superoxide dismutase (SOD), and catalase activity, and evidence of increased lipid peroxidation and protein oxidation. Furthermore, an inflammatory response (indicated by IL-1β, and TNFα) within the brain was stimulated by TiO₂ exposure.

Repeated dose toxicity: Dermal

No information identified except the dermal studies reported under acute toxicity and toxicokinetics.

Repeated dose toxicity: Other routes

No studies were identified.

Epidemiological information

The epidemiological evidence reviewed concluded, in line with other authors, that the results of available studies are unlikely to be useful for an assessment of exposure to TiO₂ nanoparticles as the size(s) of the particles in those studies were not properly measured/characterised/described.

9.6.4.6 Biological mechanisms and target organ toxicity of nano-titanium dioxide

Lung toxicity

Several authors have investigated the mechanism of lung toxicity *in vitro* (for example Park *et al.* 2008; Churg *et al.* 1999; Gurr *et al.* 2005; Simon-Deckers *et al.* 2008; Kim *et al.* 1999 and Barlow *et al.* 2005). The evidence shows that TiO₂ nanoparticles are toxic to both macrophages and epithelial lung cells with inflammation and oxidative stress possibly leading to genotoxicity and cytotoxicity.

Liver toxicity

Hussain *et al.* (2005) and Linnainmaa *et al.* (1997) show no or low toxicity of TiO₂ nanoparticles to BRL 3A liver cells and liver epithelial cells.

Cardio-toxicity

Helfenstein *et al.* (2008) showed that TiO₂ nanoparticles were able to affect cardiomyocyte electrophysiology, enhance ROS production, and reduce myofibril organisation, whereas Peters *et al.* (2004) found TiO₂ relatively low-toxic to HDMEC endothelial microvascular cells (with minimal IL-8 release).

Toxicity to the Central Nervous System (CNS)

Long *et al.* (2006, 2007) indicates that TiO₂ nanoparticles caused a ROS driven toxicity to some types of cells of the CNS *in vitro*.

Kidney toxicity

L'azou *et al.* (2008) showed different responses by different types of kidney cells *in vitro*.

Immunotoxicity

Renwick *et al.* (2001), Afaq *et al.* (1998) and Kang *et al.* (2008) suggest that TiO₂ nanoparticles may affect cell types of the immune system, including via a ROS driven response.

Oxidative, inflammatory and genotoxic toxicity

As evidenced by the description of the above *in vitro* studies and the various *in vivo* studies described in this assessment, a predominant mechanism of TiO₂ nanoparticle toxicity seems to be a ROS driven toxicity that may lead to inflammation and potentially geno- and cytotoxicity. This mechanism is also found by NIOSH (2005) to be the main mechanism, which may lead to cancer (at least in rats) following pulmonary overload.

In summary, no studies have been identified studying repeated dose toxicity following oral and dermal exposure. However, the conclusions under acute toxicity seem also relevant for repeated dose toxicity.

Several studies investigating repeated inhalation exposure have been identified. The studies complement those described under acute exposure in terms of showing that nano-sized particles are more potent than micro-sized and that the predominant toxic effect seems to be inflammation. Furthermore, there seems to be an inter-species variability with rats being more sensitive than mice and hamsters.

A No Observed Effect Concentration (NOAEC) of 0.5 mg m⁻³ (true no-effect level possibly a bit higher) was identified in a 13 week (6 hours per day, 5 days per week, 21 nm particles) inhalation study in rats (supposed to be the most sensitive species).

In a number of in vitro studies, TiO₂ nanoparticles have been shown to be toxic to various cell lines of the liver, heart, CNS, kidney and immune system. The relevance of these data have to be assessed against the ability of TiO₂ nanoparticles to reach these targets, which is, except perhaps for oral intake questionable/uncertain given the absorption patterns of TiO₂ nanoparticles.

It should however be noted that following nasal instillation, one author (in two studies) shows that TiO₂ nanoparticles may reach the brain via a neuronal mediated translocation of TiO₂. Further investigations of the possible neurotoxic behaviour of TiO₂ nanoparticles following inhalation are therefore recommended.

Altogether, in vitro and in vivo evidence indicates that the underlying mechanism driving most of the toxicity of TiO₂ nanoparticles appears to be an oxidative stress driven inflammation that may lead to cyto- and geno-toxicity, and possibly cancer (in case of pulmonary overload). It is therefore also believed that this type of toxicity has a threshold.

9.6.4.7 Mutagenicity

In vitro:

No mutagenicity or genotoxicity studies classically used in chemical regulatory setting have been identified, but several *in vitro* studies conducted indicate that TiO₂ nanoparticles themselves do not cause direct chemical/physical genotoxicity. However, as already described, several studies have indicated that TiO₂ nanoparticles may cause genotoxicity via an indirect mechanism involving oxidative stress. It should be noted that some studies did not find genotoxicity.

In vivo:

No information identified, but see the section on carcinogenicity.

TiO₂ nanoparticles are not expected to cause direct mutagenicity/genotoxicity (although further testing may be needed to fully confirm this), but may trigger genotoxicity via an indirect threshold driven mechanism involving oxidative stress.

9.6.4.8 Carcinogenicity

One study describing tumour findings following chronic inhalation (Heinrich *et al.* 1995) was described in the section on repeated dose toxicity. The study used very high doses and had a long duration (high death in the control group). NIOSH (2005) concluded, based on those data that TiO₂ is carcinogenic in rats and that it cannot be excluded to be carcinogenic in humans. It is expected that carcinogenicity occurs following pulmonary overload and thus has a threshold. It should be noted that also the International Agency for Research on Cancer have assessed TiO₂ (even the microform – if exposure is high enough) to be a Class 2B carcinogen (Possibly carcinogenic to humans) (IARC 2006).

One study found tumours at very high concentrations after a very long exposure time. TiO₂ nanoparticles may thus cause carcinogenicity, which is expected to occur via a non-chemical specific mechanism involving pulmonary overload. In terms of further testing, it is recommended to conduct a chronic study with lower dose levels.

9.6.4.9 Toxicity for reproduction

Effects on fertility:

Komatsu *et al.* (2008) showed that TiO₂ nanoparticles are taken up by and affect viability, proliferation and gene expression of Leydig cells (testosterone producing cells of the testis) *in vitro*.

Developmental toxicity:

No information identified

Fertility:

One *in vitro* study suggests that TiO₂ nanoparticles may be toxic towards Leydig cells. However, given the toxico-kinetics, it can be questioned whether TiO₂ can indeed reach these cells. No studies investigating female fertility were identified. Overall, no conclusion can be drawn.

Developmental toxicity

No studies identified and no conclusion can be drawn.

9.6.5 Derivation of DNEL(s)

As noted under repeated dose toxicity, it is expected that TiO₂ nanoparticles exhibit their toxicity via a threshold mechanism (oxidative stress driven inflammation), which may lead to other effects, even cancer. It therefore seems relevant to derive no effect levels, if possible.

However, it should be noted that there is uncertainty around whether (some) nanomaterials may have nano-specific (possibly non-threshold) mechanisms (driven e.g. by size/surface area). There is also uncertainty around the possible neurotoxicity of TiO₂ nanoparticles. Nevertheless, for the purpose of this exercise, an attempt is made to calculate a DNEL.

For short term inhalation, a NOAEL of 125 µg per rat (Renwick *et al.* 2004) corresponding to 0.5 µg kg⁻¹ assuming a rat weight of 250 g was identified. As it is difficult to relate this to any inhalation exposure, we will not attempt to derive a DNEL for short term exposure.

For repeated dose toxicity, a No Observed Effect Concentration (NOAEC) of 0.5 mg m^{-3} ($500 \text{ } \mu\text{g m}^{-3}$) was identified in a 13 week (6 hours per day, 5 days per week, 21 nm particles) inhalation study in rats (Bermudez *et al.* 2004).

As noted in the introduction, DNEL will be based on the Chapter R.8 of the REACH guidance (ECHA, 2008).

Modification of the starting point (correction for exposure time and worker light activity)

Corrected NOAEC (8 hours, worker light activity) = $6 \text{ hours} / 8 \text{ hours} \times 6.7 \text{ m}^3 / 10 \text{ m}^3 \times \text{NOAEC}$
(6 hours, rat) = $250 \text{ } \mu\text{g m}^{-3}$

Interspecies extrapolation:

As rats were shown to be the most sensitive species and as the toxic effects seen do not involve metabolism (and therefore the need for allometric scaling), it is suggested to use an assessment factor of 1.5 (instead of 2.5) for interspecies extrapolation.

Intraspecies:

The default factor of 5 (workers) is applied.

Exposure duration:

As it has been shown that exposure time is relevant, a default factor of 2 is applied to extrapolate from sub-chronic to chronic conditions. No factor is applied for severity of effect.

This gives an overall assessment factor of: $1.5 \times 5 \times 2 = 15$.

This gives a DNEL for chronic inhalation of about $250 / 15 \text{ } \mu\text{g m}^{-3} = 17 \text{ } \mu\text{g m}^{-3}$ (8 hours light activity, worker).

As noted previously, NIOSH assumes TiO_2 to be carcinogenic in rats following a non-chemical specific mechanism of pulmonary overload of the lung resulting in inflammation and ultimately cancer. On that basis, NIOSH will not exclude that nano/ultrafine TiO_2 is a human carcinogen and suggests protecting workers via a recommended exposure level until better evidence on the true carcinogenic behaviour of TiO_2 nanoparticles becomes available. Based on data from the Heinrich *et al.* (1995) and using the linearised upper bound on risk from the multistage model, a recommended exposure level (REL) for $<100\text{nm TiO}_2$ of $100 \text{ } \mu\text{g m}^{-3}$ (for worker exposure 10 hours per day, 40 hours per week) is derived (NIOSH, 2005). This value is assumed to cause a maximum of 1/1000 cancer cases in a work-life, but NIOSH note that it is likely to be much lower, possibly zero.

Although derived via different routes, the DNEL derived in this assessment and the NIOSH value are relatively close. This is perhaps not surprising as the two values in a way protect the same target, namely inflammation, which may (or may not) lead to cancer in humans. Furthermore, it should be noted that in the Bermudez *et al.* (2004) study, only minimal to mild effects were seen at the exposure level of 2 mg m^{-3} . If those effects are assumed not to be adverse, this exposure level would be the NOAEC and a DNEL of about $70 \text{ } \mu\text{g m}^{-3}$ (practically identical to the NIOSH value) would be derived.

No data are available for deriving DNELs for short-term and chronic oral and dermal exposure.

9.6.6 Risk characterisation

TiO_2 nanoparticles have been shown to be absorbed following oral exposure. The main target organ as indicated in an intra-peritoneal study seems to be the liver. There are however little data for assessing the possible toxicity following oral exposure and also no data on human oral exposure has been identified. Thus the assessment is inconclusive for oral exposures.

Following dermal exposure, there is substantial evidence that TiO_2 nanoparticles do not pass through to the viable layers of healthy skin. Thus, little toxicity is expected via this route in healthy skin. However, further studies are needed to assess the toxico-kinetics and possible

toxicity following exposure to damaged skin. A number of findings have shown TiO₂ nanoparticles to cause toxicity to skin cells in vitro. Altogether, it can qualitatively be concluded that healthy skin is most likely not at risk, whereas damaged skin may be at risk.

Following inhalation, TiO₂ nanoparticles have been shown to partly accumulate in the lungs, but there is no evidence of systemic uptake. This may need further attention. Following repeated nasal instillation, TiO₂ nanoparticles have however been found to accumulate and cause toxicity in the brain. It is hypothesised that the transport occurs as neuronal transport by-passing the blood-brain barrier. This needs further investigation.

There is substantial evidence that following inhalation, nano-sized TiO₂ is more toxic than micro-sized TiO₂. It has also been shown that there seems to be a dose-response relationship in TiO₂ nanoparticle toxicity, that different species seem to have different sensitivity (with rat being the most sensitive animal) and that the form of TiO₂ may influence toxicity with the anatase form demonstrated to be more toxic than the rutile form.

It seems that the main mechanism of toxicity of TiO₂ nanoparticles is driven by oxidative stress which may lead to inflammation and ultimately cyto- and genotoxicity and in case of pulmonary overload perhaps cancer. It is thus believed that TiO₂ nanoparticles exert their toxicity via a threshold mechanism (as actually low doses would trigger a protective response). However, it should be noted that there is uncertainty around whether (some) nanomaterials may have nano-specific (possibly non-threshold) mechanisms. Furthermore, more data on the possible direct mutagenic/genotoxic behaviour of TiO₂ nanoparticles may be warranted to exclude a non-threshold mechanism. Finally, as noted above, the neurotoxic behaviour requires further attention. Consequently, the following should be seen as an attempt to calculate a DNEL-based quantitative risk characterisation for the case that we actually deal with a threshold mechanism. The assessment should not be applied for any regulatory decision-making, given the large uncertainties associated with toxicity as well as exposure data.

For chronic worker inhalation (8 hour per day, light activity), a DNEL of 17 µg m⁻³ (in a study using 21 nm particles) has been derived. This could likely be higher depending on how the NOAEC is defined in the key study and if a shorter daily worker exposure time than 8 hours is assumed. As noted, NIOSH has derived a Recommended Exposure Level (REL) of 100 µg m⁻³, aiming at a maximum risk of 1/1000 over a work life, but noted that the risk is possibly lower, perhaps even zero. Given the uncertainties related to the data (particle sizes, exposure time/activity corrections, assessment factors, etc.), this value is not considered significant different from the DNEL(s) calculated in this assessment.

The only occupational inhalation exposure data found for this exercise are based on read-across from handling of Al₂O₃ powder, where a maximum/worst case exposure of 200000 particles cm⁻³ has been estimated. Assuming these to be representative for TiO₂ this corresponds to about 52 µg m⁻³ and 420 µg m⁻³ assuming particle sizes of 50 and 100 nm, respectively.

Due to the significant uncertainties in these data (toxicity, real surface area and exposure), one should be careful with drawing firm conclusions, but assuming chronic exposure to such concentrations, it appears that exposure may exceed the DNEL estimated in this exercise as well as the Recommended Exposure Level (REL) recommended by NIOSH. In conclusion, based on identified evidence there may be a risk associated with chronic occupational exposure to TiO₂ nanoparticles. However, it is strongly recommended to establish representative exposure values for chronic occupational exposure to TiO₂ nanoparticles (carefully monitoring the sizes and thereby surface area of the TiO₂ nanoparticles as surface area is assumed to be a key driver of toxicity).

Spray applications of TiO₂ nanoparticle containing products have been reported. A maximum value of 3.5 g m⁻³ was estimated based on modelling of application of a spray-on sunscreen. This is an acute/short term exposure that would not be able to cause the chronic inflammation/pulmonary overload phenomenon seen for chronic exposures. However, also inflammation has been reported following short term exposures. Most of the toxicity studies identified used exposure via intratracheal installation and it seems difficult to translate such data for deriving a

short term acute inhalation DNEL in mg m^{-3} . However, risk following short term exposure to spray applications cannot be excluded and further data aiming at identifying real short term exposure levels and a short-term inhalation NOAEC seems justified.

Assuming that TiO_2 nanoparticles could be encountered in other spray applications (such as paints/varnishes), more chronic exposure of workers and/or consumers could be anticipated and the modelled value is way above the long-term DNEL estimated. However, as such uses would probably be surrounded by risk management measures (at least for workers) it is difficult to conclude whether there would be a risk. However, longer term exposure to TiO_2 nanoparticles contained in spray applications should be further investigated in terms of establishing data on duration, frequency and level of exposure for such uses.

The only consumer data available (reported as $22 \text{ particles cm}^{-3}$ (55 nm)) for release from TiO_2 coatings (due to wear and tear) indicates that the exposures are very low and probably do not constitute a risk. However, it is doubtful whether it is actually possible to measure such low level as measured in the study, and so this may require further considerations.

In a number of in vitro studies, TiO_2 nanoparticles have been shown to be toxic to various cell lines of the liver, heart, CNS, kidney, male reproductive system and immune system. The relevance of these data have to be assessed against the ability of TiO_2 nanoparticles to reach these targets, which is, except perhaps for oral intake, questionable given the lack of information regarding absorption patterns of TiO_2 nanoparticles.

The main recommendations for future work involve:

- Collection/measurements of exposure data, in particular for applications causing short terms and chronic/long term occupational or consumer inhalation exposure;
- Further investigation of the possibilities for TiO_2 nanoparticles to cause cancer following chronic exposure at lower doses than tested so far. The recommended species would be the rat having shown to be sensitive to TiO_2 nanoparticle toxicity;
- Studies to identify a short term NOAEC and DNEL for inhalation;
- Studies of systemic uptake of TiO_2 nanoparticles following inhalation;
- Further studies of toxicity (including systemic toxicity) following oral exposure at relevant doses;
- Investigation of the possibility for TiO_2 nanoparticles to pass damaged (e.g. sunburnt) skin;
- Further studies of the toxicokinetics of TiO_2 nanoparticles following inhalation;
- Further studies of the possible neuronal transport of TiO_2 nanoparticles following inhalation and possible resultant CNS effects;
- Further studies on the direct mutagenic/genotoxic nature of TiO_2 nanoparticles.

9.6.7 Environmental Risk Assessment for titanium dioxide nanoparticles

9.6.7.1 Environmental fate properties

Degradation

Not relevant for metal oxides. No data is available about oxidation state modification and subsequent consequences.

Environmental distribution

Kaegi *et al.* (2008) reported leaching of TiO_2 in paints from a house façade. The leaching of TiO_2 nanoparticles from a model façade and a real façade was compared. The study using a newly painted model facade resulted in a titanium concentration of $600 \mu\text{g l}^{-1}$ with 90% of nanoparticles with a size range between 20 and 300 nm. In contrast, the real façade, exposed for two years to weather, resulted in a leaching of $10 \mu\text{g l}^{-1}$ for titanium, while the urban run-off contained $8 \mu\text{g l}^{-1}$. The size of particles in the real façade was around 20-300 nm for 90% of particles, while in urban run-off 50% of particles are $< 300 \text{ nm}$.

Concerning dispersion stability, TiO₂ in tap water showed rapid aggregation due to electric double layer compression (i.e. ionic strength effect).

Transport of TiO₂ through porous media was investigated by Lecoanet and Wieser (2004). Their findings showed that transport is a function of Darcy velocity, with 55% of particles (low flow velocity) and 77% of particles (high flow velocity) transported through the porous media. Mueller and Nowack (2008) used a life-cycle perspective to model the quantities of engineered nanoparticles released into the environment including titanium dioxide. The quantification was based on a substance flow analysis from products to air, soil, and water in Switzerland. They calculated PECs between 0.7 and 16 µg l⁻¹ TiO₂ in water, and between 0.4 and 4.8 µg kg⁻¹ TiO₂ in soil. However, given the lack of information, numerous assumptions and estimates had to be made. Some details and limitations of these calculations are discussed in Hansen (2009).

Boxall *et al.* (2007) developed a framework of simple models and algorithms for estimating nanoparticle concentrations in water, soil and air. For TiO₂ nanoparticles they estimated 24.5 µg l⁻¹ TiO₂ in water and 1030 µg kg⁻¹ TiO₂ in soil. In comparison to the values calculated by Mueller and Nowack (2008), the estimate for water is higher but in the same order of magnitude, while the estimate for soil is several orders of magnitude higher. Hansen (2009) discusses some differences of the two approaches.

Bioaccumulation

Zhang *et al.* (2007) studied the accumulation of Cd, while Sun *et al.* (2007) studied the accumulation of As in *Cyprinus carpio* in presence of TiO₂ nanoparticles. The results highlighted that TiO₂ increased the accumulation of both elements, with significant bioconcentration factor (BCF) increases. Cd accumulated in fish, both in muscles and in viscera, with BCF for the total body was 9.4 times higher than control, while the As BCF increased 2.5 times. TiO₂ accumulation, which was similar that observed for Cd and As, was also recorded. These findings highlight the relevance of TiO₂ accumulation, both for the nanomaterial itself and as a carrier for other chemicals already present in the environment.

In summary, TiO₂ nanoparticles can be released into water from different sources, as for example from house façades, or from household wastewater. However, once in water the TiO₂ particles aggregated in presence of cations, increasing the tendency to settle. As for ZnO, the stabilizing effect on dispersion of natural organic matter, clay and colloidal substances should be investigated, in order to better understand the transport potential of TiO₂ nanoparticles in water. Transport in soil is possible, although the effect of natural soil components on the transport is not yet investigated. The interaction of TiO₂ with biota may result in bioaccumulation of TiO₂ nanoparticles, but also in increased bioaccumulation of metals and metalloids already present in the water. The indirect toxicity of TiO₂ due to carrier effects should be considered.

9.6.7.2 Environmental hazard assessment

Aquatic compartment (including sediment)

Fish:

The short-term toxicity studies on fish cover different life stages and species, including whole organisms and cell cultures. Vevers *et al.* (2008) exposed gonadal tissue cells (rainbow trout) to TiO₂ nanoparticles dispersed in culture media (Table 9.23). The results highlight the possibility of genotoxicity (after UVA activation) and cytotoxicity at 50 mg l⁻¹ TiO₂. On the contrary, no effects were measured in zebrafish embryos at 500 mg l⁻¹, but TiO₂ particle size was larger (230 nm).

The exposure of rainbow trout for a longer period (14 days) to TiO₂ showed effects on the brain (histological examination indicated biochemical disturbances) and other sub-lethal effects at a relatively low concentration (0.1 mg l⁻¹), but the size of TiO₂ in exposure media was not measured (21 nm mean size in stock solution).

With the available data an LC_{50 fish} (short-term) over 500 mg l⁻¹ can be established.

Table 9.23: Short-term effects of nano TiO₂ on fish

Method	Results	Remarks	Reference
<i>Oncorhynchus mykiss</i> Gonadal tissue cells	DNA strand breakage (24h): 50 mg l ⁻¹ Cytotoxicity (24h): 50 mg l ⁻¹	Exposure to TiO ₂ dispersed in MEM and PBS, under UVA illumination, size 24.4 nm UVA illumination irrelevant, MEM or PBS media	Vevers <i>et al.</i> (2008)
<i>Danio rerio</i> Embryos and larvae	No significant mortality (96h): 500 mg l ⁻¹ No effects on hatching rate (96h): 500 mg l ⁻¹ No malformation (96h): 500 mg l ⁻¹	TiO ₂ suspended in water, size 230 nm	Zhu <i>et al.</i> (2008a)
<i>Oncorhynchus mykiss</i> adults	No mortality (14d): 1.0 mg l ⁻¹ Sublethal effects (gill pathologies, mucus secretion) (14d): 0.1 mg l ⁻¹ Biochemical disturbances in brain (14d): 0.1 mg l ⁻¹	Exposure to sonicated TiO ₂ , size 21 nm, semi-static exposure Relevant for chronic effects	Federici <i>et al.</i> (2007)

MEM = Minimum Essential Medium

PBS = Phosphate-buffered saline

Aquatic invertebrates:

Table 9.24: Short-term effects of nano TiO₂ on aquatic invertebrates

Method	Results	Remarks	Reference
<i>Daphnia pulex</i> Adults	NOEC ^a (48h): > 10 mg l ⁻¹	TiO ₂ 20% rutile and 80% anatase, size 20.5 nm	Griffitt <i>et al.</i> (2008)
<i>Ceriodaphnia dubia</i>	NOEC ^a (48h): > 10 mg l ⁻¹	TiO ₂ 20% rutile and 80% anatase, size 20.5 nm	Griffitt <i>et al.</i> (2008)
<i>Chydorus sphaericus</i> Cladocra, Crustacean	NOEC ^a (48h): > 100 mg l ⁻¹	TiO ₂ dispersion in tap water	Velzeboer <i>et al.</i> (2008)
<i>Daphnia magna</i>	LC ₅₀ (48h): 20000 mg l ⁻¹		Heinlaan <i>et al.</i> (2008)
<i>Thamnocephalus platyurus</i> ,	NOEC (24h): > 20000 mg l ⁻¹		Heinlaan <i>et al.</i> (2008)
<i>Daphnia pulex</i> Adults	40% mortality (8d): 20 ppm	No concentration-response relationship established	Adams <i>et al.</i> (2006)

^a: reported as LC₅₀ > x mg/L from author, but it is also reported that no effect were observed. Therefore, it is here listed as NOEC.

Short-term studies on aquatic invertebrates (five different crustacean species) show a NOEC and a LC₅₀ over 20 g l⁻¹ TiO₂ nanoparticles for *Thamnocephalus platyurus*, and a LC₅₀ around 20 g l⁻¹ for daphnids, which is a very high concentration compared to the predicted environmental concentration (Table 9.24). Other studies with crustaceans report NOECs over 10 mg l⁻¹ and over 100 mg l⁻¹ TiO₂. Thus, TiO₂ seems not to be acutely toxic to aquatic

invertebrates. An 8 day study reported 40% mortality in *Daphnia pulex* after exposure to 20 mg l⁻¹ TiO₂ (Adams *et al.* 2006). However, no NOEC or EC₁₀ was determined. The LC₅₀ for aquatic invertebrates (short-term) is over 20 g l⁻¹ TiO₂ nanoparticles.

Algae and aquatic plants:

Table 9.25: Effects of nano TiO₂ on algae

Method	Results	Remarks	Reference
<i>Chlamydomonas reinhardtii</i>	EC ₅₀ (72h): 10 mg l ⁻¹	Endpoint is growth inhibition.	Wang <i>et al.</i> (2008)
<i>Pseudokirchneriella subcapitata</i>	EC ₅₀ (72h): 5.83 mg l ⁻¹ NOEC (72h): 0.98 mg l ⁻¹	Toxicity suggested due to entrapping cells by particle aggregates	Aruoja <i>et al.</i> (2008)
<i>Desmodesmus subspicatus</i>	EC ₅₀ (72h): 44 mg l ⁻¹	TiO ₂ anatase, 25 nm. Pre-illumination did not change the results.	Hund-Rinke and Simon (2006)
<i>Pseudokirchneriella subcapitata</i>	No measurable effects (4.5h): 100 mg l ⁻¹		Velzeboer <i>et al.</i> (2008)

Effects of TiO₂ on different algae species were measured at concentrations between 5.83 and 44 mg l⁻¹ (EC₅₀ 72h) (Table 9.25). A suggestion about the toxicity mode of action was made by Aruoja *et al.* (2008) that considered the entrapment of algae cells by TiO₂ nanoparticles aggregates (indirect toxicity caused by physical effect).

On the basis of available data, the lowest EC_{50 algae} is 5.83 mg l⁻¹ nano-TiO₂ and a NOEC_{algae} of 0.98 mg l⁻¹ TiO₂ can be determined.

Other aquatic organisms

Table 9.26: Effects of nano TiO₂ on bacteria

Method	Results	Remarks	Reference
<i>Vibrio fischeri</i>	NOEC ^a (30 min): >20000 mg l ⁻¹		Heinlaan <i>et al.</i> (2008)
<i>Vibrio fischeri</i>	No measurable effects (30 min): 100 mg l ⁻¹	TiO ₂ size range: 50-150 nm	Velzeboer <i>et al.</i> (2008)
<i>Bacillus subtilis</i>	No measurable effects (14-20h): 500 ppm 75% growth inhibition (14-20h): 1000 ppm	Photocatalytic ROS not the only mechanism of action. TiO ₂ average size: 330 nm.	Adams <i>et al.</i> (2006)
<i>Escherichia coli</i>	No measurable effects (14-20h): 100 ppm 44% growth inhibition (14-20h): 1000 ppm 72% growth inhibition (14-20h): 5000 ppm	Photocatalytic ROS not the only mechanism of action. Less sensitive than <i>Bacillus</i> . TiO ₂ average size: 330 nm.	Adams <i>et al.</i> (2006)

a: reported as LC₅₀ > x mg/L from author, but it is also reported that no effect were observed. Therefore, here listed as NOEC.

Effects on bacteria were measured in short-term studies. Heinlaan *et al.* (2008) observed no effects up to 20 g l⁻¹TiO₂, however reported the result as LC₅₀. In Table 9.26 this is listed as a NOEC to avoid an over estimation of effects. The reported results indicate an LC₅₀ between 500 mg l⁻¹and 1000 mg l⁻¹ TiO₂, and a NOEC_{bacteria} (short-term) of 500 mg l⁻¹ TiO₂ (*B. subtilis* Adams *et al.* 2006).

Terrestrial compartment

Toxicity to soil macro organisms:

Jemec *et al.* (2008) investigated the effect of TiO₂ nanoparticles on the woodlouse (*Porcellio scaber*). No mortality as well as no effects on the feeding rate, defecation rate, food assimilation efficiency and weight was observed (Table 9.27). However, there was a decrease of the enzymatic activity related to oxidative stress, following a binary response instead of the classical dose-response relationships. As a result, an effect was observed both at very low concentration (0.5 µg g⁻¹ food) and at a concentration of 2 to 3 mg g⁻¹ of food. Therefore, the LC_{50 soil macro organisms} is over 3 mg l⁻¹, and a NOEC cannot be established.

Table 9.27: Effects of nano TiO₂ on soil macro organisms

Method	Results	Remarks	Reference
<i>Porcellio scaber</i> Woodlouse (isopoda)	No sub-lethal effects (feeding rate, defecation rate, food assimilation efficiency, weight change) (3d): 3000 µg g ⁻¹ food No mortality (3d): 3000 µg g ⁻¹ Reduced CAT and GST enzymatic activity (3d): binary response at 0.5 µg g ⁻¹ and 2000-3000 µg g ⁻¹	Sonicated and non-sonicated TiO ₂ added to food Only for treatment with non-sonicated TiO ₂	Jemec <i>et al.</i> (2008)

CAT = catalase

GST = glutathione S-transferase

Toxicity to terrestrial plants:

Table 9.28: Effects of nano TiO₂ on terrestrial plants

Method	Results	Remarks	Reference
<i>Spinacia oleracea</i> seeds	Positive effects on germination, and vigour (48h): 6.0 g l ⁻¹ solution	Seeds pre-treated in suspension illuminated with natural light	Zheng <i>et al.</i> (2005)
<i>Spinacia oleracea</i> seedlings	Positive effects on growth: 0.25-4 g l ⁻¹ solution Photosynthetic activity decrease: > 4 g l ⁻¹ solution	Suggested mode of action: photocatalytic activity resulting in antimicrobial effects	Zheng <i>et al.</i> (2005)
<i>Spinacia oleracea</i> Seeds and seedlings	Positive effects on leaf area, weights, protein content, chlorophyll content (35d): 0.25 mg l ⁻¹ solution	N deficiency in growth medium can cause negative effects on N content, chlorophyll content, protein content, chloroplast	Yang <i>et al.</i> (2007)

The results of studies which assessed the effects of TiO₂ nanoparticles on terrestrial plants are summarised in Table 9.28. TiO₂ nanoparticles showed positive effects on spinach germination and growth in concentrations up to 4 g l⁻¹ solution (used to soak seeds prior to incubation). Exposure to more than 4 g l⁻¹ solution caused a reduction of the photosynthetic activity. Moreover, since the exposure to TiO₂ solution was carried out only during seeds soaking, it is difficult to correlate the concentration in exposure solution with environmental exposure (that normally should continue also during seedlings growing phase). However, no EC value or

NOEC was calculated by Zheng *et al.* (2005). Therefore, this study is not used for a PNEC estimation.

Toxicity to soil micro-organisms:

Table 9.29: Effects of nano TiO₂ on soil micro organisms

Method	Results	Remarks	Reference
Microbial community	No measurable effects ^a (7d): > 100 mg/L	24h incubation	Velzeboer <i>et al.</i> (2008)

a: reported as EC₅₀ from authors, but it is also reported that no effect were observed.

As outlined in Table 9.29, no effects of nanoparticle TiO₂ on soil microbial community were observed up to 100 mg l⁻¹ after 7 days. The result is in line with toxicity of TiO₂ for other bacteria (Table 9.26).

9.6.8 Risk characterisation and gap analysis

Quantitative risk assessment:

Due to the limited data, a quantitative risk assessment is only carried out for the aquatic compartment, freshwater (without sediment, without considering the food chain).

PEC/PNEC comparison can provide provisional information about the potential for environmental impacts of TiO₂. However, the PEC estimated by Mueller and Nowack (2008) and Boxall *et al.* (2007) are highly uncertain; therefore, calculations for different scenarios were made to highlight the possible range of outcomes and to support the perception of the result's uncertainty by the reader (see Table 9.30).

The PNEC can be estimated for the aquatic compartment (freshwater), by using LC₅₀ values for three trophic levels (fish, *Daphnia*, algae) and an assessment factor of 1000. The lowest EC₅₀ was that for algae of 5.8 mg l⁻¹ TiO₂.

Table 9.30: Risk Characterisation for nano TiO₂ for the aquatic compartment

Compartment	PEC	PNEC	PEC/PNEC	Remarks
Freshwater	0.7 µg l ⁻¹	5.8 µg l ⁻¹	0.1	PEC corresponds to lower value from Mueller and Nowack (2008)
	16 µg l ⁻¹	5.8 µg l ⁻¹	2.8	PEC corresponds to higher value from Mueller and Nowack (2008)
	24.5 µg l ⁻¹	5.8 µg l ⁻¹	4.2	PEC from Boxall <i>et al.</i> (2007)

Depending on the PEC value chosen, the PEC/PNEC ratio for the aquatic compartment range from 0.1 to 4. Thus, it will be necessary to refine the assessment. The uncertainty of the quantitative risk assessment for TiO₂ as presented above is high, in particular as the available exposure information is weak. Additional exposure information, either through more detailed modelling based on better data (e.g. manufacture and use data) or exposure measurements in natural waters are needed. Furthermore, long-term ecotoxicity data would justify the use of a lower assessment factor to derive a PNEC, and decrease the uncertainty of the assessment.

Qualitative risk assessment:

Titanium dioxide nanomaterials are used in several consumer and industrial products, e.g. personal care products (in particular in sunscreens), protective coatings and building products. The wide-spread use of products containing TiO₂ may allow release of TiO₂ nanoparticles into the environment via wastewater release and leaching of surfaces exposed to the external ambient environment. This is particular relevant given that TiO₂ nanoparticles are produced in large amounts (over several million tonnes per annum).

Short-term studies on fish and invertebrates showed no mortality caused by TiO₂ at concentrations of up to 100 mg l⁻¹, although sublethal effects (brain activity modification) were observed in fish exposed at 0.1 mg l⁻¹. In order to refine the risk assessment, it is necessary to perform long-term studies on fish and *Daphnia*. From the studies conducted to date algae seem to be more sensitive than fish and daphnids. It would be useful to confirm the toxicity for algae with a wider range of algal species.

Thus, more data should be generated to address the emissions, environmental fate and interaction with biota of nanoparticle TiO₂. Furthermore, the correlations between particle sizes, aggregate sizes and toxicity are still not well understood.

Gaps that prevent the derivation of a robust and reliable risk assessment are:

1. Lack of information on production volumes of TiO₂ nanoparticles in their many forms;
2. Lack of information on the number of TiO₂ nanoparticle products, market penetration, and amounts of the nanoparticles in these products;
3. Lack of information on behaviour of TiO₂ nanoparticles during wastewater treatment;
4. Lack of monitoring field data on TiO₂ nanoparticles levels in environmental compartments;
5. Lack of toxicity data on several species categories for aquatic compartments, as for example long-term studies on fish and *Daphnia*;
6. Lack of fate and toxicity data for the terrestrial compartment, as well as for the aquatic sediment;
7. Lack of information concerning interaction between organisms and TiO₂ nanoparticles (adsorption, uptake, bioaccumulation, etc.).

9.6.9 Environmental risk assessment for zinc oxide nanoparticles

9.6.9.1 Environmental fate properties

No information about degradation, transport and bioaccumulation is available in the review.

Environmental distribution

Boxall *et al.* (2007) developed a framework of simple models and algorithms for estimating nanoparticle concentrations in water, soil and air. For ZnO nanoparticles, they estimated 76 µg l⁻¹ ZnO in water and 3194 µg kg⁻¹ ZnO in soil. Some limitations of these exposure estimates are discussed in Hansen (2009).

9.6.9.2 Environmental hazard assessment

Aquatic compartment (including sediment)

Fish:

Table 9.31: Short-term effects of nano ZnO on fish

Method	Results	Remarks	Reference
<i>Danio rerio</i> Embryos	LC ₅₀ (96h): 1.793 mg l ⁻¹ No observed toxicity hatching rate (96h): < 0.5 mg l ⁻¹ EC ₅₀ hatching rate (84h): 2.065 mg l ⁻¹	Toxicity of nano ZnO similar to Zn ⁺ toxicity	Zhu <i>et al.</i> (2008a)

The hazard data for fish include only short-term data concerning the early life stage of one species, zebra fish, *Danio rerio*. The study of Zhu *et al.* (2008a) identified three different toxicity thresholds for *Danio rerio* embryos (Table 9.31). A dose response relationship was observed

for mortality of embryos, with an LC₅₀ of 1.8 mg l⁻¹. Concerning hatching rate, no effect were observed at 0.5 mg l⁻¹ exposure (it is not considered a NOEC since 0.5 mg l⁻¹ it is not a statistically derived value), and an EC₅₀ fish(hatching) of 2.1 mg l⁻¹ were detected, showing for this endpoint a dose response relationship.

Aquatic invertebrates:

Table 9.32: Short-term effects of nano ZnO on aquatic invertebrates

Method	Results	Remarks	Reference
<i>Daphnia magna</i> neonates	LC50 (48h): 3.2 mg l ⁻¹ NOEC (48h): 0.5 mg l ⁻¹	Effect due to Zn ²⁺ ions	Heinlaan <i>et al.</i> (2008)
<i>Daphnia pulex</i> Adults	100% mortality (8d): 0.5 mg l ⁻¹ 73% mortality (8d): 0.2 mg l ⁻¹	No concentration-response relationship established	Adams <i>et al.</i> (2006)
<i>Thamnocephalus platyurus</i> Larvae	LC50 (24h): 0.18 mg l ⁻¹ NOEC (24h): 0.05 mg l ⁻¹	Effect due to Zn ²⁺ ions	Heinlaan <i>et al.</i> (2008)

The literature reviewed on aquatic invertebrates reports toxicity data for three crustacean species (Table 9.32). Heinlaan *et al.* (2008) studied the effects of nano ZnO on two different crustaceans. The comparison of the results highlights that *Thamnocephalus platyurus* is more sensitive (LC₅₀ and NOEC one order of magnitude lower for *Thamnocephalus platyurus* than for *Daphnia magna*) to nanoparticle ZnO. In a 8 day study with *Daphnia pulex*, Adams *et al.* (2006) reports the toxicity of nanoparticle ZnO, with 73% mortality observed at the same concentration (0.2 mg l⁻¹) as the LC₅₀ (0.18 mg l⁻¹) of *Thamnocephalus platyurus*. On the basis of these data, a NOEC_{aquatic invertebrates} (short-term) of 0.05 mg l⁻¹ for nanoparticle ZnO and a LC₅₀ aquatic invertebrates (short-term) of 0.18 mg l⁻¹ can be established. It should be highlighted that authors attribute the toxicity of ZnO in this study to solubilised Zn²⁺ ions rather than to the nanoparticulate form (Heinlaan *et al.* 2008).

Algae and aquatic plants:

Table 9.33: Effects of nano ZnO on algae

Method	Results	Remarks	Reference
<i>Pseudokirchneriella subcapitata</i>	Growth inhibition: EC ₅₀ (72h): 0.042 mg l ⁻¹ NOEC (72h): 0.02 mg l ⁻¹	Negligible shading effect. 50-70 nm ZnO nanoparticles. Toxicity attributable to Zn(II) released by ZnO	Aruoja <i>et al.</i> (2008)
<i>Pseudokirchneriella subcapitata</i>	Growth inhibition: IC ₅₀ (72h): 60 µg l ⁻¹ IC ₅₀ (72h): 44 µg l ⁻¹	Sonicated ZnO Sonicated ZnO + nonylphenol etoxylate Toxicity attributable to Zn(II) released by ZnO	Franklin <i>et al.</i> (2007)

Two studies on growth inhibition of *Pseudokirchneriella subcapitata* were carried out, by using different ZnO dispersions (Table 9.33). The values obtained by Aruoja *et al.* (2008) and by Franklin *et al.* (2007) are similar, with an EC₅₀ algae in the range 42-60 µg l⁻¹ Zn, and a NOEC_{algae} of 20 µg l⁻¹ Zn. According to both studies, the toxicity to *Pseudokirchneriella subcapitata* is caused by the release of Zn²⁺ instead of the direct effect of ZnO nanoparticles.

Other organisms:

Table 9.34: Effects of nano ZnO on bacteria

Method	Results	Remarks	Reference
<i>Vibrio fischeri</i>	EC ₅₀ growth inhibition (30 min): 1.9 mg l ⁻¹ NOEC (30 min): 0.75 mg l ⁻¹	ZnO size range: 50-70 nm. Flash assay used. Nano ZnO less toxic than Zn ions.	Heinlaan <i>et al.</i> (2008)
<i>Vibrio fischeri</i> Strain NRRL-B-11177	EC ₅₀ growth inhibition (30 min): 3.9-4.8 mg l ⁻¹	ZnO size range: 50-70 nm. Flash assay used. No effect observed using Microtox test	Mortimer <i>et al.</i> (2008)
<i>Staphylococcus aureus</i> <i>Streptococcus agalactiae</i>	95% growth inhibition (24h): 0.12 M (9.8 g l ⁻¹) 30% growth inhibition (24h): 0.0012 M (97 mg l ⁻¹) Integrity loss of cell walls observed	In <i>Streptococcus agalactiae</i>	Huang <i>et al.</i> (2008)
<i>Bacillus subtilis</i>	Growth inhibition (90-98%): 10-500 ppm	Photocatalytic ROS not the only mechanism of action	Adams <i>et al.</i> (2006)
<i>Escherichia coli</i>	Growth inhibition (14-38%): 10-500 ppm	Photocatalytic ROS not the only mechanism of action. Less sensitive than Bacillus	Adams <i>et al.</i> (2006)

The effect of nanoparticle ZnO was investigated on different bacteria (Table 9.34). Only two studies identified a NOEC or an EC₅₀ at 30 minutes (*Vibrio fischeri*). However, also the studies Huang *et al.* (2008) and Adams *et al.* (2006) provide useful information about the mechanism of action that included not only photocatalytic ROS and antibacterial activity, but also another mechanism that is not evident for now. According to the available data, the lowest EC₅₀ bacteria is 1.9 mg l⁻¹ for nanoparticle ZnO and a NOEC_{bacteria} of 0.75 mg l⁻¹ can be established.

Terrestrial compartment

Concerning the terrestrial compartment, toxicity data were available only for plants. The available data were produced by Lin and Xing in two studies of 2007 and 2008, concerning the root elongation in seedlings (Table 9.35). The endpoint reported in the papers was IC₅₀ measured at 5 and 12 days. The role of Zn²⁺ ions with respect to the observed effects is not clear. ZnO nanoparticles of 20 nm and 500-800 nm were investigated. However, no conclusions can be drawn concerning the effect of the particles size on the toxicity of ZnO. The lowest reported IC₅₀ plants (short-term) was 20 mg l⁻¹ for nanoparticle ZnO.

Table 9.35: Effects of nano ZnO on terrestrial plants

Method	Results	Remarks	Reference
<i>Lolium sp.</i> (ryegrass) seedlings	IC ₅₀ root growth inhibition (12d): 64 mg l ⁻¹	ZnO aggregates 500-800 nm. Zn ²⁺ more toxic than NP	Lin and Xing (2008)
<i>Raphanus sativus</i> (radish) seedlings	IC ₅₀ root growth inhibition (5d): 50 mg l ⁻¹	ZnO size: 20 nm. Effect occurred during seed incubation rather than seed soaking stage. No toxicity of Zn ²⁺ (4 mg l ⁻¹).	Lin and Xing (2007)
<i>Brassica napus</i> (rape) and <i>Lolium sp.</i> (ryegrass) seedlings	IC ₅₀ root growth inhibition (5d): 20 mg l ⁻¹	ZnO size: 20 nm. Effect occurred during seed incubation rather than seed soaking stage. No toxicity of Zn ²⁺ (4 mg l ⁻¹).	Lin and Xing (2007)

9.6.10 Risk characterisation and gap analysis

Quantitative risk assessment:

Due to the limited data, a quantitative risk assessment is only carried out for the aquatic compartment, freshwater (without sediment, without considering any food chain effects).

The water and soil PECs estimated by Boxall *et al.* (2007) are highly uncertain; however the PEC/PNEC comparison can provide provisional information about the potential for environmental impacts of ZnO nanoparticles.

A PNEC for the freshwater compartment can be estimated according to the REACH guidance. There are acute toxicity values for three trophic levels available: LC_{50 fish} = 1.8 mg l⁻¹ nanoparticle ZnO, EC_{50 invertebrates} = 0.18 mg l⁻¹ nanoparticle ZnO, and EC_{50 algae} = 0.042 mg l⁻¹ nanoparticle ZnO. The lowest value is that of the algae to which an assessment factor of 1000 has to be applied.

Table 9.36: Risk Characterisation for nano ZnO for the aquatic compartment

Compartment	PEC	PNEC	PEC/PNEC	Remarks
Freshwater	76 µg l ⁻¹	0.042 µg l ⁻¹	1800	PEC from Boxall <i>et al.</i> (2007)

The PEC/PNEC ratio for the aquatic compartment is above 1000 (Table 9.36). Taking into account that a PNEC estimated from LC_{50 fish} or EC_{50 Daphnia} would also give a RCR higher than 1, the result raises a concern about the environmental effects of nanoparticle ZnO, even considering the uncertainty related to the PEC estimation. The RCR is also similar to that which could be calculated from algae toxicity data available for dissolved zinc (EC₅₀ = 135 µg l⁻¹ dissolved zinc EU risk assessment report). Given that in several studies the toxicity of nanoparticle ZnO has been traced to Zn²⁺ ions, the effects of different coatings on ions release (e.g. coating stability, coating effectiveness, etc.) should be the focus of further investigations.

Taking into account the high level of uncertainty with respect to the PEC, improved exposure information would be useful. Furthermore, long-term ecotoxicity data estimating NOEC or EC₁₀ for fish and invertebrates would be valuable to refine the assessment, and reduce the uncertainty of the estimate. However, from the reviewed studies, the most sensitive group were the microalgae. Therefore, an algae test with a second algae species would be advisable.

Despite all uncertainties, this provisional assessment shows that care should be taken to minimise emissions of zinc nanoparticles to the environment.

Qualitative risk assessment:

ZnO as a nanomaterial used in several industrial applications and consumer products can be released into the environment, and especially into aquatic systems, from several sources e.g.

by wastewaters emissions, as well as by industrial processes and by recycling and disposal processes of electronic items.

Once in natural water, however, ZnO may aggregate rapidly and thus the transport should be reduced. ZnO aggregates can settle to sediments posing a risk for filter feeders and sediment organisms. However, the stabilisation effect of natural organic matter on ZnO was not investigated.

ZnO shows highest toxicity to algae, followed by aquatic invertebrates, fish, bacteria and terrestrial plants, in decreasing order. Therefore, the first trophic levels of the aquatic community are the most affected. The investigation of ZnO uptake, bioaccumulation and biomagnification, and the indirect effect on the food chain due to food source depletion should be investigated.

Since it has been widely mentioned in the literature that the toxicity of ZnO nanoparticles seems to be related more to Zn²⁺ ions released in water rather than to the 'nanoparticle' nature of the substance, the comparison of these considerations with the 'bulk' ZnO risk assessment results can provide additional information. ZnO is classified as very toxic for aquatic organisms (Directive 67/548/EEC), possibly causing long-term effects in the aquatic environment (N;R50-53)².

Moreover, the EU risk assessment (http://ecb.jrc.ec.europa.eu/DOCUMENTS/Existing-Chemicals/RISK_ASSESSMENT/SUMMARY/zincoxideENVsum073.pdf accessed: 16/10/2009) highlighted that the PNEC is exceeded in surface water, sediments, and STP, as well as in soil, for different production processes and sources: i.e. formulation of lubricants, glass industry, varistor industry, catalysts processing, paints formulation, and cosmetics formulation and private use. Among these sectors, ZnO in nanosize is used especially in catalyst processing and in cosmetic products, which could be the most relevant sources for the environment. Appropriate risk reduction measures to limit the emission of ZnO nanoparticles are required.

Nanoparticle ZnO should be treated as a potential threat for the aquatic environment, since the uncertainty of exposure levels and environmental transport is very high, and because it is most toxic for the first levels of the aquatic food chain.

In order to perform a full risk assessment for nanoparticle ZnO, the following data gaps need to be addressed:

1. Lack of information on production volumes of nanoparticle ZnO, in its many forms;
2. Lack of information on the number of nanoparticle ZnO products, market penetration, and amounts of the nanoparticles in these products;
3. Lack of information on behaviour of nanoparticle ZnO during wastewater treatment;
4. Lack of exposure data and monitoring field data on nanoparticle ZnO levels in environmental compartments;
5. Very little information about environmental fate, limited to aggregation in natural water;
6. Lack of toxicity long-term studies for the aquatic compartment, i.e. long-term studies on fish and *Daphnia*;
7. Limited ecotoxicity data and lack of fate data for the terrestrial compartment, as well as for the aquatic sediment;
8. Lack of information concerning interaction between organisms and nanoparticle ZnO (adsorption, uptake, bioaccumulation, etc.).

² According the new Globally Harmonised System of Classification and Labelling of Chemicals (GHS), N; R50-53 can be translated to **Hazardous to aquatic environment** in **Acute Toxicity Category 1** and **Chronic Toxicity Category 1** and assigned **H400** (*very toxic to aquatic life*) and **H410** (*Very toxic to aquatic life with long lasting effects*).

9.7 CONCLUSIONS AND RECOMMENDATIONS

9.7.1 General Issues

Exposure data

The risk assessments show a significant lack of measured and modelled exposure data of nanomaterials, for humans (occupational and consumer exposure) and for the environment. The limited amount of published measured data for human exposure may be due to the difficulties associated with the measurement of ultrafine or nanoparticles (and the decision which metric(s) to use) and their distinction from background particles. For the environment, this is further complicated by the challenges of "identifying" nanoparticles in environmental matrices. A few relatively simple exposure models have been used (see e.g. Boxall *et al.* 2007 and Mueller and Nowack 2008). However, more sophisticated reliable models for predicting exposure to nanomaterials have not been identified.

For risk assessment purposes, it is in general highly recommended to further establish good exposure data for all relevant exposure routes and targets, via measurements as well as to develop validated exposure models. Establishment of exposure data should address the issues related to a proper characterisation, as discussed in the following. It is also important to further study the interaction of nanoparticles with environmental matrices (e.g. natural organic matter, sediments, etc.), affecting the environmental fate and transport, and thus the exposure for aquatic and soil organisms. The surface chemistry of the nanomaterial (i.e. coating and functionalisation) may play a relevant role in the fate of the nanomaterial.

Characterisation and metrics

As noted, the choice of metric(s) seems of key importance for assessing risks (hazard and exposure) of nanomaterials. This finding is in line with many other authors (see for example SCENIHR 2009 and Chapter 3 of this report, which notes that a proper characterisation of nanoparticles is fundamental for exposure assessment as well for toxicity testing. Work is ongoing in various *fora* (in particular OECD and ISO) to define/outline a proper set of characteristics for nanomaterials. It therefore seems premature to draw within this review a definitive conclusion regarding this issue. However, a few considerations will be given.

For human health, it seems that the risk of metal and metal oxides is largely driven by the size and therefore surface area of the nanoparticles, and it seems that chemistry may (e.g. silver) or may not (e.g. TiO₂) influence the toxicity, possibly depending (at least partly) on the formation and toxicity of free metal ions. For the carbon-based nanomaterials investigated in this report it seems very relevant in addition to consider the three dimensional structure of the nanomaterial (e.g. fibre-like characteristics), the chemical composition (e.g. impurities from their production) and not the least the various surface modifications, which are often added deliberately to promote a certain effect (e.g. increase water solubility). A particular challenge (both in terms of measuring exposure and assessing risks) is introduced by the fact that exposure data often refers to a distribution of particles of different characteristics and different sizes, whereas toxicity tests are often performed for mono-sized nanoparticles of one type. In addition, nanoparticles will often aggregate to agglomerates (both relevant for exposure assessment and toxicity testing) and as evidenced in the risk assessments, it is not always clear what the agglomeration state was in the relevant studies. Even if known, it is difficult to make general conclusions on how this will influence the toxicity. This issue is related to sample preparation, which is a key phase in toxicity testing that has not been sufficiently addressed in studies, with different nanoparticles being prepared in different ways in order to take into account inherent properties (e.g. physico-chemical parameters) and exposed matrix/animals.

For the environment, it was not possible to determine an influence of the size or the shape on the ecotoxicity for any of the groups of investigated nanoparticles. The effects may however be affected by agglomeration and aggregation. Toxicity of metals and metal oxides seems to be driven by chemical composition, but the effect of coatings (e.g. in consumer products mostly coated nanomaterials are used) on their toxicity was not sufficiently studied. For example, coating can reduce or block the release of toxic ions from silver nanoparticles thus reducing

their toxicity. Moreover, coating and surface functionalisation may improve the metal and metal oxide nanoparticle dispersion stability and hydrophilicity and consequently may increase the possibility of transport over long distances in the environment. The effects of carbon-based nanomaterials on organisms are influenced by functionalisation and the level of impurities (especially in CNT).

In conclusion, the risk assessment supports strongly the further development of a proper characterisation scheme (including proper considerations of agglomeration/aggregation) for nanoparticles in the exposure media, when conducting exposure assessment, as well as in the generation of data for assessing hazardous properties. This seems a key prerequisite for doing a proper assessment of the risks. It should be noted that this characterisation does not necessarily need to be the same for different types of materials. There is an urgent need for the development of reference nanomaterials for the evaluation of both the quality of measurement techniques and to compare biological responses.

Scaling from bulk substances

It is often discussed to which degree the risks of nanoparticles can be assessed based on the toxicity of the bulk/normal substances, i.e. whether the risk of the bulk/normal substances can simply "be scaled" to the nanoform taking into account the smaller size of the particle or whether the small sizes triggers "nano-specific" behaviour/effects. To date, no firm conclusion can be drawn which would be applicable to all nanoparticles. However, when considering whether scaling is possible, it seems to be a prerequisite that if the 'chemistry' (at least partly) drives the toxicity, it needs to be the same chemistry in the bulk/normal form as well as in the nanoform. This already introduces some reservations for carbon-based nanomaterials, which have surface modifications deliberately added to give them specific properties. For more chemically 'inert' particles, it may be possible to draw conclusions on their behaviour and scaled from larger inert particles simply based on the shape. However, this needs further investigation beyond the possibilities in this review.

For human health, there are indications that (some of) the toxic effects of nanosized TiO₂ can simply be scaled based on surface area considerations from the toxicity of the micro-sized TiO₂. For silver, there is still too little known about the toxico-kinetics to give a fair judgement of this question. For the carbon-based nanomaterials, it does not seem obvious that the toxicity observed for the nanoforms could be found based on scaling from any normal/bulk state of carbon-materials. These observations should rather be seen as reflections than firm conclusions.

For the environment, it is interesting to note that the toxicity seen for nanosized ZnO is indicated by some authors to be related to the release of zinc ions (Zn⁺⁺) just as the toxicity of the bulk form of ZnO. Scaling may therefore be possible for this substance. The influence of increased surface area of nano-forms with respect to the bulk form is to be verified on the ion leaching amount and efficiency. However this does not include coated nanomaterials, which have peculiar properties. Concerning silver nanoparticles, no conclusion can be drawn yet, even if toxicity of silver seems to be related to Ag⁺ ions.

In conclusion, it seems possible to predict (part of) the toxicity of some nanomaterials based on the toxicity of the bulk/normal form, but this is not possible for all types of nanomaterials.

Toxico-kinetics

For human health, although scattered, there are some useful investigations available describing the toxic behaviour of nanomaterials (see the significant toxicity reviews of this report). Despite this there are still significant gaps related to the knowledge of the toxico-kinetic behaviour of nanomaterials. For example there are significant uncertainties associated with the possible pulmonary uptake of nanoparticles (TiO₂ and carbon-based nanomaterials) and a lack of knowledge in which form they are absorbed (e.g. nano-silver, is it absorbed as particle, ion, complex or a combination?). Several distribution studies have been conducted on the various nanomaterials' ability to spread to various organs following intravenous or intraperitoneal injection. These results indicate a propensity for wide distribution to several organs, which may

be very relevant for medical applications, however currently difficult to apply in a risk assessment for workers and consumers (as conducted in this study) as it is not known whether the materials will be absorbed following the exposure routes relevant for these populations (inhalation, dermal and oral). The same problem holds true for a proper interpretation of *in vitro* findings. As evidenced in the toxicity reviews, a significant amount of *in vitro* data showing toxicity to tissues/cells in various organs have been identified. It is difficult to judge the value of such findings without knowing whether the nanomaterials will ever reach those organs, and if so, at which concentrations.

For the environment, ecotoxicity testing often does not deal with toxico-kinetics. However, it is relevant to further investigate the possible uptake, distribution, and excretion of nanoparticles in the species tested, and to identify target organs (e.g. do nanoparticles reach the brains of fish? Are ingested nanoparticles adsorbed?). Biotransformation of ingested coated/modified nanoparticles in the environment should be investigated, since nanoparticle properties (including toxicity) may be changed (e.g. less hydrophilic). Another issue related to nanomaterials' toxico-kinetics is the carrier effect of organic nanomaterials by increasing bioaccumulation of organic and inorganic chemicals.

Thus it is highly recommended to further intensify the research on the toxico-kinetic behaviour of the various nanomaterials. This relates to testing of both toxicity and ecotoxicity.

9.7.2 Human health specific issues

Genotoxicity

As evidenced in the toxicity reviews, several/most of the nanomaterials investigated seem to trigger an oxidative stress driven inflammatory response, which may lead to genotoxicity. If this occurs it is a threshold-driven secondary genotoxicity. However, there seems to be a lack of investigations of the direct (possibly non-threshold) genotoxicity of the nanomaterials.

It is recommended to conduct further testing of possible direct genotoxicity of nanomaterials.

9.7.3 Environmental specific issues

Chronic toxicity

Up until now, ecotoxicity data are mainly focused on short-term tests reporting acute endpoints, i.e. mortality. Only a few studies reporting sub-lethal endpoints were carried out, but often using short-term exposure studies. A general lack of data was observed for long-term studies on fish, *Daphnia*, and algae as well as for sediment and terrestrial organisms. Moreover, exposure concentrations in acute tests are often unrealistically high, while effects related to very low concentrations are possible (e.g. reduced aggregation).

*It is recommended that more chronic toxicity studies should be conducted, especially on *Daphnia* and fish species, supporting the prediction of more reliable environmental no effect concentrations (PNEC). Studies about algae should also be performed, as often algae are the most sensitive organisms, and they can provide information about mechanism of action, and can substitute fish studies that are more complex and subjected to ethical issues. Studies should be supported by a thorough nanomaterial characterisation during all exposure phases. Concerning exposure concentrations, chronic studies may allow the investigation of realistic concentrations showing relevant sub-lethal effects (e.g. reproduction, behaviour).*

Mechanism of action

There is a general lack of knowledge about the mechanism of action of nanoparticles. Some toxic actions such as reactive oxygen species generation are well understood, but some studies suggest the existence of other mechanisms that are still not explained. The identification of the mechanism of action may help to correlate the results of laboratory studies to the environment. Moreover, more attention should be given to the identification of indirect effects. For example, a study on algae highlighted that TiO₂ toxicity was exerted by algae entrapment in aggregates.

It is recommended that studies are designed and conducted to identify the mode of action of nanomaterials, also using 'omics' systems. It is important to understand if the toxic action is exerted after uptake into cells, or whether they already occur via extracellular exposure (e.g. generation of reactive oxygen species (ROS) outside the cell) or at the cell membrane level. Moreover, indirect toxic effects should be better investigated, such as physical effects (e.g. algae entrapment into agglomerates, light shadowing) or chemical effects (e.g. nutrient adsorption and sequestration, transport of toxic chemicals into the cell).

9.7.4 Outlook/Perspectives on future hazard and risk assessment methodology

Human Health

To date the database on exposure and effects of nanomaterials is not sufficient to make conclusive decisions on their risks. As already mentioned before, the main priority for further information will be good quality and representative exposure data and substantial information on toxico-kinetics, as well as properly characterised exposure and toxicity data. Based on that information, further testing strategies should be set up to cover all relevant endpoints needed for a risk assessment. In the respective sections of the risk assessment, suggested information requirements for each of the studied nanomaterials were listed.

It has to be carefully analysed if available testing methods (as used for bulk/normal chemical substances) need to be adapted to reliably identify and investigate toxic effects of nanoparticles. An effort should be made to minimise animal testing, however keeping in mind that the data generated are considered appropriate to identify risks relevant for humans and are accepted by regulators.

The current conclusion to carry out risk assessment for nanoparticles could only be to do it on a case-by-case basis. When more data becomes available it might be possible to group nanomaterials according to their physical, chemical and/or biological properties and testing could be done to be representative for a group. Based on such future knowledge, it may also be possible to develop (Q)SAR and priority setting models as further discussed below.

Environment

From the ecotoxicity and environmental fate studies published so far, it is evident that each nanomaterial, also within the same group (e.g. CNT), should be assessed on a case-by-case basis, given the inherent nanomaterial properties (e.g. different chemical composition, coating/functionalisation, size) and the interaction with the environment (e.g. aggregation, ions leaching, transport, uptake by biota, sorption). Bearing in mind the many different nanomaterials available on the market, it might be useful to identify the nanomaterials of concern, i.e. priority nanomaterials. Prioritisation is generally the first step before risk assessment, in order to focus the resources to more relevant cases. The identification of the most important risk hypotheses including the nanomaterials of concern can be done by applying what is called the 'problem formulation' (PF) in the ecological risk assessment framework. The PF allows the definition of the conceptual model, linking chemicals to sources, pathway, target and impacts, and thus allowing the identification of priority nanomaterials.

The actual knowledge about nanomaterials, their ecotoxicity, and fate and behaviour in the environment is not sufficient to carry out a full risk assessment or to depict a full conceptual model. However, the available data could be used to evaluate each of the conceptual model elements to prioritise nanomaterials, balancing the risks posed to environment and human health and the potential benefits.

Linkov *et al.* (2007) suggested the application of a multi-criteria decision analysis (MCDA) as one of the most promising ways to rank nanomaterials. MCDA is an approach which is part of the weight-of-evidence family, where a set of evidences (i.e. parameters measuring the attributes of a chemical) are weighted and aggregated to formulate a judgment about the risk (or benefit) posed by a chemical. The relationship between information/data and risk/benefits, as well as the relative importance of each parameter for risks/benefits assessment, are evaluated according to professional judgment, and the MCDA result is a relative ranking, showing the level of concern of one nanomaterial with respect to another.

The application of the MCDA approach by using the DART (Decision Analysis by Ranking Techniques) software (<http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c=DART>, accessed 16th October 2009) represent a way to use, in an organised way, the information collected within the ENRHES review, as well as new data published after the cut-off date for the literature review of ENRHES, and to obtain a nanomaterial ranking. The possibility to identify benefit parameters to be included in the ranking exercise should be explored.

In conclusion, it seems that currently available test methods and risk assessment methodologies might not be sufficient to effectively assess the possible risk of nanomaterials. Therefore it is recommended to assess and improve already available methods and methodologies which maybe not be used on a regular basis yet for their applicability to test and/or prioritise nanomaterials. Furthermore, new methods and methodologies will have to be developed that take into account the specific properties of nanomaterials. In future appropriate hazard and risk assessment methodologies would be required that allow a prioritisation of nanomaterials with a higher expected risk and facilitate effective testing of groups of nanomaterials of similar properties.

9.8 REFERENCES

- Adams, L.K., Lyon, D.Y. and Alvarez, P.J.J. 2006. Comparative eco-toxicity of nanoscale TiO₂, SiO₂, and ZnO water suspensions. *Water Res.*, vol. 40, no. 19, pp. 3527-3532.
- Afaq, F., Abidi, P., Matin, R. and Rahman, Q. 1998, "Cytotoxicity, pro-oxidant effects and antioxidant depletion in rat lung alveolar macrophages exposed to ultrafine titanium dioxide", *Journal of Applied Toxicology*, vol. 18, no. 5, pp. 307-312.
- Ahamed, M., Karns, M., Goodson, M., Rowe, J., Hussain, S.M., Schlager, J.J. and Hong, Y. 2008, "DNA damage response to different surface chemistry of silver nanoparticles in mammalian cells", *Toxicology and Applied Pharmacology*, vol. 233, no. 3, pp. 404-410.
- Ahn, M. H., Kang, C. M., Park, C. S., Park, S. J., Rhim, T., Yoon, P. O., Chang, H. S., Kim, S. H., Kyono, H. and Kim, K. C. 2005, "Titanium dioxide particle-induced goblet cell hyperplasia: association with mast cells and IL-13", *Respiratory Research*, vol. 6, pp. 34-43
- Arora, S., Jain, J., Rajwade, J. M., and Paknikar, K. M. 2008, "Cellular responses induced by silver nanoparticles: *In vitro* studies", *Toxicol.Lett.*, vol. 179, no. 2, pp. 93-100.
- Arora, S., Jain, J., Rajwade, J. M., and Paknikar, K. M. 2009, "Interactions of silver nanoparticles with primary mouse fibroblasts and liver cells", *Toxicol.Appl.Pharmacol.*, vol. 236, no. 3, pp. 310-318.
- Aruoja, V., Dubourguier, H.C., Kasemets, K. and Kahru, A. 2008. Toxicity of nanoparticles of CuO, ZnO and TiO₂ to microalgae *Pseudokirchneriella subcapitata*. *Science of the Total Environment*, vol. 407, no. 4, pp. 1461-1468.
- AshaRani, P. V., Low Kah, M. G., Hande, M. P., and Valiyaveetil, S. 2009, "Cytotoxicity and genotoxicity of silver nanoparticles in human cells", *ACS Nano.*, vol. 3, no. 2, pp. 279-290.
- Asharani, P.V., Wu, Y.L., Gong, Z., Valiyaveetil, S. 2008. Toxicity of silver nanoparticles in zebrafish models. *Nanotechnology*, 19, 255102 (8pp). doi:10.1088/0957-4484/19/25/255102.
- Baker, G.L., Gupta, A., Clark, M.L., Valenzuela, B.R., Staska, L.M., Harbo, S.J., Pierce, J.T. and Dill, J.A. 2008, "Inhalation toxicity and lung toxicokinetics of C₆₀ fullerene nanoparticles and microparticles", *Toxicological Sciences*, vol. 101, no. 1, pp.122-131.
- Bar-Ilan, O., Albrecht, R.M., Fako, V.E. and Furgeson, D.Y. 2009, "Toxicity assessments of multisized gold and silver nanoparticles in zebrafish embryos", *Small*. Epub before print.
- Barlow, P. G., Clouter-Baker, A., Donaldson, K., MacCallum, J. and Stone, V. 2005, "Carbon black nanoparticles induce type II epithelial cells to release chemotaxins for alveolar macrophages", *Particle and Fibre Toxicology*, vol. 2, pp. 11-25.
- Bello, D., Wardle, B.L., Yamamoto, N., deVilloria, R.G., Garcia, E.J., Hart, A.J., Ahn, K., Ellenbecker, M.J. and Hallock, M. 2009, "Exposure to nanoscale particles and fibers during machining of hybrid advanced composites containing carbon nanotubes", *Journal of Nanoparticle Research*, vol. 11, no.1, pp. 231-249.
- Bermudez, E., Mangum, J. B., Wong, B. A., Asgharian, B., Hext, P. M., Warheit, D. B. and Everitt, J. I. 2004, "Pulmonary responses of mice, rats, and hamsters to subchronic inhalation of ultrafine titanium dioxide particles", *Toxicological Sciences*, vol. 77, no. 2, pp. 347-357
- Blickley, T.M. and McClellan-Green, P. 2008. Toxicity of Aqueous Fullerene in Adult and Larval *Fundulus heteroclitus*. *Environ.Toxicol.Chem*, vol. 27, no. 9, pp. 1964-71.
- Bogdanovic, V., Stankov, K., Icevic, I., Zikic, D., Nikolic, A., Solajic, S., Djordjevic, A. and Bogdanovic, G. 2008, "Fullerenol C₆₀(OH)₂₄ effects on antioxidative enzymes activity in irradiated human erythroleukemia cell line", *Journal of Radiation Research*, vol. 49, no. 3, pp. 321-327.
- Boxall, A., Chaudhry, Q., Sinclair, C., Jones, A., Aitken, R., Jefferson, B., Watts, C. 2007. Current and future predicted environmental exposure to engineered nanoparticles. Final client report by the Central Science Laboratory for Department of Environment Food and Rural Affairs (DEFRA)

- Braydich-Stolle, L., Hussain, S., Schlager, J.J. and Hofmann, M.C. 2005, "In Vitro cytotoxicity of nanoparticles in mammalian germline stem cells", *Toxicological Sciences*, vol. 88, no.2, pp. 412–419
- Brown, D.M., Kinloch, I.A., Bangert, U., Windle, A.H., Walter, D.M., Walker, G.S., Scotchford, C.A., Donaldson, K. and Stone, V. 2007, "An *in vitro* study of the potential of carbon nanotubes and nanofibres to induce inflammation mediators and frustrated phagocytosis", *Carbon*, vol. 45, no. 9, pp. 1743-1756.
- Bullard-Dillard, R., Creek, K.E., Scrivens, W.A. and Tour, J.M. 1996, "Tissue sites of uptake of ^{14}C labelled C_{60} ", *Bioorganic Chemistry*, vol. 24, no. 4, pp. 376-385.
- Canas, J.E., Long, M.Q., Nations, S., Vadan, R., Dai, L., Luo, M.X., Ambikapathi, R., Lee, E.H. and Olszyk, D. 2008. Effects of functionalised and nonfunctionalised single-walled carbon nanotubes on root elongation of select crop species. *Environmental Toxicology and Chemistry*, vol. 27, no. 9, pp. 1922-1931.
- Carlson, C., Hussain, S., Schrand, A., Braydich-Stolle, L., Hess, K., Jones, R., Schlager, J. 2008. Unique Cellular Interaction of Silver Nanoparticles: Size-Dependent Generation of Reactive Oxygen Species, *J. Phys. Chem. B*, vol. 112, pp. 13608–13619
- Casey, A., Davoren, M., Herzog, E., Lyng, F.M., Byrne, H.J. and Chambers, G. 2007b, "Probing the interaction of single walled carbon nanotubes within cell culture medium as a precursor to toxicity testing", *Carbon*, vol. 45, no.1, pp. 34-40.
- Casey, A., Herzog, E., Davoren, M., Lyng, F.M., Byrne, H.J. and Chambers G. 2007a, "Spectroscopic analysis confirms the interactions between single walled carbon nanotubes and various dyes commonly used to assess cytotoxicity", *Carbon*, vol.45, no.7, pp. 1425-1432.
- Cha, K., Hong, H. W., Choi, Y. G., Lee, M. J., Park, J. H., Chae, H. K., Ryu, G., and Myung, H. 2008, "Comparison of acute responses of mice livers to short-term exposure to nano-sized or micro-sized silver particles", *Biotechnol.Lett.*, vol. 30, no. 11, pp. 1893-1899.
- Chang, A.L.S., Khosravi, V. and Egbert, B. 2006, "A case of argyria development after colloidal silver digestion", *Journal of Cutaneous Pathology*, vol. 33, no. 12, pp. 809-811.
- Chen, B.X., Wilson, S.R., Das, M., Coughlin, D.J. and Erlanger, B.F. 1998a, "Antigenicity of fullerenes: antibodies specific for fullerenes and their characteristics", *Proceedings of the National Academy of Sciences USA*, vol. 95, no.18, pp.10809-10813.
- Chen, C., Xing, G., Wang, J., Zhao, Y., Li, B., Tang, J., Jia, G., Wang, T., Sun, J., Xing, L., Yuan, H., Gao, Y., Meng, H., Chen, Z., Zhao, F., Chai, Z. and Fang, X. 2005, "Multihydroxylated $[\text{Gd}@C_{82}(\text{OH})_{22}]_n$ nanoparticles: antineoplastic activity of high efficiency and low toxicity", *Nano Letters*, vol. 5, no.10, pp. 2050-2057.
- Chen, H. W., Su, S. F., Chien, C. T., Lin, W. H., Yu, S. L., Chou, C. C., Chen, J. J. and Yang, P. C. 2006, "Titanium dioxide nanoparticles induce emphysema-like lung injury in mice", *FASEB Journal*, vol. 20, no. 13, pp. 2393-2395.
- Chen, H.H., Yu, C., Ueng, T.H., Chen, S., Chen, B.J., Huang, K.J. and Chiang, L.Y. 1998b, "Acute and subacute toxicity study of water-soluble polyalkylsulfonated C_{60} in rats", *Toxicologic Pathology*, vol. 26, no. 1, pp.143-151.
- Chen, J., Dong, X., Zhao, J. and Tang, G, 2008, "In vivo acute toxicity of titanium dioxide nanoparticles to mice after intraperitoneal injection", *Journal of Applied Toxicology*, vol. 29, no. 4, pp. 330-337.
- Cheng, J., Chan, C.M., Veca, L.M., Poon, W.L., Chan, P.K., Qu, L., Sun, Y.P. and Cheng, S.H. 2009, "Acute and long-term effects after single loading of functionalised multi-walled carbon nanotubes into zebrafish (*Danio rerio*)", *Toxicology and Applied Pharmacology*, vol. 235, no. 2, pp. 216-225.
- Cheng, J., Flahaut, E. and Cheng, S.H. 2007, "Effect of carbon nanotubes on developing zebrafish (*Danio rerio*) embryos", *Environmental Toxicology and Chemistry*, vol, 26, no. 4, pp. 708-716.

- Cherukuri, P., Gannon, C.J., Leeuw, T.K., Schmidt, H.K., Smalley, R.E., Curley, S.A. and Weisman, R.B. 2006, "Mammalian pharmacokinetics of carbon nanotubes using intrinsic near-infrared fluorescence", *Proceedings of the National Academy of Science USA*, vol. 103, no. 50, pp. 18882-18886.
- Chi, Z., Liu, R., Zhao, L., Qin, P., Pan, X., Sun, F., and Hao, X. 2009, "A new strategy to probe the genotoxicity of silver nanoparticles combined with cetylpyridine bromide", *Spectrochim. Acta A Mol. Biomol. Spectrosc.*, vol. 72, no. 3, pp. 577-581.
- Cho, W-S., Cho, M., Jeong, J., Choi, M., Cho, H-Y., Han, B.S., Kim, S.H., Kim, H.O., Lim, Y.T., Chung, B.H. and Jeong, J. 2009, " Acute toxicity and pharmacokinetics of 13 nm-sized PEG-coated gold nanoparticles", *Toxicology and Applied Pharmacology*, vol. 236, no.1, pp. 16–24.
- Choi, O. and Hu, Z.Q. 2008. Size dependent and reactive oxygen species related nanosilver toxicity to nitrifying bacteria. *Environ.Sci. Technol.*, vol. 42, no. 12, pp. 4583-4588.
- Choi, O., Deng, K.K., Kim, N.J., Ross, L., Surampalli, R.Y. and Hu, Z.Q. 2008. The inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth. *Water Res.*, vol. 42, no. 12, pp. 3066-3074.
- Churg, A., Gilks, B. and Dai, J. 1999. "Induction of fibrogenic mediators by fine and ultrafine titanium dioxide in rat tracheal explants". *American Journal of Physiology*, vol. 277, no.5, pp. L975-L982
- Davoren, M., Herzog, E., Casey, A., Cottineau, B., Chambers, G., Byrne, H.J. and Lyng, F.M. 2007, "In vitro toxicity evaluation of single walled carbon nanotubes on human A549 lung cells", *Toxicology In Vitro*, vol. 21, no. 3, pp. 438-448.
- De Jong, W. H., Hagens, W.I, Krystek, P., Burger, M.C., Sips, A. J.A.M., Geertsma, R.E., 2008, "Particle size-dependent organ distribution of gold nanoparticles after intravenous administration", *Biomaterials*, vol. 29, no. 12, pp. 1912-1919.
- Demou, E., Peter, P. and Hellweg, S. 2008, "Exposure to Manufactured Nanostructured Particles in an Industrial Pilot Plant", *Ann.Occup.Hyg.*, vol. 52, no. 8, pp. 695-706.
- Deng, X., Jia, G., Wang, H., Sun, H., Wang, X., Yang, S., Wang, T. and Liu, Y. 2007, "Translocation and fate of multi-walled carbon nanotubes *in vivo*", *Carbon*, vol. 45, no. 7, pp. 1419-1424.
- Dhawan, A., Taurozzi, J.S., Pandey, A.K., Shan, W., Miller, S.M., Hashsham, S.A. and Tarabara, V.V. 2006, "Stable colloidal dispersions of C₆₀ fullerenes in water: evidence for genotoxicity", *Environmental Science and Technology*, vol. 40, no. 23, pp. 7394-7401.
- Di, S.A., Chiaretti, M., Carru, G.A., Bellucci, S. and Mazzanti, G. 2009, "Multi-walled carbon nanotubes: Lack of mutagenic activity in the bacterial reverse mutation assay", *Toxicology Letters*, vol, 184, no. 3, pp. 192-197.
- Donaldson, K. and Stone, V. 2003, "Current hypotheses on the mechanisms of toxicity of ultrafine particles", *Annali dell 'Istituto Superiore di Sanita*, vol. 39, no. 3, pp. 405-410.
- Dugan, L.L., Gabrielsen, J.K., Yu, S.P., Lin, T.S. and Choi, D.W. 1996, "Buckminsterfullerene free radical scavengers reduce excitotoxic and apoptotic death of cultured cortical neurons", *Neurobiology of Disease*, vol. 3, no.2, pp. 129-135.
- Dussert, A. S., Gooris, E. and Hemmerle, J. 1997, "Characterization of the mineral content of a physical sunscreen emulsion and its distribution onto human stratum corneum", *International Journal of Cosmetic Science*, vol. 19, no. 3, pp. 119-129.
- ED (Environmental Defense – Dupont). *NANO Risk Framework*. June 2007
- Erlanger, B.F., Chen, B.X., Zhu, M. and Brus, L. 2001, "Binding of an anti-fullerene IgG monoclonal antibody to single wall carbon nanotubes", *Nano Letters*, vol. 1, no.9, pp. 465-467.
- European Chemicals Agency, 2008. *REACH Guidance on Information Requirements and Chemicals Safety Assessment*.

- Fabian, E., Landsiedel, R., Ma-Hock, L., Wiench, K., Wohlleben, W., and Van, R. B. 2008, "Tissue distribution and toxicity of intravenously administered titanium dioxide nanoparticles in rats", *Archives of Toxicology*, vol. 82, no. 3, pp. 151-157.
- Federici, G., Shaw, B.J. and Handy, R.D. 2007. Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): Gill injury, oxidative stress, and other physiological effects. *Aquatic Toxicology*, vol. 84, pp. 415-430.
- Ferguson, P.L., Chandler, G.T., Templeton, R.C., DeMarco, A., Scrivens, W.A. and Englehart, B.A. 2008. Influence of sediment-amendment with single-walled carbon nanotubes and diesel soot on bioaccumulation of hydrophobic organic contaminants by benthic invertebrates. *Environ.Sci.Technol.*, vol. 42, no. 10, pp. 3879-3885.
- Ferin, J., Oberdorster, G. and Penney, D. P. 1992, "Pulmonary retention of ultrafine and fine particles in rats", *American Journal of Respiratory Cell and Molecular Biology*, vol. 6, no.5, pp. 535-542
- Fortner, J.D., Kim, D., Boyd, A.M., Falkner, J.C., Moran, S., Colvin, V.L., Hughes, J.B., Kim, J. 2007. Reaction of Water-Stable C60 Aggregates with Ozone. *Environmental Science and Technology* 2007 41 (21), 7497-7502
- Franco, A., Hansen, S.F., Olsen, A.I., Butti, L. 2007. Limits and prospects of the incremental approach and the European legislation on the management of risks related to nanomaterials. *Regulatory Toxicology and Pharmacology*, 48, 171-183
- Franklin, N.M., Rogers, N.J., Apte, S.C., Batley, G.E., Gadd, G.E. and Casey, P.S. 2007. Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and ZnCl₂ to a freshwater microalga (*Pseudokirchneriella subcapitata*): The importance of particle solubility. *Environ.Sci.Technol.*, 41 (24), 8484-8490.
- Fujita, K., Morimoto, Y., Ogami, A., Myojyo, T., Tanaka, I., Shimada, M., Wang, W.N., Endoh, S., Uchida, K., Nakazato, T., Yamamoto, K., Fukui, H., Horie, M., Yoshida, Y., Iwahashi, H. and Nakanishi, J. 2009, "Gene expression profiles in rat lung after inhalation exposure to C(60) fullerene particles" *Toxicology*, vol. 258, no. 1, pp. 47-55.
- Fujitani, Y., Kobayashi, T., Arashidani, K., Kunugita, N. and Suemura, K. 2008, "Measurement of the physical properties of aerosols in a fullerene factory for inhalation exposure assessment", *Journal of occupational and environmental hygiene*, vol. 5, no. 6, pp. 380-389.
- Gamer, A. O., Leibold, E. and Van, R. B. 2006, "The *in vitro* absorption of microfine zinc oxide and titanium dioxide through porcine skin", *Toxicology In Vitro*, vol. 20, no. 3, pp. 301-307
- Gao, J., Youn, S., Hovsepyan, A., Vernica, L.L., Wang, Y., Bitton, G., Bonzongo, J.J. 2009. Dispersion and Toxicity of Selected Manufactured Nanomaterials in Natural River Water Samples: Effects of Water Chemical Composition. *Environmental Science and Technology* 2009 43 (9), 3322-3328
- Ghafari, P., St-Denis, C.H., Power, M.E., Jin, X., Tsou, V., Mandal, H.S., Bols, N.C. and Tang, X.W. 2008. Impact of carbon nanotubes on the ingestion and digestion of bacteria by ciliated protozoa. *Nature Nanotechnology*, vol. 3, no. 6, pp. 347-351.
- Gharbi, N., Pressac, M., Hadchouel, M., Szwarc, H., Wilson, S.R. and Moussa, F. 2005. "[60]Fullerene is a powerful antioxidant *in vivo* with no acute or subacute toxicity" *Nano Letters*, vol. 5, no. 12, pp. 2578-2585.
- Grassian, V. H., Adamcakova-Dodd, A., Pettibone, J. M., O'shaughnessy, P. T. and Thorne, P. S. 2007, "Inflammatory response of mice to manufactured titanium dioxide nanoparticles: comparison of size effects through different exposure routes", *Nanotoxicology*, vol. 1, no. 3, pp. 211-226
- Griffitt, R.J., Luo, J., Gao, J., Bonzongo, J.C. and Barber, D.S. 2008. Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. *Environmental Toxicology and Chemistry*, vol. 27, no. 9, pp. 1972-1978.

- Gurr, J. R., Wang, A. S., Chen, C. H. and Jan, K. Y. 2005, "Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells", *Toxicology*, vol. 213, no.1-2, pp.66-73
- Han, B. and Karim, M.N. 2009, Cytotoxicity of aggregated fullerene C₆₀ particles on CHO and MDCK cells", *Scanning*, vol. 30, no.2, pp. 213-220.
- Han, J.H., Lee, E.J., Lee, J.H., So, K.P., Lee, Y.H., Bae, G.N., Lee, S.B., Ji, J.H., Cho, M.H. and Yu, I.J. 2008, " Monitoring multiwalled carbon nanotube exposure in carbon nanotube research facility ", *Inhal.Toxicol.*, vol. 20, no. 8, pp. 741-749.
- Han, Z., Zhang, F., Lin, D., Xing, B. 2008. Clay Minerals Affect the Stability of Surfactant-Facilitated Carbon Nanotube Suspensions. *Environmental Science and Technology* 42 (18), 6869-6875
- Handy, R.D., Owen, R., Valsami-Jones, E. 2008. The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges, and future needs. *Ecotoxicology*, 17, 315-325.
- Hansen S.F. *Regulation and Risk Assessment of Nanomaterials - too little, too late?* Technical University of Denmark, Department of Environmental Engineering, PhD thesis, February 2009.
- Harrod, K.S., Jaramillo R.J., Berger, J.A., Gigliotti, A.P., Seilkop, S.K. and Reed, M.D. 2005, "Inhaled diesel engine emissions reduce bacterial clearance and exacerbate lung disease to *Pseudomonas aeruginosa* infection *in vivo*", *Toxicological Sciences*, vol. 83, no. 1, pp. 155-165.
- Harrod, K.S., Jaramillo R.J., Rosenberger, C.L., Wang, S.Z., Berger, J.A., McDonald, J.D. and Reed, M.D. 2003, "Increased susceptibility to RSV infection by exposure to inhaled diesel engine emissions", *American Journal of Respiratory Cell and Molecular Biology* vol. 28,, no. 4 pp. 451-463.
- Heinlaan, M., Ivask, A., Blinova, I., Dubourguier, H.C. and Kahru, A. 2008. Toxicity of nanosized and bulk ZnO, CuO and TiO₂ to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. *Chemosphere*, vol. 71, no. 7, pp. 1308-1316.
- Heinrich, U., Fuhst, R., Rittinghausen, S., Creutzenberg, O., Bellmann, B., Koch, W., and Levsen, K. 1995, "Chronic inhalation exposure of wistar rats and two different strains of mice to diesel engine exhaust, carbon black, and titanium dioxide", *Inhalation Toxicology*, vol. 7, no. 4, pp. 533-556.
- Helfenstein, M., Miragoli, M., Rohr, S., Muller, L., Wick, P., Mohr, M., Gehr, P. and Rothen-Rutishauser, B. 2008, "Effects of combustion-derived ultrafine particles and manufactured nanoparticles on heart cells *in vitro*", *Toxicology*, vol. 253,no. 1-3, pp. 70-78
- Henry, T.B., Menn, F.M., Fleming, J.T., Wilgus, J., Compton, R.N. and Saylor, G.S. 2007. Attributing effects of aqueous C₆₀ nano-aggregates to tetrahydrofuran decomposition products in larval zebrafish by assessment of gene expression. *Environ.Health Perspect.*, vol. 115, no. 7, pp. 1059-1065.
- Hsin, Y.H., Chen, C.F., Huang, S., Shih, T.S., Lai, P.S. and Chueh, P.J. 2008, "The apoptotic effect of nanosilver is mediated by a ROS- and JNK dependent mechanism involving the mitochondrial pathway in NIH3T3 cells", *Toxicology Letters*, vol. 179, no. 3, pp. 130-139.
- Hsu, L.Y. and Chein, H.M. 2007, "Evaluation of nanoparticle emission for TiO₂ nanopowder coating materials", *Journal of Nanoparticle Research*, vol. 9, no. 1, pp. 157-163.
- Huang, Z.B., Zheng, X., Yan, D.H., Yin, G.F., Liao, X.M., Kang, Y.Q., Yao, Y.D., Huang, D. and Hao, B.Q. 2008. Toxicological effect of ZnO nanoparticles based on bacteria. *Langmuir*, vol. 24, no. 8, pp. 4140-4144.
- Huczko, A., Lange, H. and Calko, E. 1999, "Fullerenes: experimental evidence for a null risk of skin irritation and allergy", *Fullerene Science and Technology*, vol. 7, no. 5, pp. 935-939.
- Hund-Rinke, K. and Simon, M. 2006. Ecotoxic effect of photocatalytic active nanoparticles (TiO₂) on algae and daphnids. *Environ.Sci.Pollut.Res.Int.*, vol. 13, no. 4, pp. 225-232.

- Hussain, S. M., Hess, K. L., Gearhart, J. M., Geiss, K. T. and Schlager, J. J. 2005, "In vitro toxicity of nanoparticles in BRL 3A rat liver cells", *Toxicology In Vitro*, vol. 19, no. 7, pp. 975-983
- Hyun, J. S., Lee, B. S., Ryu, H. Y., Sung, J. H., Chung, K. H., and Yu, I. J. 2008, "Effects of repeated silver nanoparticles exposure on the histological structure and mucins of nasal respiratory mucosa in rats", *Toxicol.Lett.*, vol. 182, no. 1-3, pp. 24-28.
- Hyung and Kim. 2008. Natural Organic Matter (NOM) Adsorption to Multi-Walled Carbon Nanotubes: Effect of NOM Characteristics and Water Quality Parameters. *Environmental Science and Technology* 42 (12), 4416-4421
- IARC (International Agency for Research on Cancer) 2006. Overall evaluations of carcinogenicity to humans, Group 2B: Possibly carcinogenic to humans. IARC Monograph. 93, 1-5
- Injac, R., Perse, M., Cerne, M., Potocnik, N., Radic, N., Govedarica, B., Djordjevic, A., Cerar, A. and Strukelj, B. 2009, "Protective effects of fullereneol C₆₀(OH)₂₄ against doxorubicin-induced cardiotoxicity and hepatotoxicity in rats with colorectal cancer", *Biomaterials*, vol. 30, no. 6, pp. 1184-1196.
- Inoue, K., Takano, H., Ohnuki, M., Yanagisawa, R., Sakurai, M., Shimada, A., Mizushima, K. and Yoshikawa, T. 2008, "Size effects of nanomaterials on lung inflammation and coagulatory disturbance", *International Journal of Immunopathology and Pharmacology*, vol. 21, no. 1, pp. 197-206
- Jacobsen, N.R., Pojana, G., White, P., Moller, P., Cohn, C.A., Korsholm, K.S., Vogel, U., Marcomini, A., Loft, S. and Wallin, H. 2008, "Genotoxicity, cytotoxicity, and reactive oxygen species induced by single-walled carbon nanotubes and C(60) fullerenes in the FE1-Mutatrade mark Mouse lung epithelial cells", *Environmental and Molecular Mutagenesis*, vol. 49, no. 6, pp. 476-487.
- Jaisi, D.P., Saleh, N.B., Blake, R.E. and Elimelech, M. 2008. Transport of Single-Walled Carbon Nanotubes in Porous Media: Filtration Mechanisms and Reversibility. *Environ. Sci. Technol*, 42 (22): 8317–8323
- Jemec, A., Drobne, D., Remskar, M., Sepcic, K. and Tisler, T. 2008. Effects of Ingested Nano-Sized Titanium Dioxide on Terrestrial Isopods *Porcellio Scaber*. *Environ.Toxicol.Chem.*, 2:1, pp. 1.
- Ji, J.H., Jung, J.H., Kim, S.S., Yoon, J.U., Park, J.D., Choi, B.S., Chung, Y.H., Kwon, I.H., Jeong, J., Han, B.S., Shin, J.H., Sung, J.H., Song, K.S. and Yu, I.J. 2007, "Twenty-eight-day inhalation toxicity study of silver nanoparticles in sprague-dawley rats", *Inhalation Toxicology*, vol. 19, no. 10, pp. 857-871.
- Jia, G., Wang, H., Yan, L., Wang, X., Pei, R., Yan, T., Zhao, Y. and Guo X. 2005, "Cytotoxicity of carbon nanomaterials: single-wall nanotube, multi-wall nanotube, and fullerene", *Environmental Science and Technology*, vol. 39, no. 5, pp. 1378-1383.
- Jin, C. Y., Zhu, B. S., Wang, X. F. and Lu, Q. H. 2008, "Cytotoxicity of titanium dioxide nanoparticles in mouse fibroblast cells", *Chemical Research in Toxicology*, vol. 21, no. 9, pp. 1871-1877.
- Johansen, A., Pedersen, A., Karlson, U., Hansen, B.M., Scott-Fordsmand, J. and Winding, A. 2008. Effects of C60 fullerene nanoparticles on soil bacteria and protozoans. *Environ.Toxicol.Chem*, 1:1. pp. 1.
- Kaegi, R., Ulrich, A., Sinnet, B., Vonbank, R., Wichser, A., Zuleeg, S., Simmler, H., Brunner, S., Vonmont, H., Burkhardt, M., Boller, M. 2008. Synthetic TiO₂ nanoparticle emission from exterior facades into the aquatic environment. *Environ. Pollut.* 156, 233–239.
- Kamat, J.P., Devasagayam, T.P.A., Priyadarsini, K.I. and Mohan, H. 2000, "Reactive oxygen species mediated membrane damage induced by fullerene derivatives and its possible biological implications", *Toxicology*, vol. 155, no. 1-3, pp. 55-61.

- Kang, S. J., Kim, B. M., Lee, Y. J. and Chung, H. W. 2008, "Titanium dioxide nanoparticles trigger p53-mediated damage response in peripheral blood lymphocytes", *Environmental and Molecular Mutagenesis*, vol. 49, no. 5, pp. 399-405.
- Kang, S., Mauter, M.S. and Elimelech, M. 2008. Physicochemical determinants of multiwalled carbon nanotube bacterial cytotoxicity. *Environ.Sci.Technol.*, vol. 42, no. 19, pp. 7528-7534.
- Kennedy, A.J., Hull, M.S., Steevens, J.A., Dontsova, K.M., Chappell, M.A., Gunter, J.C., et al. 2008. Factors influencing the partitioning and toxicity of nanotubes in the aquatic environment. *Environ Toxicol Chem* 27:1932-41.
- Kim YS, Kim JS, Cho HS, Rha DS, Park JD, Choi BS, Lim R, Chang HK, Chung YH, Kwon IH, Jeong J, Han BS, Yu IJ. 2008. Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats. *Inhal. Toxicol.* 20: 575–583.
- Kim, J. K., Lee, W. K., Lee, E. J., Cho, Y. J., Lee, K. H., Kim, H. S., Chung, Y., Kim, K. A. and Lim, Y. 1999, "Mechanism of silica- and titanium dioxide-induced cytotoxicity in alveolar macrophages", *Journal of Toxicology and Environmental Health Part A*, vol. 58, no. 7, pp. 437-450
- Kim, Y., Suh, H.S., Cha, H.J, Kim, S.H., Jeong, K.S., Kim, D.H. 2009, "A case of generalised argyria after ingestion of colloidal silver solution", *American Journal of Industrial Medicine*, vol. 52, no. 3, pp. 246-250.
- Kisin, E.R., Murray, A.R., Keane, M.J., Shi, X.C., Schwegler-Berry, D., Gorelik, O., Arepalli, S., Castranova, V., Wallace, W.E., Kagan, V.E. and Shvedova, A.A. 2007, "Single-walled carbon nanotubes: geno- and cytotoxic effects in lung fibroblast V79 cells", *Journal of Toxicology and Environmental Health Part A*, vol. 70, no. 24, pp. 2071-2079.
- Kiss, B., Biro, T., Czifra, G., Toth, B. I., Kertesz, Z., Szikszai, Z., Kiss, A. Z., Juhasz, I., Zouboulis, C. C. and Hunyadi, J. 2008, "Investigation of micronized titanium dioxide penetration in human skin xenografts and its effect on cellular functions of human skin-derived cells", *Experimental Dermatology*, vol. 17, no. 8, pp. 659-667
- Klimisch HJ, Andreae E and Tillmann U (1997). A systematic approach for evaluating the quality of experimental and ecotoxicological data. *Reg.Tox. and Pharm.* 25:1-5
- Komatsu, T., Tabata, M., Kubo-Irie, M., Shimizu, T., Suzuki, K.I., Nihei, Y. and Takeda K. 2008, "The effects of nanoparticles on mouse testis Leydig cells *in vitro*", *Toxicology in Vitro*, vol. 22, no. 8, pp. 1825–1831
- Koyama, S., Endo, M., Kim, Y-A., Hayashi, T., Yanagisawa, T., Osaka, K., Koyama, H., Hania, H. and Kuroiwa, N. 2006, "Role of systemic T-cells and histopathological aspects after subcutaneous implantation of various carbon nanotubes in mice", *Carbon*, vol. 44, no. 6, pp. 1079-1092.
- Lacerda, L., li-Boucetta, H., Herrero, M.A., Pastorin, G., Bianco, A., Prato, M. and Kostarelos, K. 2008, "Tissue histology and physiology following intravenous administration of different types of functionalised multiwalled carbon nanotubes", *Nanomedicine*, vol. 3, no. 2, pp.149-161.
- Lam, C.W., James, J.T., McCluskey, R, and Hunter, R.L.. 2004, "Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation" *Toxicological Sciences*, vol. 77, no. 1, pp. 126-134.
- Larese, F.F., D'Agostin, F., Crosera, M., Adami, G., Renzi, N., Bovenzi, M. and Maina, G. 2009, "Human skin penetration of silver nanoparticles through intact and damaged skin", *Toxicology*, vol. 255, no. 1-2, pp.33-37.
- L'azou, B., Jorly, J., On, D., Sellier, E., Moisan, F., Fleury-Feith, J., Cambar, J., Brochard, P. and Ohayon-Court, C. 2008, "*In vitro* effects of nanoparticles on renal cells", *Particle and Fibre Toxicology*, vol. 5, pp. 22-.36
- Lecoanet, H.F., Wiesner, M.R. 2004. Velocity effects on fullerene and oxide nanoparticle deposition in porous media. *Environ Sci Technol.* 38(16):4377-82.

- Lee, K.J., Nallathamby, P.D., Browning, L.M., Osgood, C.L. and Xu XHN. 2007, "*In vivo* imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. *ACS Nano*, vol. 1, no. 2, pp. 133–143
- Li, J.G., Li, W.X., Xu, J.Y., Cai, X.Q., Liu, R.L., Li, Y.J., Zhao, Q.F. and Li, Q.N. 2007a, "Comparative study of pathological lesions induced by multiwalled carbon nanotubes in lungs of mice by intratracheal instillation and inhalation", *Environmental Toxicology*, vol. 22, no. 4, pp. 415-421.
- Li, Z. Hulderman, T., Salmen, R., Chapman, R., Leonard, S.S., Young, S.H., Shvedova, A.A., Luster, M.I. and Simeonova, P.P. 2007b, "Cardiovascular effects of pulmonary exposure to single-wall carbon nanotubes", *Environmental Health Perspectives*, vol. 115, no. 3, pp. 377-382.
- Lin, A.M., Fang, S.F., Lin, S.Z., Chou, C.K., Luh, T.Y. and Ho, L.T. 2002, "Local carboxyfullerene protects cortical infarction in rat brain" *Neuroscience Research*, vol. 43, no. 4, pp. 317-321.
- Lin, D.H. and Xing, B.S. 2007. Phytotoxicity of nanoparticles: Inhibition of seed germination and root growth. *Environmental Pollution*, vol. 150, no. 2, pp. 243-250.
- Lin, D.H. and Xing, B.S. 2008. Root uptake and phytotoxicity of ZnO nanoparticles. *Environ.Sci.Technol.*, vol. 42, no. 15, pp. 5580-5585.
- Linkov, I., Satterstrom, F.K., Steevens, J., Ferguson, E., Pleus, R.C. 2007. Multi-criteria decision analysis and environmental risk assessment for nanomaterials. *Journal of Nanoparticle Research*, 9 543-554
- Linnainmaa, K., Kivipensas, P. and Vainio, H. 1997 "Toxicity and cytogenetic studies of ultrafine titanium dioxide in cultured rat liver epithelial cells", *Toxicology In Vitro*, vol. 11, no.4, pp. 329-335.
- Long, T. C., Saleh, N., Tilton, R. D., Lowry, G. V. and Veronesi, B. 2006, "Titanium dioxide (P25) produces reactive oxygen species in immortalized brain microglia (BV2): implications for nanoparticle neurotoxicity", *Environmental Science and Technology*, vol. 40, no. 14, pp. 4346-4352.
- Long, T. C., Tajuba, J., Sama, P., Saleh, N., Swartz, C., Parker, J., Hester, S., Lowry, G. V. and Veronesi, B. 2007, "Nanosize titanium dioxide stimulates reactive oxygen species in brain microglia and damages neurons *in vitro*", *Environmental Health Perspectives*, vol. 115, no. 11, pp. 1631-1637.
- Lovern, S.B., Klaper, R. 2006. *Daphnia magna* mortality when exposed to titanium dioxide and fullerene (C₆₀) nanoparticles. *Environ. Toxicol. Chem.*, vol. 25, pp. 1132-1137.
- Luoma, S. N. 2008, *Silver Nanotechnologies and The Environment: Old Problems or New Challenges*, The Pew Charitable Trusts and the Woodrow Wilson International Center for Scholars.
- Lyon, D.Y. and Alvarez, P.J.J. 2008. Fullerene water suspension (nC60) exerts antibacterial effects via ROS-independent protein oxidation. *Environ. Sci. Technol.* 42:8127-8132.
- Magrez, A., Kasas, S., Salicio, V., Pasquier, N., Seo, J.W., Celio, M., Catsicas, S., Schwaller, B. and Forro, L. 2006, "Cellular toxicity of carbon-based nanomaterials", *Nano Letters*, vol. 6, no. 6, pp. 1121-1125.
- Manna, S.K., Sarkar, S., Barr, J., Wise, K., Barrera, E.V., Jejelowo, O., Rice-Ficht, A.C. and Ramesh, G.T. 2005, "Single-walled carbon nanotube induces oxidative stress and activates nuclear transcription factor-kappaB in human keratinocytes", *Nano Letters*, vol. 5. no. 9, pp.1676-1684.
- Mavon, A., Miquel, C., Lejeune, O., Payre, B. and Moretto, P. 2007, "*In vitro* percutaneous absorption and *in vivo* stratum corneum distribution of an organic and a mineral sunscreen", *Skin Pharmacology and Physiology*, vol. 20, no.1, pp. 10-20.
- Maynard, A.D., Baron, P.A., Foley, M., Shvedova, A.A., Kisin, E.R. and Castranova V. 2004, "Exposure to carbon nanotube material: aerosol release during the handling of unrefined single-

walled carbon nanotube material", *Journal of Toxicology and Environmental Health Part A*, vol. 67, no. 1, pp. 87-107.

Methner, M.M., 2008, "Engineering case reports. Effectiveness of local exhaust ventilation (LEV) in controlling engineered nanomaterial emissions during reactor cleanout operations", *Journal of occupational environmental hygiene*, vol. 5, no. 66, pp. 63-69.

Mitchell, L.A., Gao, J., Wal, R.V., Gigliotti, A., Burchiel, S.W. and McDonald, J.D. 2007, "Pulmonary and systemic immune response to inhaled multiwalled carbon nanotubes", *Toxicological Sciences*, vol. 100, no. 1, pp. 203-214.

Monteiro-Riviere, N. and Inman, A.O. 2006, "Challenges for assessing carbon nanomaterial toxicity to the skin", *Carbon*, vol. 44, no. 6, pp/ 1070-1078.

Monteiro-Riviere, N.A., Inman, A.O., Wang, Y.Y. and Nemanich, R.J. 2005, "Surfactant effects on carbon nanotube interactions with human keratinocytes", *Nanomedicine*, vol. 1, no. 4, pp.293-299.

Monteiro-Riviere, N.A., Nemanich, R.J., Inman, A.O., Wang, Y.Y., Riviere, J.E.. 2005, "Multi-walled carbon nanotube interactions with human epidermal keratinocytes", *Toxicology Letters*, vol. 155, no. 3, pp. 377-384.

Mori, T., Takada, H., Ito, S., Matsubayashi, K., Miwa, N. and Sawaguchi, T. 2006, "Preclinical studies on safety of fullerene upon acute oral administration and evaluation for no mutagenesis", *Toxicology*, vol. 225, no. 1, pp. 48-54.

Mortimer, M., Kasemets, K., Heinlaan, M., Kurvet, I. and Kahru, A. 2008. High throughput kinetic *Vibrio fischeri* bioluminescence inhibition assay for study of toxic effects of nanoparticles. *Toxicol.In.Vitro.*, vol. 22, no. 5, pp. 1412-1417.

Mossman, B.T., Kamp, D.W. and Weitzman, S.A. 1996, "Mechanisms of carcinogenesis and clinical features of asbestos-associated cancers", *Cancer Investigation*, vol. 14, no. 5, pp. 466-480.

Mouchet, F., Landois, P., Flahaut, E., Pinelli, E. and Gauthier, L. 2007. Assessment of the potential in vivo ecotoxicity of Double-Walled Carbon Nanotubes (DWNTs) in water, using the amphibian *Ambystoma mexicanum*. *Nanotoxicology*, vol. 1, no. 2, pp. 149-156.

Mouchet, F., Landois, P., Sarremejean, E., Bernard, G., Puech, P., Pinelli, E., Flahaut, E. and Gauthier, L. 2008. Characterisation and in vivo ecotoxicity evaluation of double-wall carbon nanotubes in larvae of the amphibian *Xenopus laevis*. *Aquatic Toxicology*, vol. 87, no. 2, pp. 127-137.

Mueller, N.C. and Nowack, B. 2008, "Exposure modeling of engineered nanoparticles in the environment" *Environ.Sci.Technol.*, vol. 42, no. 12, pp. 4447-4453.

Muller, J., Decordier, I., Hoet, P.H., Lombaert, N., Thomassen, L., Huaux, F., Lison, D. and Kirsch-Volders, M. 2008, "Clastogenic and aneugenic effects of multi-wall carbon nanotubes in epithelial cells", *Carcinogenesis*, vol. 29, no. 2, pp. 427-433.

Muller, J., Delos, M., Panin, N., Rabolli, V., Huaux, F. and Lison, D. 2005, "Absence of carcinogenic response to multiwall carbon nanotubes in a 2-year bioassay in the peritoneal cavity of the rat", *Toxicological Sciences*, vol. 110, no. 2, pp. 442-448.

Muller, J., Huaux, F., Moreau, N., Misson, P., Heilier, J.F., Delos, M., Arras, M., Fonseca, A., Nagy, J.B. and Lison, D. 2005, "Respiratory toxicity of multi-wall carbon nanotubes", *Toxicology and Applied Pharmacology*, vol. 207, no. 3, pp. 221-231.

Murr, L.E., Garza, K.M., Soto, K.F., Carrasco, A., Powell, T.G., Ramirez, D.A., Guerrero, P.A., Lopez, D.A. and Venzor, J. III. 2005, "Cytotoxicity assessment of some carbon nanotubes and related carbon nanoparticle aggregates and the implications for anthropogenic carbon nanotube aggregates in the environment", *International Journal of Environmental Research and Public Health*, vol. 2, no. 1, pp. 31-42.

Murray, A.R., Kisin, E., Leonard, S.S., Young, S.H., Kommineni, C., Kagan, V.E., Castranova, V. and Shvedova, A.A. 2009, "Oxidative stress and inflammatory response in dermal toxicity of single-walled carbon nanotubes", *Toxicology*, vol. 257. no. 3, pp. 161-171.

Navarro, E., Piccapietra, F., Wagner, B., Marconi, F., Kaegi, R., Odzak, N., Sigg, L. and Behra R. 2008. Toxicity of Silver Nanoparticles to *Chlamydomonas reinhardtii*, *Environ. Sci. Technol.*, vol. 42, no. 23, pp. 8959–8964.

Nielsen, G.D., Roursgaard, M., Jensen, K.A., Poulsen, S.S, and Larsen, S.T. 2008, "In vivo biology and toxicology of fullerenes and their derivatives", *Basic and Clinical Pharmacology and Toxicology*, vol. 103, no. 3, pp. 197-208.

NIOSH (National Institute for Occupational Safety and Health) 2005. NIOSH Current Intelligence bulletin: Evaluation of Health Hazard and Recommendations for Occupational Exposure to Titanium Dioxide. DRAFT. NIOSH.
Accessed at: <http://www.cdc.gov/niosh/review/public/TIO2/pdfs/TIO2Draft.pdf> (16th October 2009)

Nurkiewicz, T. R., Porter, D. W., Barger, M., Millecchia, L., Rao, K. M., Marvar, P. J., Hubbs, A. F., Castranova, V. and Boegehold, M. A. 2006, "Systemic microvascular dysfunction and inflammation after pulmonary particulate matter exposure", *Environmental Health Perspectives*, vol. 114, no.3, pp. 412-419.

Oberdorster, E., Zhu, S.Q., Blickley, T.M., McClellan-Green, P. and Haasch, M.L. 2006. Ecotoxicology of carbon-based engineered nanoparticles: Effects of fullerene (C-60) on aquatic organisms. *Carbon*, vol. 44, no. 6, pp. 1112-1120.

Pacurari, M., Yin, X.J., Zhao, J., Ding, M., Leonard, S.S., Schwegler-Berry, D., Ducatman, B.S., Sbarra, D., Hoover, M.D., Castranova, V. and Vallyathan V. 2008, "Raw single-wall carbon nanotubes induce oxidative stress and activate MAPKs, AP-1, NF-kappaB, and Akt in normal and malignant human mesothelial cells", *Environmental Health Perspectives*, vol. 116. no. 9, pp. 1211-1217.

Paik, S.Y., Zalk, D.M., Swuste, P. 2008. Application of a pilot control banding tool for risk level assessment and control of nanoparticle exposure. *Ann. Occ. Hyg.*, 52 (6), 419-428.

Park, E. J., Yi, J., Chung, K. H., Ryu, D. Y., Choi, J., and Park, K. 2008, "Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells", *Toxicology Letters*, vol. 180, no. 3, pp. 222-229.

Peters, K., Unger, R. E., Kirkpatrick, C. J., Gatti, A. M. and Monari, E. 2004, "Effects of nano-scaled particles on endothelial cell function *in vitro*: studies on viability, proliferation and inflammation", *Journal of Materials in Science. Materials in Medicine*, vol. 15, no. 4, pp. 321-325.

Petersen, E.J., Huang, Q.G. and Weber, W.J. 2008a. Bioaccumulation of radio-labeled carbon nanotubes by *Eisenia foetida*. *Environ.Sci.Technol.*, vol. 42, no. 8, pp. 3090-3095.

Petersen, E.J., Huang, Q.G. and Weber, W.J. 2008b. Ecological uptake and depuration of carbon nanotubes by *Lumbriculus variegates*. *Environ.Health Perspect.*, vol. 116, no. 4, pp. 496-500.

Pflucker, F., Hohenberg, H., Holzle, E., Will, T., Pfeiffer, S., Wepf, R., Diembeck, W., Wenck, H. and Gers-Barlag, H. 1999, "The outermost stratum corneum layer is an effective barrier against dermal uptake of topically applied micronized titanium dioxide", *International Journal of Cosmetic Science*, vol. 21, no.6, pp. 399-411.

Poland, C.A., Duffin, R., Kinloch, I.A., Maynard, A., Wallace, W.A.H., Seaton, A., Stone, V., Brown, S., MacNee, W. and Donaldson, K. 2008, "Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study", *Nature Nanotechnology*, vol, 3, no.7, pp. 423-428.

Radomski, A., Jurasz, P., Alonso-Escolano, D., Drews, M., Morandi, M., Malinski, T. and Radomski, M.W. 2005, "Nanoparticle-induced platelet aggregation and vascular thrombosis", *British Journal of Pharmacology*, vol. 146, no.6, pp. 882-893.

- Renwick, L. C., Brown, D., Clouter, A. and Donaldson, K. 2004, "Increased inflammation and altered macrophage chemotactic responses caused by two ultrafine particle types", *Occupational and Environmental Medicine*, vol. 61, no.5, pp. 442-447.
- Renwick, L. C., Donaldson, K. and Clouter, A. 2001, "Impairment of alveolar macrophage phagocytosis by ultrafine particles", *Toxicology and Applied Pharmacology*, vol. 172, no. 2, pp. 119-127.
- Roberts, A.P., Mount, A.S., Seda, B., Souther, J., Qiao, R., Lin, S.J., Ke, P.C., Rao, A.M. and Klaine, S.J. 2007. In vivo biomodification of lipid-coated carbon nanotubes by *Daphnia magna*. *Environ.Sci.Technol.*, vol. 41, no. 8, pp. 3025-3029.
- Roberts, J.E., Wielgus, A.R., Boyes, W.K., Andley, U. and Chignell, C.F. 2008, "Phototoxicity and cytotoxicity of fullerol in human lens epithelial cells", *Toxicology and Applied Pharmacology*, vol. 228, no 1, pp. 49-58.
- Rotoli B.M., Bussolati O., Bianchi M.G., Barilli A., Balasubramanian C., Bellucci, S. and Bergamaschi, E. 2008, "Non-functionalised multi-walled carbon nanotubes alter the paracellular permeability of human airway epithelial cells", *Toxicology Letters*, vol. 178, no. 2, pp. 95-102.
- Roursgaard, M., Poulsen, S.S., Kepley, C.L., Hammer, M., Nielsen, G.D. and Larsen, S.T. 2008, "Polyhydroxylated C₆₀ fullerene (fullerenol) attenuates neutrophilic lung inflammation in mice", *Basic and Clinical Pharmacology and Toxicology*, vol. 103, no. 4, pp. 386-388.
- Rouse, J.G., Yang, J., Barron, A.R. and Monteiro-Riviere, N.A. 2006, "Fullerene-based amino acid nanoparticle interactions with human epidermal keratinocytes", *Toxicology In Vitro*, vol. 20, no. 8, pp. 1313-1320.
- Rouse, J.G., Yang, J., Ryman-Rasmussen, J.P., Barron, A.R. and Monteiro-Riviere, N.A. 2007, "Effects of mechanical flexion on the penetration of fullerene amino acid-derivatized peptide nanoparticles through skin", *Nano Letters*, vol. 7, no. 1, pp. 155-160.
- Sakamoto, Y., Nakae, D., Fukumori, N., Tayama, K., Maekawa A., Imai, K., Hirose, A., Nishimura, T., Ohashi N. and Ogata, A. 2009, "Induction of mesothelioma by a single intrascrotal administration of multi-wall carbon nanotube in intact male Fischer 344 rats", *The Journal of Toxicological Sciences*, vol. 34, no. 1, pp.65-76.
- Sato, Y., Yokoyama, A., Shibata, K., Akimoto, Y., Ogino, S., Nodasaka, Y., Kohgo, T., Tamura, K., Akasaka, T., Uo, M., Motomiya, K., Jeyadevan, B., Ishiguro, M., Hatakeyama, R., Watari, F. and Tohji, K. 2005, "Influence of length on cytotoxicity of multi-walled carbon nanotubes against human acute monocytic leukemia cell line THP-1 *in vitro* and subcutaneous tissue of rats *in vivo*", *Molecular Biosystems*, vol. 1, no. 2, pp.176-182.
- Sayes, C.M., Fortner, J.D., Guo, W., Lyon, D.Y., Boyd, A.M., Ausman, K., Tao, Y.J., Sitharaman, B., Wilson, L.J., Hughes, J.B., West, J.L. and Colvin, V.L. 2004, "The differential cytotoxicity of water-soluble fullerenes", *Nano Letters*, vol. 4, no. 10, pp. 1881-1887.
- Sayes, C.M., Gobin, A.M., Ausman, K.D., Mendez, J, West, J,L. and Colvin, V.L. 2005, "Nano-C₆₀ cytotoxicity is due to lipid peroxidation", *Biomaterials*, vol. 26, no. 36, pp. 7587-7595.
- Sayes, C.M., Marchione, A.A., Reed, K.L. and Warheit D.B. 2007, "Comparative pulmonary toxicity assessments of C₆₀ water suspensions in rats: few differences in fullerene toxicity *in vivo* in contrast to *in vitro* profiles", *Nano Letters*, vol. 7, no. 8, pp. 2399-2406.
- SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks). 2007. The appropriateness of the risk assessment methodology in accordance with the technical guidance documents for new and existing substances for assessing the risk of nanomaterials, *Scientific Committee on Emerging and Newly Identified Health Risks*, 21-22 June 2007.
- SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks). 2009. Risk Assessment of Products of Nanotechnologies, *Scientific Committee on Emerging and Newly Identified Health Risks*, 19 January 2009.

- Schulz, J., Hohenberg, H., Pflucker, F., Gartner, E., Will, T., Pfeiffer, S., Wepf, R., Wendel, V., Gers-Barlag, H. and Wittern, K. P. 2002, "Distribution of sunscreens on skin", *Advanced Drug Delivery Reviews*, vol. 54, no. 1, pp. S157-S163.
- Scott-Fordsmand, J.J., Krogh, P.H., Schaefer, M. and Johansen, A. 2008. The toxicity testing of double-walled nanotubes-contaminated food to *Eisenia veneta* earthworms. *Ecotoxicol. Environ. Saf.*, vol. 71, no. 3, pp. 616-619.
- Scrivens, W.A., Tour, J.M., Creek, K.E. and Pirisi, L. 1994, "Synthesis of ¹⁴C-labelled C₆₀, its suspension in water, and its uptake by human keratinocytes", *Journal of the American Chemical Society*, vol. 116, no.10, pp. 4517-4518.
- Seagrave J.C., McDonald, Gigliotti A.P., Nikula, K.J., Seilkop, S.K, Gurevich, M. and Mauderly, J.L. 2002, "Mutagenicity and *in vivo* toxicity of combined particulate and semivolatile organic fractions of gasoline and diesel engine emissions", *Toxicol. Sci*, vol. 70 no. 2, pp. 1212-226.
- Semmler-Behnke, M., Kreyling, W.G., Lipka, J., Fertsch, S., Wenk, A., Takenaka, S., Schmid, G. and Brandau, W. 2008, "Biodistribution of 1.4- and 18-nm Gold Particles in Rats", *Small*, vol. 4, no. 12, pp. 2108-2111.
- Sera, N., Tokiwa, H. and Miyata, N. 1996, "Mutagenicity of the fullerene C₆₀-generated singlet oxygen dependent formation of lipid peroxides", *Carcinogenesis*, vol. 17, no. 10, pp. 2163-2169.
- Shay, D.K., Holman, R.C., Newman, R.D., Liu, L.L., Stout, J.W. and Anderson, L.J. 1999, "Bronchiolitis-associated hospitalizations among US children, 1980-1996", *JAMA*, vol. 282 no. 2, pp. 1440-1446.
- Shvedova, A.A., Castranova, V., Kisin, E.R., Schwegler-Berry, D., Murray, A.R., Gandelsman, V.Z., Maynard, A., and Baron, P. 2003, "Exposure to carbon nanotube material: assessment of nanotube cytotoxicity using human keratinocyte cells", *Journal of Toxicology and Environmental Health Part A*, vol. 66, no. 20, pp. 1909-1926.
- Shvedova, A.A., Kisin, E., Murray, A.R., Johnson, V.J., Gorelik, O., Arepalli, S., Hubbs, A.F., Mercer, R.R., Keohavong, P., Sussman, N., Jin, J., Yin, J., Stone, S., Chen, B.T., Deye, G., Maynard, A., Castranova, V., Baron, P.A. and Kagan, V.E. 2008a, "Inhalation vs. aspiration of single-walled carbon nanotubes in C57BL/6 mice: inflammation, fibrosis, oxidative stress, and mutagenesis", *American Journal of Physiology- Lung Cellular and Molecular Physiology*, vol. 295, no. 4, pp. L552-L565.
- Shvedova, A.A., Kisin, E.R., Mercer, R., Murray, A.R., Johnson, V.J., Potapovich, A.I., Tyurina, Y.Y., Gorelik, O., Arepalli, S., Schwegler-Berry, D., Hubbs, A.F., Antonini, J., Evans, D.E., Ku, B.K., Ramsey, D., Maynard, A., Kagan, V.E., Castranova, V. and Baron, P.. 2005, "Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice", *American Journal of Physiology- Lung Cellular and Molecular Physiology*, vol. 289, no. 5, pp. L698-L708.
- Shvedova, A.A., Kisin, E.R., Murray, A.R., Gorelik, O., Arepalli, S., Castranova, V., Young, S.H., Gao, F., Tyurina, Y.Y., Oury, T.D. and Kagan, V.E.. 2007, "Vitamin E deficiency enhances pulmonary inflammatory response and oxidative stress induced by single-walled carbon nanotubes in C57BL/6 mice", *Toxicology and Applied Pharmacology*, vol. 221, no. 3, pp. 339-348.
- Simon-Deckers, A., Gouget, B., Mayne-L'hermite, M., Herlin-Boime, N., Reynaud, C. and Carriere, M. 2008, "*In vitro* investigation of oxide nanoparticle and carbon nanotube toxicity and intracellular accumulation in A549 human pneumocytes", *Toxicology*, vol. 253, no. 1-3 pp. 137-146.
- Singh, R., Pantarotto, D., Lacerda, L., Pastorin, G., Klumpp, C., Prato, M., Bianco, A. and Kostarelos, K. 2006, "Tissue biodistribution and blood clearance rates of intravenously administered carbon nanotube radiotracers", *Proceedings of the National Academy of Science USA*, vol. 103, no. 9, pp. 3357-3362.
- Smith, C.J., Shaw, B.J. and Handy, R.D. 2007. Toxicity of single walled carbon nanotubes to rainbow trout, (*Oncorhynchus mykiss*): Respiratory toxicity, organ pathologies, and other physiological effects. *Aquatic Toxicology*, vol. 82, no. 2, pp. 94-109.

Sonavane, G., Tomoda, K. and Makino, K. 2008b, "Biodistribution of colloidal gold nanoparticles after intravenous administration: Effect of particle size", *Colloids and Surfaces. B Biointerfaces*, vol. 66, no. 2, pp. 274-280 .

Sonavane, G., Tomoda, K., Sano, A., Ohshima, H., Terada, H. and Makino, K. 2008a, "*In vitro* permeation of gold nanoparticles through rat skin and rat intestine: effect of particle size", *Colloids and Surfaces. B Biointerfaces*, vol. 65, no. 1, pp. 1-10.

Soto, K., Garza, K.M. and Murr, L.E. 2007, "Cytotoxic effects of aggregated nanomaterials *Acta Biomaterialia*. vol. 3, no. 3, pp. 351-358.

Sun, H.W., Zhang, X.Z., Niu, Q., Chen, Y.S. and Crittenden, J.C. 2007. Enhanced accumulation of arsenate in carp in the presence of titanium dioxide nanoparticles. *Water Air and Soil Pollution*, vol. 178, no. 1-4, pp. 245-254.

Sung, J.H., Ji, J.H., Park, J.D., Yoon, J.U., Kim, D.S., Jeon, K.S., Song, M.Y., Jeong, J., Han, B.S., Han, J.H., Chung, Y.H., Chang, H.K., Lee, J.H., Cho, M.H., Kelman, B.J. and Yu, I.J. 2009, "Subchronic inhalation toxicity of silver nanoparticles", *Toxicological Sciences*, vol. 108, no.2, 452-461.

Tabata, Y., Murakami, Y. and Ikada, Y. 1997, "Photodynamic effect of polyethylene glycol-modified fullerene on tumour", *Japan Journal of Cancer Research*, vol. 88, no. 11, pp. 1108-1116.

Takagi, A., Hirose, A., Nishimura, T., Fukumori, N., Ogata, A., Ohashi, N., Kitajima, S. and Kanno, J. 2008, "Induction of mesothelioma in p53[±] mouse by intraperitoneal application of multi-wall carbon nanotube", *Journal of Toxicological Science*, vol. 33, no. 1, pp.105-116.

Takenaka, S., Karg, E., Roth, C., Schulz, H., Ziesenis, A., Heinzmann, U., Schramel, P., and Heyder, J. 2001, "Pulmonary and systemic distribution of inhaled ultrafine silver particles in rats", *Environ.Health Perspect.*, vol. 109 Suppl 4, pp. 547-551.

Templeton, R.C., Ferguson, P.L., Washburn, K.M., Scrivens, W.A. and Chandler, G.T. 2006. Life-cycle effects of single-walled carbon nanotubes (SWNTs) on an estuarine meiobenthic copepod. *Environ.Sci.Technol.*, vol. 40, no. 23, pp. 7387-7393.

Tong, Z., Bischoff, M., Nies, L., Applegate, B. and Turco, R.F. 2007. Impact of fullerene (C₆₀) on a soil microbial community. *Environ.Sci.Technol.*, vol. 41, no. 8, pp. 2985-2991.

Torchilin V. P. (Editor). 2006, "Nanoparticulated as drug carriers", *Imperial College Press, ISBN 1-86094-630-5*. Chapter 14: Drug nanocrystals/nanosuspensions for the delivery of poorly soluble drugs.

Trimble, T.A., You, J., Lydy, M.J. 2008. Bioavailability of PCBs from field-collected sediments: application of Tenax extraction and matrix-SPME techniques. *Chemosphere*, 71, 337-344.

Trop, M., Novak, M., Rodl, S., Hellbom, B., Kroell, W., Goessler, W., 2006, "Silver coated dressing Acticoat caused raised liver enzymes and argyria-like symptoms in burn patient", *The Journal of Trauma*, 60 (3), 648-652.

Tsai, S., Ada, E., Isaacs, J.A. and Ellenbecker, M.J. 2009, "Airborne nanoparticle exposures associated with the manual handling of nanoalumina and nanosilver in fume hoods", *Journal of Nanoparticle Research*, vol. 11, pp. 147-161.

Tsuchiya, T., Oguri, I., Yamakoshi, Y.N. and Miyata, N. 1996, "Novel harmful effects of [60]fullerene on mouse embryos *in vitro* and *in vivo*", *FEBS Letters*, vol. 393, no. 1, pp. 139-145.

Usenko, C.Y., Harper, S.L. and Tanguay, R.L. 2008, "Fullerene C₆₀ exposure elicits an oxidative stress response in embryonic zebrafish", *Toxicology and Applied Pharmacology*, vol. 229, no. 1, pp. 44-55.

Velzeboer, I., Hendriks, A.J., Ragas, A.M.J. and Van de Meent, D. 2008. Aquatic ecotoxicity tests of some nanomaterials. *Environmental Toxicology and Chemistry*, vol. 27, no. 9, pp. 1942-1947.

Engineered Nanoparticles: Review of Health and Environmental Safety

Vevers, W.F. and Jha, A.N. 2008. Genotoxic and cytotoxic potential of titanium dioxide (TiO₂) nanoparticles on fish cells in vitro. *Ecotoxicology*, vol. 17, no. 5, pp. 410-420.

Vlachou, E., Chipp, E., Shale, E., Wilson, Y.T., Papini, R. and Moiemmen, N.S. 2007, "The safety of nanocrystalline silver dressings on burns: A study of systemic silver absorption", *Burns*, vol. 33, no. 8, pp. 979-985.

Wadhwa, A., and Fung, M. (2005). Systemic argyria associated with ingestion of colloidal silver. *Dermatology Online Journal*, **11**(1): 12.

Wang J., Zhang X., Chen Y., Sommerfield M. and Hu, Q. 2008. Toxicity assessment of manufactured nanomaterials using the unicellular green alga *Chlamydomonas reinhardtii*. *Chemosphere*, vol. 73, no. 7.

Wang, H., Wang, J., Deng, X., Sun, H., Shi, Z., Gu, Z., Liu, Y. and Zhao, Y. 2004, "Biodistribution of carbon single-walled nanotubes in mice", *Journal of Nanoscience and Nanotechnology*, vol. 4, no.8, pp. 1019-1024.

Wang, I.C., Tai, L.A., Lee, D.D., Kanakamma, P.P., Shen, C.K., Luh, T.Y., Cheng, C.H. and Hwang, K.C. 1999, "C(60) and water-soluble fullerene derivatives as antioxidants against radical-initiated lipid peroxidation", *Journal of Medicinal Chemistry*, vol. 42, no. 22, pp. 4614-4620.

Wang, J., Chen, C., Liu, Y., Jiao, F., Li, W., Lao, F., Li, Y., Li, B., Ge, C., Zhou, G., Gao, Y., Zhao, Y. and Chai, Z. 2008a, "Potential neurological lesion after nasal instillation of TiO₂ nanoparticles in the anatase and rutile crystal phases", *Toxicology Letters*, vol. 183, no. 1-3, pp. 72-80.

Wang, J., Liu, Y., Jiao, F., Lao, F., Li, W., Gu, Y., Li, Y., Ge, C., Zhou, G., Li, B., Zhao, Y., Chai, Z. and Chen, C. 2008b, "Time-dependent translocation and potential impairment on central nervous system by intranasally instilled TiO₂ nanoparticles", *Toxicology*, vol. 254, no. 1-2, pp. 82-90.

Wang, J., Zhou, G., Chen, C., Yu, H., Wang, T., Ma, Y., Jia, G., Gao, Y., Li, B., Sun, J., Li, Y., Jiao, F., Zhao, Y. and Chai, Z. 2007, "Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration", *Toxicology Letters*, vol. 168, no. 2, pp. 176-185.

Warheit, D. B., Webb, T. R., Reed, K. L., Frerichs, S. and Sayes, C. M. 2007, "Pulmonary toxicity study in rats with three forms of ultrafine-TiO₂ particles: differential responses related to surface properties. *Toxicology*, vol. 230, no.1, pp. 90-104.

Warheit, D.B., Laurence, B.R., Reed, K.L., Roach, D.H., Reynolds, G.A. and Webb, T.R. 2004, "Comparative pulmonary toxicity assessment of single-wall carbon nanotubes in rats", *Toxicological Sciences*, vol. 77, no. 1, pp. 117-125.

Wick, P., Manser, P., Limbach, L.K., Dettlaff-Weglikowska, U., Krumeich, F., Roth, S., Stark, W.J. and Bruinink, A. 2007, "The degree and kind of agglomeration affect carbon nanotube cytotoxicity", *Toxicological Letters*, vol. 168, no. 2, pp. 121-131.

Witzmann, F.A. and Monteiro-Riviere, N.A. 2006, "Multi-walled carbon nanotube exposure alters protein expression in human keratinocytes", *Nanomedicine*, vol. 2, no. 3, pp.158-168.

Wiwanitkit. V., Sereemasapun A. and Rojanathanes R. 2009, "Effect of gold nanoparticles on spermatozoa", *Fertility and Sterility*, vol. 91, no.1, pp.7-8

World Health Organization (WHO). (1997). Determination of airborne fibre number concentrations. A recommended method, by phase-contrast optical microscopy (membrane filter method). Geneva

Xia, T., Kovochich, M., Brant, J., Hotze, M., Sempf, J., Oberley, T., Sioutas, C., Yeh, J.I., Wiesner, M.R. and Nel, A.E. 2006, "Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm", *Nano Letters*, vol. 6, no. 8, pp. 1794-1807.

- Xiao, L., Takada, H., Gan, X., Miwa, N. 2006, "The water-soluble fullerene derivative "Radical Sponge" exerts cytoprotective action against UVA irradiation but not visible-light-catalyzed cytotoxicity in human skin keratinocytes", *Bioorganic and Medicinal Chemistry Letters*, vol. 16, no. 6, pp. 1590-1595.
- Xie, B., Zhihua, X., Wenhua, G., Oilin, L. 2008. Impact of Natural Organic Matter on the Physicochemical Properties of Aqueous C60 Nanoparticles. *Environmental Science and Technology* 42 (8), 2853-2859
- Yamago, S., Tokuyama, H., Nakamura, E., Kikuchi, K., Kananishi, S., Sueki, K., Nakahara, H., Enomoto, S. and Ambe F. 1995, "In vivo biological behaviour of a water-miscible fullerene: ¹⁴C labelling, absorption, distribution, excretion and acute toxicity", *Chemistry and Biology*, vol. 2, no.6, pp. 385-389.
- Yamawaki, H. and Iwai, N. 2006, "Cytotoxicity of water-soluble fullerene in vascular endothelial cells" *American Journal of Physiology-Cell Physiology*, vol. 290, no. 6, pp. C1495-C1502.
- Yang, F., Liu, C., Gao, F., Su, M., Wu, X., Zheng, L., Hong, F. and Yang, P. 2007. The improvement of spinach growth by nano-anatase TiO₂ treatment is related to nitrogen photoreduction. *Biol.Trace Elem.Res.*, vol. 119, no. 1, pp. 77-88.
- Yang, S.T., Guo, W., Lin, Y., Deng, X-Y., Wang, H-F., Sun, H-F., Liu, X-F., Wang, X., Chen, M., Huang, Y-P. and Sun, Y-P. 2007, "Biodistribution of pristine single-walled carbon nanotubes *in vivo*", *Journal of Physical Chemistry C*, vol. 111, no. 48, pp. 17761-17764.
- Yang, S.T., Wang, X., Jia, G., Gu, Y., Wang, T., Nie, H., Ge, C., Wang, H. and Liu Y. 2008, "Long-term accumulation and low toxicity of single-walled carbon nanotubes in intravenously exposed mice", *Toxicology Letters*, vol. 181, no. 3, pp. 182-189.
- Ye, S.F., Wu, Y.H., Hou, Z.Q. and Zhang, Q.Q. 2009, "ROS and NF-kappaB are involved in upregulation of IL-8 in A549 cells exposed to multi-walled carbon nanotubes", *Biochemical and Biophysical Research Communications*, vol. 379, no. 2, pp. 643-648.
- Yeganeh, B., Kull, C.M., Hull, M.S. and Marr, L.C. 2008, "Characterization of airborne particles during production of carbonaceous nanomaterials", *Environmental science and technology*, vol. 42, no. 12, pp. 4600-4606.
- Yeo, M.K. and Kang, M. 2008. Effects of nanometer sized silver materials on biological toxicity during zebrafish embryogenesis. *Bulletin of the Korean Chemical Society*, vol. 29, no. 6, pp. 1179-1184.
- Yin, J.J., Lao, F., Meng, J., Fu, P.P., Zhao, Y., Xing, G., Gao, X., Sun, B., Wang, P.C., Chen, C. and Liang, X.J. 2008, "Inhibition of tumor growth by endohedral metallofullerenol nanoparticles optimized as reactive oxygen species scavenger", *Molecular Pharmacology*, vol. 74, no. 4, pp. 1132-1140.
- Yin, J-J., Lao, F., Fu, P.P., Wamer, W.G., Zhao, Y., Wang, P.C., Qiu, Y., Sun, B., Xing, G., Dong, J., Liang, X-J. and Chen, C. 2009, "The scavenging of reactive oxygen species and the potential for cell protection by functionalised fullerene materials", *Biomaterials*, vol. 30, no.4, pp. 611-612.
- Yokoyama, A., Sato, Y., Nodasaka, Y., Yamamoto, S., Kawasaki, T., Shindoh, M., Kohgo, T., Akasaka, T., Uo, M., Watari, F., and Tohji, K. 2005, "Biological behavior of hat-stacked carbon nanofibers in the subcutaneous tissue in rats", *Nano Letters*, vol. 5, no. 1, pp. 157-161.
- Zhang, L.W., Zeng, L., Barron, A.R. and Monteiro-Riviere, N.A. 2007, "Biological interactions of functionalised single-wall carbon nanotubes in human epidermal keratinocytes", *International Journal of Toxicology*, vol, 26, no. 2, pp. 103-113.
- Zhang, X., Sun, H., Zhang, Z., Niu, Q., Chen, Y., Crittenden, J.C. 2007. Enhanced bioaccumulation of cadmium in carp in the presence of titanium dioxide nanoparticles. *Chemosphere*, vol. 67, pp. 160-166.
- Zheng, L., Hong, F.S., Lu, S.P. and Liu, C. 2005. Effect of nano-TiO₂ on strength of naturally and growth aged seeds of spinach. *Biol.Trace Elem.Res.*, vol. 104, no. 1, pp. 83-91.

Engineered Nanoparticles: Review of Health and Environmental Safety

Zhu, J.T., He, J., Chen, J.Y., Lu, D.R. and Zhou, L.W. 2008, "Fast differential interference contrast imaging combined with autocorrelation treatments to measure the heart rate of embryonic fish" *Journal of Biomedical Optics*, vol. 13, no. 2, pp. 020503.

Zhu, L., Chang, D.W., Dai, L. and Hong, Y. 2007, "DNA damage induced by multiwalled carbon nanotubes in mouse embryonic stem cells", *Nano Letters*, vol. 7, no. 12, pp. 3592-3597.

Zhu, S.Q., Oberdorster, E. and Haasch, M.L. 2006a. Toxicity of an engineered nanoparticle (fullerene, C-60) in two aquatic species, *Daphnia* and fathead minnow. *Mar. Environ. Res.*, vol. 62, pp. S5-S9.

Zhu, X.S., Zhu, L., Duan, Z.H., Qi, R.Q., Li, Y. and Lang, Y.P. 2008a. Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to Zebrafish (*Danio rerio*) early developmental stage. *Journal of Environmental Science and Health Part A-Toxic/hazardous Substances and Environmental Engineering*, vol. 43, no. 3, pp. 278-284.

Zhu, X.S., Zhu, L., Lang, Y.P. and Chen, Y.S. 2008b. Oxidative stress and growth inhibition in the freshwater fish *Carassius auratus* induced by chronic exposure to sublethal fullerene aggregates. *Environmental Toxicology and Chemistry*, vol. 27, no. 9, pp. 1979-1985.

Zhu, Y., Zhao, Q.F., Li, Y.G., Cai, X.Q. and Li, W. 2006b. The interaction and toxicity of multi-walled carbon nanotubes with *Styloynchia mytilus*. *Journal of Nanoscience and Nanotechnology*, vol. 6, no. 5, pp. 1357-1364.

10 SUMMARY OF CONCLUSIONS AND RECOMMENDATIONS

10.1 INTRODUCTION

The ENRHES report consists of a series of reviews based upon four nanomaterial types, fullerenes, carbon nanotubes, metals and metal oxides. For each material the report summarises state of the art knowledge with respect to potential exposures, human toxicology and environmental toxicology. This information has been summarised and assimilated in order to generate a risk assessment for each classification. The following sections provide an overview of the major conclusions generated from this exercise, along with recommendations generated, often due to gaps in the existing knowledge which prevented a complete and thorough risk assessment from being conducted.

10.2 MATERIALS PRODUCTION AND USE

Current data suggests that in 2007 the fullerenes market was worth \$58.5 million and the market for carbon nanotubes was approximately \$168.5 million in 2008. The market for nanoparticles as a whole was worth around \$1.6 billion in 2007. This market is anticipated to expand further since many nanoscale materials are currently being produced solely at the laboratory scale with further work required for scale-up before many nanomaterial manufacturing techniques and applications can be fully commercialised. For example, carbon nanotubes have a wide range of potential applications, however high volume manufacture for this nanomaterial is still to be realised by many companies.

Once such nanomaterials can be generated in sufficient volumes, there is also a need to integrate the nanomaterial into products or applications, and at present there is a lack of fundamental knowledge regarding the ability to process such nanomaterials. There are also technological challenges in the areas of molecular manufacturing, quality assurance and the eventual programmability of nanodevices.

In terms of developing from raw nanomaterials to usable systems/devices, issues include the stability and durability of materials and reproducibility of the system for mass production. It is necessary to improve both the synthetic control in producing nanomaterials for applications and to improve selectivity in their mode of action. In terms of up scaling to industrial economic processing, the costs of production, when developed, also need to be kept low. Price and cost of the end unit is driven by efficient and effective cheap synthetic chemistry that can be scaled to industrial production. Validation of reliability is also important as this gives a "usable" end product. However there is a problem in correlating limited testing with assurance of total quality. Breakthroughs are expected in uniformity of composition and self assembled systems compositional purity, this relates to better and more consistent end-product performance.

As part of the ENRHES review, a survey was carried out to determine the quantities of various types of nanomaterials produced and used, the type of products in which they are used, any exposure data gathered and risk assessment practices employed. The survey was open from 04/02/2009 – 06/03/2009 and announcements and invitations to participate were sent to industry associations including CEFIC, the CIA, the NIA, ENTA, the Institute of Nanotechnology's database and its media contacts. However many of the responses were incomplete and missing vital data for use. Only 13 survey responses were deemed to have relevant information. The survey data was provided primarily from commercial organisations with less than 250 employees, from a cross section of business areas. The industry associations had suggested that the survey was very similar to the UK's voluntary reporting scheme and that many companies were hesitant to provide information. There was concern that at least some of the information requested was company confidential and so the information received was limited, and was based on products of limited commercial quantity and value. The survey also highlighted that many companies do not describe their products generally as nano, preferring often to describe them as ultrafine.

The survey data does not provide a complete overview of nanomaterial production and use worldwide. Although the survey suggests that CNT dominate the market, this does not reflect the actual predominance of silica and carbon black products currently in the market place.

10.3 CHARACTERISATION

Effort is being made towards improving the characterisation basis for toxicological studies, such as identifying the key physico-chemical characteristics of nanoparticles and how they can be measured. It is important to emphasise that multiple techniques should be used wherever possible to develop a more complete understanding of particle characteristics. This is particularly important with respect to particle size and dispersion.

The review of Powers *et al.* (2006), amongst others, concluded that a consensus may be emerging about the importance of characterisation. The body of literature published since confirms that there is now a consensus that thorough and accurate particle characterisation is an essential part of assessing the potential toxicity of nanoparticles in biological systems. Appropriate and common characterisation of test materials is important to ensure that results are reproducible, and also to provide the basis for understanding the properties of nanoparticles that determine their biological effects. Some of the key parameters influencing the biological activity of nanoparticles remain unknown or to be fully understood at this point. Hence, the characterisation of test materials should be as comprehensive as possible and broad in scope. A study conducted with a material that has not been characterised with respect to a property later found to be critical for toxicity will ultimately be of little value.

Thorough characterisation of test materials is time consuming, expensive, complex and may never be fully available. To some extent, the characterisation required depends on the objectives of the study. However, there are a number of fundamental properties that researchers in the field generally agree must be addressed. This subset forms the basis of a minimum set of characteristics that should be measured for test materials used in toxicity studies. In addition to composition, these include size and shape, state of dispersion, surface area, and surface chemistry.

10.4 EXPOSURE

In general, there is a paucity of published data concerning exposure to nanoparticles. Various types of studies have been used in an attempt to provide the maximum information available.

There are a few studies which have speculated on possible or plausible exposure scenarios. While these studies are useful in mapping the exposure landscape, they do not in themselves lead to numerical estimates of exposure. Exposures are clearly plausible in occupational, consumer and environmental settings throughout the lifecycle of materials and products.

We have also considered modelling studies and have identified two which provide useful information relating to environmental and consumer exposure.

For the materials of interest we have identified only 11 studies which have reported measured exposure data. All of these are in the occupational setting, while no studies have reported consumer exposures or exposures in the environment. All but one of the studies have reported inhalation exposure only; one study reported dermal exposure and no studies reported ingestion exposure. Most of the studies were carried out in university settings, however, some industrial settings were also found. A wide range of instruments and approaches were used and exposures were reported in terms of number, mass and surface area concentrations, as totals and differentiated as a function of size. Most studies showed some evidence of elevated exposures although these were often associated with ineffective or deliberately disabled control systems. Studies which have assessed the effectiveness of respiratory filters have shown that, as theory predicts, they are efficient collectors of nanoparticles.

In order for such companies to assess potential exposure of their workers to the manufactured nanomaterials, there is a requirement for devices to measure exposure such as airborne mass

concentration. At present there is a lack of systems for measuring primary particle sizes in real-time during the production process, and the lack of smart units which are capable of separating particles by their size, magnetic, or electrical properties during the production process. Up scaling in order to manufacture new nanomaterials in a controllable, reproducible, and cost-efficient manner is necessary to allow such monitoring of workers and therefore to allow effective occupational control strategies to be developed and implemented.

More information on occupational, consumer and environmental exposure is urgently needed to support effect risk assessment and characterisation.

10.5 ENVIRONMENTAL FATE AND BEHAVIOUR

The general paucity of data in the area of environmental fate and behaviour represents a major obstacle in developing a holistic view of the fate and transport of nanomaterials within the environment to date and therefore environmental exposure. It may even be stated that the paucity of data relevant for soil and sediment behaviour of nanomaterials is so pronounced, particularly for metal oxides and carbon nanotubes, that no general conclusions can be drawn. Much work is underway, and it is likely that in the coming years more relevant scientific information will emerge on which to develop further understanding of the area (Aitken *et al.* 2009; SCENIHR 2009). The following section represents those key conclusions and recommendations the ENRHES consortium consider as key developments for an informed and structured approach to this task.

Most of our current knowledge of transport of nanomaterials within air, soil and water compartments remains rooted in aerosol and colloid science. This background provides preliminary information from which to develop further understanding of nanoparticles' fate and transport. With respect to the aquatic environment, one consistent finding is that most nanomaterials interact with natural organic matter and other materials found in the environment, and that this influences the fate and transport of nanomaterials in water and may also be of significance for their biological effects.

There is therefore a need for systematic studies to be undertaken on different types of nanomaterials using a range of physico-chemical parameters (e.g. size, shape, form, surface area), in order to generate data which will support development of reliable and truly relevant models. Predictive modelling of emission scenarios and subsequent transport pathways will play an important role in furthering understanding this area. In concordance with this, the development of more sophisticated predictive models is currently underway in some studies, but this information is not yet available in the peer reviewed, public domain.

Most applications of engineered nanomaterials require functionalisation of their surface (e.g. to ensure successful incorporation into the product for which they are intended). Examination of the literature clearly shows that functionalisation of nanomaterials also has an important effect on their subsequent transport and fate. Considerable further work is therefore required to develop a full understanding of the effect different functionalisations have on the transport and fate of nanomaterials in air, water and soil systems.

However, a recurring problem with the research conducted to date is its lack of comparability due to, for example, the use of nanomaterials with different functionalisation, different experimental approaches, different levels of attention to characterisation of the nanomaterials used, and variation in timescale of studies conducted. Although it is well known that different types of nanomaterials, with different functionalisations will have different fates and behaviour in the environment, it is also important that consistent data become available via rigorous and comparable experimental approaches. As the body of work in the area expands, such variation in approaches may result in a lack of comparability of data generated by different research groups. The EMERGNANO report (Aitken *et al.* 2009) states that much work is ongoing to assess transport of nanomaterials in the environment, from which it is expected valuable output should be generated. However, the study's authors also note that there is much overlap in the work being undertaken by different groups (an observation perhaps symptomatic of a wider-scale lack of coordination in research effort between funding bodies and government). As such

the scope of outcomes is likely to be lesser than had the research effort as a whole been more coordinated.

Development of valid real-time methods by which to selectively detect and quantify specific nanomaterials within air, water and soil systems has been identified as crucial. Likewise, characterisation of sources and release mechanisms for nanomaterials into the environment, according to amounts being released and nanomaterial physico-chemical characteristics is a key task to address (Wu *et al.* 2008), as is development of metrological strategies for monitoring transport and fate of nanomaterials within different environmental compartments. This must be done carefully should, as is predicted, the nanomaterials alter in size rapidly via processes such as agglomeration.

A final observation stems from the literature identified as being specifically relevant to the transport of nanomaterials in soil and sediment, which focus on remediation of contaminated media. Results from these studies can offer some information on transport and, due to the use of engineered NPs for remediation purposes, there is a strong incentive to gain knowledge on transport properties of selected nanomaterials which should lead to valuable contributions to the field.

10.6 TOXICITY

10.6.1 Carbon fullerenes

There are a number of factors that appear to be implicated in fullerene behaviour and toxicity, including chemical structure, surface modifications, and preparation procedures. Ultimately these factors drive fullerene water solubility which appears to be related to antioxidant/cytoprotective or pro-oxidant/cytotoxic properties. Generally the greater water solubility exhibited by a fullerene sample, the lesser the toxicity associated with exposure. However, the situation is complicated by the findings that residual solvents (or their derivatives) used within the preparation of fullerene samples are able to contribute to the observed toxicity, which negates improving water solubility by particular methods. In addition, the preparation of surface modified fullerenes is conducted to improve an aspect of fullerene function, and also modifies the toxicity of fullerenes. However, the fact that fullerene derivatives do not always behave similarly to their unmodified counterparts is expected, and may allow for the safe integration of fullerenes into products, by revealing which attributes of particles are most influential in driving toxicological findings.

The studies conducted so far suggest that fullerene toxicity involves an oxidant driven response, suggesting that toxicity assessments should evaluate the potential of fullerenes to cause oxidative stress and related consequences such as inflammation or genotoxicity. The studies conducted with fullerenes thus far are rather limited in terms of models used, targets investigated and mechanisms of toxicity. Much more work is required to generate sufficient knowledge to inform a risk assessment. Accordingly, it is unrealistic to make generalisations about the behaviour of fullerenes from the limited number of studies that have been conducted, as investigations into the toxicity of fullerenes via specific routes of delivery, or at particular cell and organ targets, are often too few in number to make definite conclusions about fullerene behaviour. In addition, the quality (including the concentrations used, experimental model), of conducted experiments are questionable, which is of vital importance when considering the risk associated with fullerene exposure.

10.6.2 Carbon nanotubes

Revealing the toxic potential of CNT has been motivated by the number of applications proposed to exploit CNT, and the benefits they could provide. However CNT exploitation may be limited by the concern regarding their toxicity, and uncertainty regarding the consequences surrounding human exposure. There has been a focus on the properties of CNT that might account for toxicity, with SWCNT often being shown to be more toxic than MWCNT, although this is difficult to confirm due to other parameters that differ between CNT samples such as length. In fact longer length (>20µm) has been demonstrated to result in greater pathogenicity

in some *in vivo* models and frustrated phagocytosis *in vitro*. Increased functionalisation of the surface chemistry and reduced metallic impurities have both been associated with a relative decrease in toxicity, while the consequences of aggregation/agglomeration are highly dependent upon the model used. While physico-chemical characterisation of CNT samples is clearly important for such studies, evaluating the attributes of CNT that are responsible for driving CNT toxicity is also complicated by the experimental design. While some studies indicate an ability of CNT to elicit oxidative stress and inflammation, which ultimately culminate in cytotoxicity *in vitro* or disease *in vivo*, much work is required to establish whether this is applicable for all routes of exposure, target organs and different types of CNT.

There are a number of gaps within the literature that require addressing in future experiments. Firstly, much of the research conducted to date relates to the toxicity of CNT to the skin and lung. In contrast there is a paucity of data regarding the liver, kidneys and other organs which require consideration due to studies that have highlighted accumulation of CNT in various target sites within the body, following intravenous exposure. However, it is necessary to highlight that information regarding the systemic availability of CNT, following the exposure of the lungs, skin and GIT is severely lacking, and necessary to direct appropriate investigations, regarding the relevant targets of CNT toxicity. For pulmonary studies, intratracheal instillation and aspiration of CNT have been the primary mode of delivery, with limited inhalation studies due to cost and technical difficulty. Inhalation studies are required due to the questionable relevance of the instillation and aspiration models.

It is also relevant to highlight that a number of assays have been utilised to assess the toxicity of CNT. However, it is known that CNT can interfere with the assays that assess their toxicity, which necessitates that appropriate controls are conducted and that different assays measuring the same endpoint are used in order to confirm any findings of toxicity. It is also necessary to consider the quality (including the concentrations and experimental models used), of conducted experiments, due to their vital importance to relevantly determining the risk associated with CNT exposure.

Finally, taking all of this information into account, the disparate data provided to date suggests that CNT can represent a hazard, but that hazard potential can also be manipulated, and potentially controlled by varying a number of physico-chemical characteristics. Such information, when provided in more detail, will allow for a more effective risk assessment as well as improved management of risk for workers, consumers and the environment.

10.6.3 Metals

It is evident that silver and gold metal nanoparticles represent a potential hazard to human health. However, investigations purporting to study the toxicity of metal particulates are still in their infancy at this time, and have concentrated on revealing the toxicity, tissue distribution and antibacterial properties of silver NPs, and in addition the tissue distribution and cellular uptake of gold NPs. Consequently, more comprehensive studies are required to more fully understand the toxicity associated with metal particulate exposure. Silver and gold can become systemically available following exposure to nanoparticles made of these materials, which is evidenced by the preferential accumulation of these metals within the liver. The appearance of argyria caused by accumulation of silver within the skin, has transpired following silver nanoparticle ingestion, which further emphasises the propensity for silver nanoparticles and/or ions to become systemically available. The liver appears to be the primary site of silver accumulation, and so the consequences of this require investigation, as this organ may enable particle elimination from the body, within bile, or particle presence within the liver may be associated with toxicity, and inflammatory responses. It is apparent that the toxicity of metal particulates is reliant on their internalization and oxidative nature, which drives inflammatory, genotoxic and cytotoxic events. However, the specific properties of particles driving the observed toxicity is uncertain. Specifically, it is of relevance to determine where the toxicity derives from the small size of NPs, or whether toxicity is mediated through the release of ions from particles, or perhaps a combination of both concepts is responsible, as smaller particles are likely to allow for the greater release of Ag⁺ ions. Further studies are required to improve understanding of the toxicokinetics for metal nanoparticles such as silver.

10.6.4 Metal Oxides

Due to historical reasons, a focus on the size (and surface area) dependence of metal oxide (particularly TiO₂) toxicity has been persistently illustrated, and confirmation that particle toxicity increases as particle size decreases has been consistent within wide ranging investigations. However, it has become evident that other physico-chemical factors are able to contribute to metal oxide toxicity; including particle aggregation, crystal phase, surface modification, and particle dissolution. The exposure method, dose administered, species used, cell type under investigations and light conditions also have the potential to impact on the toxicity of metal oxide particles, indicating that the experimental set up is also very influential. However, much of the current work has concentrated on revealing the toxicity of TiO₂, and therefore the applicability of the findings to other metal oxide types is unsure at this time, and should be approached with caution.

The toxicity of metal oxides (with most studies relating to TiO₂) has been demonstrated to be inflammogenic, oxidative, and genotoxic in nature; with all endpoints considered to be inherently linked. It is also of interest that the biological mechanisms identified as being responsible for driving the toxicity of metal oxides is replicated within *in vivo* and *in vitro* settings. Cytotoxicity is also a common end point that is evaluated within studies, although the relevance of this is questionable (in terms of human exposure levels), except when establishing sub-lethal concentrations for subsequent studies that allow the identification of mechanistic processes that are responsible for toxicity. Many of the studies identified have concentrated on TiO₂ and ZnO toxicity, due to their extensive exploitation in nanomaterial containing products. The ability of particles to exert toxicity at a variety of target sites is reliant on their transfer into blood, and this should therefore be a focus of future experimentation, as at this time, the systemic availability of metal oxide particles following exposure is uncertain. Accordingly, investigations into the toxicity of metal oxides via specific routes of delivery, or at particular cell and organ targets, are often insufficient in number to make definite conclusions about particle behaviour. In addition, the quality (including the concentrations used, experimental model), of conducted experiments is an important consideration, which is of vital importance when considering the risk associated with metal oxide exposure.

10.7 EPIDEMIOLOGY AND HUMAN STUDIES

Due to the paucity of data relating to CNT, fullerene, metal and metal oxide nanoparticles, a broader approach has been applied for the epidemiology review. This therefore allows us to draw upon the depth and breadth of knowledge available for a small number of nanoparticles which have been manufactured at the industrial scale for decades.

10.7.1 Epidemiology Studies on the Carbon Black Industry

The results from the discussed epidemiology studies of the carbon black industry do indicate some adverse effects of exposure to carbon black dust on respiratory health. However, the main implications, summarised below, are reassuring:

- With respect to respiratory symptoms and lung function (FEV₁ and mid-flow rather than FVC), there is some consistent evidence of effects on these parameters, which appear to be primarily associated with current exposure rather than being caused by cumulative exposures;
- Using radiological techniques, there is clear evidence of dust retention within the respiratory system, but limited evidence of disease such as small opacities of category 2 or above;
- A mortality study by Sorahan *et al.* (2001) clearly indicates no strong and little suggestive evidence of excess non-malignant respiratory disease associated with working in the carbon black industry. Despite the fact that at two of the five factories investigated generated evidence that there was excess mortality from lung cancer, however, the study has failed to link this disease to carbon black exposure;

- The morbidity studies identified indicate relationships between exposure and respiratory health endpoints, but they do not signify an extreme response to the workplace dust exposure.

10.7.2 Epidemiology Studies on the Titanium Dioxide Industry

TiO₂ industry epidemiology studies provide little information to evaluate the health risks associated with ultrafine particle manufacture as most work has focused on larger particles. It is unlikely that exposure to a true ultrafine dust explains the variations in lung cancer mortality between studies and factories.

10.7.3 Epidemiology Studies on Welding

It is difficult to extrapolate from the epidemiology of welders and welding fumes to that of the nanoparticles generated within the nanotechnology industries, because the type of metals welded are different to those considered for the engineered nanoparticles of this study.

10.7.4 Particle Number Studies on Particles in Ambient Air

There exists a limited number of relevant epidemiological studies that assess particle number in ambient air. The studies published to date conclude that; (i) there are adverse health effects associated with the ultrafine fraction of respirable particles, with effects indicated on mortality in the general population and panels of susceptible individuals and (ii) death was related to particle numbers in the nano-size range.

10.7.5 Human Exposure Studies

Overall, the findings of human studies suggest that nanoparticles are capable of inducing physiological and inflammatory responses in humans. Such effects include a neutrophilic inflammation mediated through the chemokine IL8, as well as endothelial activation which was measured directly in studies using concentrated air pollution particles (CAPs). Some studies, but not all, have also demonstrated changes in heart rate variability.

The majority of the subjects used in the human exposure studies have been young, healthy volunteers, although there have been some studies which have employed mildly asthmatic subjects. Epidemiology investigating air pollution indicate that older subjects with pre-existing cardiopulmonary disease are the portion of the population who are most affected by everyday air pollution changes. The meaning of these results remains unclear despite the fact that the exposures used in these types of studies are typical of those of severe pollution days. It is likely that availability of volunteers and ethical concerns over potential risky exposure of subjects with coronary artery or pulmonary disease has limited the range of subjects available for such studies.

10.8 ECOTOXICITY

It is clear from the information available that neither metal nor metal oxide nanoparticles should not be considered as single groups in terms of hazard and risk assessment and therefore no general conclusion on their general ecotoxicity can, or should be made. Just like “regular forms” of metals and metal oxides there are large differences in speciation, behaviour, fate and effects, even in standardized test systems. The sections pertaining the metals and metal oxides have therefore addressed studies pertaining to specific elements rather than considering them as one group of substances.

10.8.1 Carbon fullerenes

Since 2004, a range of studies have been carried out with aquatic species and C₆₀. However, in total, less than ten studies have been carried out on fullerene toxicity towards the base-set organisms used in the REACH risk assessment procedures for chemicals (fish, crustacean and algae). More studies are available using bacterial groups and, though they do not report the

findings in traditional ecotoxicological endpoints, these studies may be of value for mechanistic interpretations of fullerene ecotoxicity in both the aquatic and the terrestrial environment.

Initial studies used different solvents to suspend C₆₀, but more recent studies have avoided the use of any solvents since, as for the mammalian toxicology studies, it has been demonstrated that not only C₆₀/solvent interactions may affect toxicity, but also solvent degradation products may be responsible for some of the observed effects.

For fish, the studies by Zhu *et al.* (2008b) resulted in a NOEC of 0.04 mg l⁻¹ and a LOEC of 1 mg l⁻¹, in terms of reduced lengths and body weights, after 32 days of exposure. In the study by Usenko *et al.* (2008) an LC_{50, 96h} of 0.19 ppm can be proposed from their results, however in this study the stock solutions of C₆₀ were prepared in pure DMSO and the solvent concentration in the tests were as high as 1% (vol/vol).

Less information is available for crustaceans: the only reported LC₅₀-value is the LC_{50,48h} of 7.9 ppm for sonicated C₆₀ found by Lovern and Klaper (2006). They also observed a LOEC of 0.45 mg l⁻¹ and a NOEC of 0.18 mg l⁻¹ in the 48-h acute toxicity test for the sonicated suspensions of C₆₀. In a long-term study with *Daphnia magna*, Oberdörster *et al.* (2006) reported a LOEC of 2.5 mg l⁻¹ for the number of offspring after 21 days for water stirred C₆₀, but the validity of this result is questionable due to too high mortality in the exposed organisms.

There is not enough data for other invertebrates or algae to draw any conclusions regarding the toxicity of fullerenes to these phyla.

In the three studies currently published on the terrestrial toxicity of fullerenes significant effects are only reported by Johansen *et al.* (2008) who found that exposure to 50 µg g⁻¹ yielded a three- to four-fold inhibition of the number of bacterial colony forming units in clay loam soil 3 hours after incorporation of 99.5% pure C₆₀ aggregates.

Major knowledge gaps are identified within persistence and bioaccumulation of fullerenes since no structured studies, aimed to investigate this, have been reported in the reviewed literature.

10.8.2 Carbon nanotubes

Only a few ecotoxicological studies of the effects of CNT to aquatic species have been carried out. Until now there has not been a strong focus on taxa belonging to the base set of organisms used for risk assessment of chemicals (fish, crustacean, and algae). Only two studies have been carried out on fish and four studies on crustaceans. To date, no algal studies have been published. Several studies exist on other taxa (ranging from bacteria and protozoans to amphibians) but due to the high variability in these studies it is not possible to draw any common conclusion on the effects of CNT on this basis. It should however be noted that a number of studies do not find adverse effects after exposure to CNT in often very high concentrations.

For aquatic toxicity the findings of Smith *et al.* (2007) raise several severe concerns that need to be addressed by future studies. These findings indicate new modes of toxicity that have not been identified in fish before, i.e., subtle neurotoxic or cardiovascular effects of SWCNT that affect fish behaviour. Furthermore, the findings of cellular pathologies in the liver (which indicate genotoxicity or cell cycle defects) give rise to concerns about whether carcinogenicity may be observed after long-term exposure to SWCNT (Smith *et al.* 2007).

So far only three studies have reported on the terrestrial toxicity of CNT. While one study finds no effects on seed germination and root growth after exposure to up to MWCNT 2000 mg l⁻¹ (Lin and Xing 2007), another study finds that addition of SWCNT significantly reduces the root elongation of tomato plants. However, this may be attributed to the very high exposure concentrations (up to 1750 mg l⁻¹) leading to a CNT attachment especially to root hairs (Cañas *et al.* 2008). In this study no decrease in root elongation was found for cabbage, carrot, cucumber, lettuce and onion. Dietary exposure to DWCNT resulted in EC_{50, 28d} values of 176±150 mg kg⁻¹ food for reproduction of earthworms (Scott-Fordsman *et al.* 2008). However,

considering the large variability, this result is not likely to contribute to risk assessment of CNT in soil.

For worms living in soil and sediments low Bioaccumulation Factor (BAF) have been found for SWCNT and MWCNT. In soil the maximum BAF was 0.02 for *E. foetida* exposed to 0.03 and 0.3 mg g⁻¹ soil for 14 days (Petersen *et al.* 2008a). In sediments it was observed that after two days of depuration in clean sediments CNT could not be detected in *L. variegates* (Petersen *et al.* 2008b)

Degradability of CNT still remains to be studied, though indications of biomodification of functionalised CNT have been reported by Roberts *et al.* (2007).

Testing difficulties in relation to obtaining, handling, purification and solubilisation are likely to have an influence in the very limited number of studies available for environmental risk assessment (i.e. ecotoxicity, persistency, and bioaccumulation).

10.8.3 Metals

The major part of the scientific literature published deals with toxic effects of silver and copper nanoparticles and some general conclusions on the toxicity of these are listed below.

Only very few studies have dealt with bioaccumulation of metal nanoparticles. However, accumulation of metals is a topic of high concern when looking at past experiences with “regular” metals. By definition metals, and hence also metal nanoparticles, are not degradable. However, changes in the metal speciation can occur depending on redox conditions, salt content etc. These changes in speciation are as complex as they are for conventional metal forms and no general conclusion can be made in this regard. Also functionalisation of metal nanoparticles is an issue of high relevance for the effects of metal nanoparticles, but so far the number of studies is too limited to draw conclusion on the influence of functionalisation on ecotoxicity, speciation, and accumulation.

10.8.3.1 Silver nanoparticles

Silver is known as a highly ecotoxic metal. A range of studies with fish, crustaceans and algae confirms that also when silver is tested as AgNP low effect concentrations are found. For fish and crustaceans the lowest reported LC_{50, 48h} values are 7 mg l⁻¹ (*Danio rerio*) and 0.040 mg l⁻¹ (*Daphnia pulex*), respectively (Griffitt *et al.* 2008). In the fish studies by Yeo and Kang (2008) exposure to 10 ppt AgNP resulted in adverse effects. For algae, an EC_{50, 5h} of 0.092 mg l⁻¹ was found for *Chlamydomonas reinhardtii* (Navarro *et al.* 2008). For silver the issue of dissolution is crucial to understanding the mechanisms of ecotoxicity since toxic effects usually can be linked to the concentration of the free mono-valent silver ion. However, both the studies by Asharani *et al.* (2008) and Navarro *et al.* (2008) show that higher effect levels than those stemming from the free Ag⁺, were found for fish and algae, respectively. This was also found for nitrifying bacteria in the studies by Choi and Hu (2008). Neither degradability nor bioaccumulation of AgNP has been addressed in the literature published before 12 December 2008.

10.8.3.2 Copper nanoparticles

The study by Griffitt *et al.* (2008) provides evidence that copper nanoparticles are highly toxic to fish, daphnids, and algae. The 50%-effect levels are below 1 mg l⁻¹, with a LC_{50,48h} of 0.060 mg l⁻¹ towards adult *Daphnia pulex* as the lowest reported effect value. However, Griffitt *et al.* (2007) found that copper sulphate was six times more toxic towards adult female zebrafish (*D. rerio*) than copper nanoparticles, when comparing LC_{50,48h}-values. It was found that aggregation and sedimentation significantly reduced the exposure concentration of copper nanoparticles. For terrestrial plants, Lee *et al.* (2008) found high EC_{50, 48 hour} values (>300 mg l⁻¹) for seedling and shoot growth of mung beans (*P. radiatus*) and wheat (*T. aestivum*) when using very high exposure concentrations (from 200-1000 mg l⁻¹).

A single study of bioaccumulation of copper nanoparticles in plants has been reported (Lee *et al.* 2008). However, due to a very high exposure concentration (1000 mg l^{-1}) further studies are needed to make conclusions on the accumulation behaviour of copper nanoparticles.

10.8.3.3 Other metal nanoparticles

Ecotoxicity studies of nanoparticles of aluminium, gold, cobalt, and nickel have been reported. However, the literature on these metals can best be described as extremely limited. In fact, only one study has dealt with aquatic toxicity of Al, Co, and Ni (Griffitt *et al.* 2008), one study focussed on the importance of gold nanoparticles functionalisation for fish toxicity (Harper *et al.* 2008), and one study documented accumulation of cobalt nanoparticles in earthworms (Oughton *et al.* 2008). Nevertheless no general conclusion on the ecotoxicity or accumulation of these metals can be drawn based on these studies, though all three studies deal with important issues related to environmental effects of metal nanoparticles

10.8.4 Metal oxides

When going through the literature, it is apparent that the major part of the scientific papers published compare different metal oxide nanoparticles. While this may be important in terms of hazard ranking and benchmarking, it is of limited relevance when it comes to actual application of nanoparticles in products. This is due to the fact that substitution of a toxic nanoparticle with a less toxic one is only possible if the two nanoparticles have similar beneficial properties for the product (SiO_2 will for instance not be a potential substitute for TiO_2 in sunscreens). However, the data produced for each individual nanoparticle are of high importance for establishing ecotoxicity dossiers for risk assessment purposes.

While a number of toxicity tests have been carried out with metal oxide nanoparticles, no studies focussed specifically on bioaccumulation have been described in the literature published before 12 December 2008. For TiO_2 nanoparticles a carrier effect has however been observed in bioaccumulation of cadmium and arsenic in fish (Zhang *et al.* (2007); Sun *et al.* (2007)) indicating that the bioavailability of other contaminants may be affected by the presence of TiO_2 nanoparticles. Both these studies also demonstrate an accumulation of TiO_2 in different parts of the carp, with highest concentrations detected in the viscera. Lower concentrations were found in skin, scales and muscles.

General conclusions on metal oxide ecotoxicity are hampered by the large diversity of materials. However, it was found that for three individual metal oxides types (TiO_2 , ZnO and SiO_2), a number of trends could be outlined:

10.8.4.1 Titanium dioxide nanoparticles

Titanium dioxide nanoparticles are among the most frequently tested nanoparticles in ecotoxicological tests. Thus, tests are available for the whole range of base set organisms (fish, crustaceans, and algae) and for a number of other species.

However, the properties of the tested TiO_2 NPs (e.g. size, crystallinity, surface coating) differ from study to study. Comparisons between different types of TiO_2 may therefore not be valid. While a number of studies find low or no effects of TiO_2 (e.g. Hund-Rinke and Simon 2006; Adams *et al.* 2006; Griffitt *et al.* 2008; Heinlaan *et al.* 2008; Jemec *et al.* 2008), the results of Federici *et al.* (2007), showed that fish exposed to 0.1 mg l^{-1} TiO_2 (P25) for 14 days showed signs of respiratory toxicity (as evidenced by gill pathologies and mucus secretion). Histological examination of the brain of exposed fish indicated biochemical disturbances at this relatively low exposure concentration. For algae (*P. subcapitata*) an $\text{EC}_{50, 72\text{h}}$ of 5.83 mg l^{-1} was found by Aruoja *et al.* (2008). Since TiO_2 is an effective photocatalyst, a number of studies have investigated the influence of light on the toxicity response of TiO_2 . Vevers *et al.* (2008) found that UVA irradiation of TiO_2 increased the DNA strand breakage in gonadal tissue cells of rainbow trout (*O. mykiss*) when exposed to 50 mg L^{-1} TiO_2 nanoparticles for 24h. However, also significant cytotoxicity was observed at this concentration and this may have influenced the results of the Comet assay. Hund-Rinke and Simon (2006) did not observe any effects of pre-

illumination of TiO₂ in algal tests and Adams *et al.* (2006a) found that antibacterial effects also occurred under dark conditions (cell death was less pronounced under dark compared to light conditions). Therefore photocatalytic production of ROS cannot alone be responsible for the inhibition observed and additional modes of action for TiO₂ nanoparticles remains to be disclosed.

10.8.4.2 Zinc oxide nanoparticles

As it is the case for silver nanoparticles, dissolution of zinc is one of the major issues addressed in the studies of ZnO nanoparticles. For ZnO nanoparticles Zhu *et al.* (2008a) found hatching and survival of zebrafish embryos to be affected with an LC_{50, 96h} of 1.79 mg l⁻¹. However, the effect levels were not different from results obtained with bulk ZnO. For crustaceans, Heinlaan *et al.* (2008) found an LC_{50, 48 h}-value of 3.2 mg l⁻¹ ZnO-NP and a NOEC of 0.5 mg l⁻¹ for *Daphnia magna*. In the same study, it was found that the LC_{50,24h} for *Thamnocephalus platyurus* was 0.18 mg l⁻¹ and the NOEC 0.05 mg l⁻¹. In the studies of algal toxicity of ZnO NP, bulk ZnO and ZnSO₄ no statistically significant difference in EC₅₀ values for *P. subcapitata* could be observed (Aruoja *et al.* 2008). In accordance with this, Franklin *et al.* (2007) concluded that the toxicity of ZnO nanoparticles to *P. subcapitata* was due to dissolved zinc. Thus, in contrast to what was found for silver nanoparticles, the toxicity of zinc oxide nanoparticles seems to be equivalent to that of the released free ion (in this case Zn²⁺).

10.8.4.3 Silicon dioxide nanoparticles

The aquatic toxicity of SiO₂ has only been addressed by a two studies of growth inhibition of algae. For the freshwater green algae *P. subcapitata*, van Hoecke *et al.* (2008) reported EC_{10, 72h} values of 10.9 mg l⁻¹ and 15.0 mg l⁻¹ for 12.5 nm and 27.7 nm SiO₂ nanoparticles, respectively. For both particle types the NOEC was 4.6 mg l⁻¹ and LOEC was 10 mg l⁻¹. In the study by Fujiwara *et al.* (2008) effect concentrations for the inhibition of *Chlorella kessleri* were in the same range for 5 nm SiO₂ nanoparticles with an IC_{50, 96h} of 8 mg l⁻¹. Effect concentrations were significantly higher for 26 nm and 78 nm SiO₂ nanoparticles with IC_{50, 96 h}-values of 71 mg l⁻¹ and 91 mg l⁻¹, respectively.

10.8.5 General ecotoxicity conclusions and recommendations

Attention should be drawn to the fact, that while many ecotoxicity studies are directed towards the core-particles, most real-world application of engineered nanoparticles require surface functionalisation. The effect of functionalisation on bioavailability and hence toxicity and bioaccumulation of nanoparticles remains to be studied. As a range of nanoparticles are non-miscible with aquatic medium, solvents have been used for dispersing nanoparticles in aqueous media. This has especially been the case in a range of the “early studies” (i.e. studies carried out before 2007) e.g. Oberdörster (2004) and Lyon *et al.* (2005). While this may result in higher throughput of tests (as long and tedious mixing procedures can be avoided) and may ensure a more uniform distribution throughout the water phase, it also raises serious problems with the validity of the results obtained due to testing artefacts introduced by solvent-medium-nanoparticle interactions (Henry *et al.* 2007). Due to the risk of producing testing artefacts, and keeping an eye on the environmental relevance of the tests carried out, the use of solvents are therefore at present not recommended.

While some studies have carried out some characterisation of the nanoparticles tested, most studies only report on chemical composition, sizes of nanoparticles (as purchased), and in some cases sizes of nanoparticles in suspension. It is evident that much more research is needed before specific properties, or combinations of properties, can be linked to the effects observed in ecotoxicity tests. For the time being only a few studies have documented links between characteristics and toxicity, e.g. van Hoecke *et al.* (2008) who found for SiO₂ nanoparticles that when results were expressed in terms of surface area instead of mass units, the apparent differential toxicity related to nanoparticle size was eliminated. The characterisation and quantification of nanoparticles in stock solutions, in media, and in biological tissues remains one of the biggest challenges in nano-ecotoxicology. This is not only needed for linking characteristics with toxicity, but also for determination of actual exposure levels and for

quantification of uptake, depuration and decay of nanoparticles. Going through the literature on environmental effects of nanomaterials, the lack of studies addressing degradability and accumulation is indeed striking. While these two properties, along with ecotoxicity, are fundamental for determining how environmentally hazardous a chemical is, it seems that most research efforts have been directed towards the potential toxicity of engineered nanomaterials.

In the reviewed ecotoxicological literature, it is obvious that the majority of the studies have used concentrations far above what is believed to be environmentally realistic. However, since we are in the beginning of ecotoxicological testing of nanoparticles this type of information is valid for risk assessment purposes in terms of ranking and benchmarking. Still it is important to emphasise that at present there are no studies supporting extrapolations from the effect levels documented in laboratory tests to environmental scenarios. Since some types of nanoparticles are not truly dissolved (and may not even be evenly dispersed in the test systems), it may be questioned whether dilution will always lead to lower effects and/or lower potential for accumulation. Dilution might result in a breakage of the agglomerates, which are described in most aquatic toxicity tests, to smaller colloid sizes for which neither toxicity nor uptake is known.

Furthermore, most of the studies reviewed have focussed at short-term effects while long-term exposures to lower concentrations aimed at chronic endpoints have been far less studied. In a few cases (e.g. C₆₀ and TiO₂) data is now available for the test species in the base set for risk assessment in REACH (i.e., fish, crustacean, algae). However, even in these cases there is a need for replication of the test results obtained due to testing difficulties with regards to preparation, handling, and quantification of nanoparticle exposure. A range of environmentally relevant species have been used, but due to the large number of different nanoparticles tested no clear pattern on species sensitivity, suitability as test organism in nano-ecotoxicity or relevance of endpoints is seen. A number of studies are published on bacterial effects, mainly in pure cultures of either *E. coli* or *B. subtilis*. While these studies may be of interest from a mechanistic point of view, e.g. in relation to nanoparticles' interaction with cell membranes, they are at present of limited value for ecotoxicological effects assessment for other taxa. If future studies succeed in disclosing mechanistic reasons to NP ecotoxicity, bacterial studies along with cellular studies may however be important screening tools.

10.9 RISK ASSESSMENT APPRAISAL

10.9.1 General issues

Lack of exposure data

The risk assessments show a significant lack of measured and modelled exposure data of nanomaterials, for humans (occupational and consumer exposure) and for the environment. The limited amount of published measured data for human exposure may be due to the difficulties associated with the measurement of ultrafine or nanoparticles (and the decision which metric(s) to use) and their distinction from background particles. For the environment, this is further complicated by the challenges of "identifying" nanoparticles in environmental matrices. A few relatively simple exposure models have been used (see e.g. Boxall *et al.* 2007; Mueller and Nowack 2008). However, more sophisticated reliable models for predicting exposure to nanomaterials have not been identified.

For risk assessment purposes, it is in general highly recommended to further establish good exposure data for all relevant exposure routes and targets, via measurements as well as to develop validated exposure models. Establishment of exposure data should address the issues related to a proper characterisation, as discussed in the following. It is also important to further study the interaction of nanoparticles with environmental matrices (e.g. natural organic matter, sediments, etc.), affecting the environmental fate and transport, and thus the exposure for aquatic and soil organisms. The surface chemistry of the nanomaterial (i.e. coating and functionalisation) may play a relevant role in the fate of the nanomaterial.

Characterisation and metrics

As noted, the choice of metric(s) seems of key importance for assessing risks (hazard and exposure) of nanomaterials. This finding is in line with many other authors who note that a proper characterisation of nanoparticles is fundamental for exposure assessment as well for toxicity testing. Work is on-going in various fora (in particular OECD and ISO) to define/outline a proper set of characteristics for nanomaterials. It therefore seems premature to draw within this review definitive conclusion on that. However, a few considerations will be given.

For human health, it seems that the risk of metal and metal oxides is largely driven by the size and therefore surface area of the nanoparticles, and it seems that chemistry may (e.g. silver) or may not (e.g. TiO₂) influence the toxicity, possibly depending (at least partly) on the formation and toxicity of free metal ions. For the carbon-based nanomaterials it seems very relevant in addition to consider the three dimensional structure of the nanomaterial (e.g. fibre-like characteristics), the chemical composition (e.g. impurities from their production) and not the least the various surface modifications, which are often added deliberately to promote a certain effect (e.g. increase water solubility). A particular challenge (both in terms of measuring exposure and assessing risks) is introduced by the fact that exposure data often refer to a distribution of particles of different characteristics and different sizes, whereas toxicity tests are often performed for mono-sized nanoparticles of one type. In addition, nanoparticles will often aggregate to agglomerates (both relevant for exposure assessment and toxicity testing) and as evidenced in the risk assessments, it is not always clear what the agglomeration state was in the relevant studies. Even if known it is difficult to make general conclusions on how this will influence the toxicity.

For the environment, it was not possible to determine an influence of the size or the shape on the ecotoxicity for any of the groups of investigated nanoparticles. The effects may however be affected by agglomeration and aggregation, especially at the very high concentrations used in the tests. Toxicity of metals and metal oxides seems to be driven by chemical composition, but the effect of coatings (e.g. in consumer products mostly coated nanomaterials are used) on their toxicity was not sufficiently studied. For example, coating can reduce or block the release of toxic ions from silver nanoparticles thus reducing their toxicity. Moreover, coating and surface functionalisation may improve the metal and metal oxide nanoparticle dispersion stability and hydrophilicity and consequently may increase the possibility of transport over long distances in the environment. The effects of carbon-based nanomaterials on organisms are influenced by functionalisation and the level of impurities (especially in CNT).

In conclusion, the findings of the ENRHES risk assessment appraisal heavily support the further development of a proper characterisation scheme (including proper considerations of agglomeration/aggregation) of the nanoparticles in the exposure media, when conducting exposure assessment, as well as in the generation of data for assessing hazardous properties. This seems a key prerequisite for doing a proper assessment of the risks. It should be noted that this characterisation does not necessarily need to be the same for different types of materials. There is an urgent need for the development of reference nanomaterials for the evaluation of both the quality of measurement techniques and to compare biological responses.

Scaling from bulk substances

It is often discussed to which degree the risks of nanoparticles can be assessed based on the toxicity of the bulk/normal substances; i.e. whether the risk of the bulk/normal substances can simply "be scaled" to the nanoform taking into account the smaller size of the particle or whether the small sizes triggers "nano-specific" behaviour/effects. To date no firm conclusion can be drawn which would be applicable to all nanoparticles. However, when considering whether scaling is possible, it seems to be a prerequisite that if the 'chemistry' (at least partly) drives the toxicity, it needs to be the same chemistry in the bulk/normal form as well as in the nanoform. This already introduces some reservations for carbon-based nano-materials, which have surface modifications deliberately added to give them specific properties. For more chemically 'inert' particles, it may be possible to draw conclusions on their behaviour and scaled from larger inert particles simply based on the shape. However, this needs further investigation beyond the possibilities in this review.

For human health, there are indications that (some of) the toxic effects of nanosized TiO₂ can simply be scaled based on surface area considerations from the toxicity of the micro-sized TiO₂. For silver, there is still too little known about the toxico-kinetics to give a fair judgement of this question. For the carbon-based nanomaterials, it does not seem obvious that the toxicity observed for the nanoforms could be found based on scaling from any normal/bulk state of carbon-materials. These observations should rather be seen as reflections than firm conclusions.

For the environment, it is interesting to note that the toxicity seen for nanosized ZnO is indicated by some authors to be related to the release of zinc ions (Zn²⁺) just as the toxicity of the bulk form of ZnO. Scaling may therefore be possible for this substance. The influence of increased surface area of nano-forms with respect to the bulk form is to be verified on the ion leaching amount and efficiency. However this does not include coated nanomaterials, which have peculiar properties. Concerning silver nanoparticles, no conclusion can be drawn yet, even if toxicity of silver seems to be related to Ag⁺ ions.

In conclusion, it seems possible to predict (part of) the toxicity of some nanomaterials based on the toxicity of the bulk/normal form, but this is not possible for all types of nanomaterials.

Toxico-kinetics

For human health, although scattered, there are quite some investigations available of the toxic behaviour of nanomaterials (see the significant toxicity reviews of this report). Despite this there are still significant gaps related to the knowledge of the toxico-kinetic behaviour of nanomaterials. For example there are significant uncertainties associated with the possible pulmonary uptake of nanoparticles (TiO₂ and carbon-based nanomaterials) and a lack of knowledge in which form they are absorbed (e.g. nano-silver, is it absorbed as particle, ion, complex or a combination?). Several distribution studies have been conducted on the various nanomaterials' ability to spread to various organs following intravenous or intraperitoneal injection. These results indicate a propensity for wide distribution to several organs, which may be very relevant for medical applications, however currently difficult to apply in a risk assessment for workers and consumers (as conducted in this study) as it is not known whether the materials will be absorbed following the exposure routes relevant for these populations (inhalation, dermal and oral). The same problem holds true for a proper interpretation of *in vitro* findings. As evidenced in the toxicity reviews, a significant amount of *in vitro* data showing toxicity to tissues/cells in various organs have been identified. It is difficult to judge the value of such findings without knowing whether the nanomaterials will ever reach those organs, and if so, at which concentrations.

For the environment, ecotoxicity testing often does not deal with toxico-kinetics. However, it is relevant to further investigate the possible uptake, distribution, and excretion of nanoparticles in the species tested, and to identify target organs (e.g. do nanoparticles reach the brains of fish? Are ingested nanoparticles adsorbed?). Biotransformation of ingested coated/modified nanoparticles in the environment should be investigated, since nanoparticle properties (including toxicity) may be changed (e.g. less hydrophilic). Another issue related to nanomaterials' toxico-kinetics is the carrier effect of organic nanomaterials by increasing bioaccumulation of organic and inorganic chemicals.

Thus it is highly recommended to further intensify the research on the toxico-kinetic behaviour of the various nanomaterials. This relates to testing of both toxicity and eco-toxicity.

10.9.2 Human health specific issues

Genotoxicity

As evidenced in the toxicity reviews, several/most of the nanomaterials investigated seem to trigger an oxidative stress driven inflammatory response, which may lead to genotoxicity. If this occurs it is a threshold-driven secondary genotoxicity. However, there seems to be a lack of investigations of the direct (possibly non-threshold) genotoxicity of the nanomaterials.

It is recommended to conduct further testing of possible direct genotoxicity of nanomaterials.

10.9.3 Environmental specific issues

Chronic toxicity

Up until now, ecotoxicity data have been mainly focused on short-term tests reporting acute endpoints, i.e. mortality. Only a few studies reporting sub-lethal endpoints were carried out, but often using short-term exposure studies. A general lack of data was observed for long-term studies on fish and *Daphnia*, as well as for sediment and terrestrial organisms. Moreover, exposure concentrations in acute tests are often unrealistic high, while effects related to very low concentrations are possible (e.g. reduced aggregation).

It is recommended that more chronic toxicity studies should be conducted, especially on *Daphnia* and fish species, supporting the prediction of more reliable environmental no effect concentrations (PNEC). Studies should be supported by a thorough nanomaterial characterization during all exposure phases. Concerning exposure concentrations, chronic studies may allow the investigation of realistic concentrations showing relevant sub-lethal effects (e.g. reproduction, behaviour).

Mechanism of action

There is a general lack of knowledge about the mechanism of action of nanoparticles. Some toxic actions such as reactive oxygen species generation are well understood, but some studies suggest the existence of other mechanisms that are still not explained. The identification of the mechanism of action may help to correlate the results of laboratory studies to the environment. Moreover, more attention should be given to the identification of indirect effects. For example, a study on algae highlighted that TiO₂ toxicity was exerted by algae entrapment in aggregates.

It is recommended that studies are designed and conducted to identify the mode of action of nanomaterials, also using 'omics' systems. It is important to understand if the toxic action is exerted after uptake into cells, or whether they already occur via extracellular exposure (e.g. generation of reactive oxygen species (ROS) outside the cell) or at the cell membrane level. Moreover, indirect toxic effects should be better investigated, such as physical effects (e.g. algae entrapment into agglomerates, light shadowing) or chemical effects (e.g. nutrient adsorption and sequestration, transport of toxic chemicals into the cell).

10.9.4 Outlook/Perspectives on future hazard and risk assessment methodology

Human Health

To date the database on exposure and effects of nanomaterials is not sufficient to make conclusive decisions on their risks. As already mentioned before, the main priority for further information will be good quality and representative exposure data and substantial information on toxico-kinetics, as well as properly characterised exposure and toxicity data. Based on that information, further testing strategies should be set up to cover all relevant endpoints needed for a risk assessment. In the respective chapters of the risk assessment, suggested information requirements for each of the studied nanomaterials were listed.

It has to be carefully analysed if available testing methods (as used for bulk/normal chemical substances) need to be adapted to reliably identify and investigate toxic effects of nanoparticles. An effort should be made to minimise animal testing, however keeping in mind that the data generated are considered appropriate to identify risks relevant for humans and are accepted by regulators.

The current conclusion to carry out risk assessment for nanoparticles could only be to do it on a case by case basis. When more data becomes available it might be possible to group nanomaterials according to their physical, chemical and/or biological properties and testing could be done representative for a group. Based of such future knowledge, it may also be possible to develop (Q)SAR and priority setting models as further discussed below.

Environment

From ecotoxicity and environmental fate studies published so far, it is evident that each nanoparticle, also within the same group (e.g. CNT), should be assessed on a case-by-case basis, because of inherent nanomaterial properties (e.g. different chemical composition, coating/functionalisation, size) and the interaction with the environment (e.g. aggregation, ions leaching, transport, uptake by biota, sorption). Having in mind the many different nanoparticles on the market, it might be useful to identify the nanomaterials of concern, i.e. priority nanomaterials. Prioritisation is generally the first step before risk assessment, in order to focus the resources to more relevant cases. The identification of the most important risk hypotheses including the nanomaterials of concern can be done by applying what is called the 'problem formulation' (PF) in the ecological risk assessment framework. The PF allows the definition of the conceptual model, linking chemicals to sources, pathway, target and impacts, and thus allowing the identification of priority nanomaterials.

The actual knowledge about nanomaterials, their ecotoxicity, and fate and behaviour in the environment is not sufficient to carry out a full risk assessment or to depict a full conceptual model. However, the available data could be used to evaluate each of the conceptual model elements to prioritize nanomaterials, balancing the risks posed to environment and human health and the potential benefits.

Linkov *et al.* (2007) suggested the application of a multi-criteria decision analysis (MCDA) as one of the most promising ways to rank nanomaterials. MCDA is an approach which is part of the weight-of-evidence family, where a set of evidences (i.e. parameters measuring the attributes of a chemical) are weighted and aggregated to formulate a judgment about the risk (or benefit) posed by a chemical. The relationship between information/data and risk/benefits, as well as the relative importance of each parameter for risks/benefits assessment, are evaluated according to professional judgment, and the MCDA result is a relative ranking, showing the level of concern of one nanomaterial with respect to another.

The application of the MCDA approach by using the DART (Decision Analysis by Ranking Techniques) software (<http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c=DART> accessed 16/10/2009) represents an organised method to use the information collected within the ENRHES review, as well as new data published after the cut-off date for the literature review of ENRHES, and to obtain a nanomaterial ranking. The possibility of identifying benefit parameters to be included in the ranking exercise should be explored.

10.10 REFERENCES

Adams, L.K., Lyon, D.Y. and Alvarez, P.J.J. 2006, "Comparative eco-toxicity of nanoscale TiO₂, SiO₂, and ZnO water suspensions", *Water Res.*, vol. 40, no. 19, pp. 3527-3532

Aitken, R.J., Hankin, S.M., Ross, B., Tran, C.L., Stone, V., Fernandes, T.F., Donaldson, K., Duffin, R., Chaudhry, Q., Wilkins, T.A., Wilkins, S.A., Levy, L.S., Rocks, S.A. and Maynard, A. 2009, *EMERGNANO: A review of completed and near completed environment, health and safety research on nanomaterials and nanotechnology*, Report on DEFRA project CB0409.

Aruoja, V., Dubourguier, H.C., Kasemets, K. and Kahru, A. 2008, "Toxicity of nanoparticles of CuO, ZnO and TiO₂ to microalgae *Pseudokirchneriella subcapitata*", *Science of the Total Environment*, vol. 407, no. 4, pp. 1461-1468.

Asharani, P.V., Wu, Y.L., Gong, Z., Valiyaveetil, S. (2008). Toxicity of silver nanoparticles in zebrafish models. *Nanotechnology*, 19, 255102 (8pp). doi:10.1088/0957-4484/19/25/255102

Boxall, A., Chaudhry, Q., Sinclair, C., Jones, A., Aitken, R., Jefferson, B., Watts, C. 2007. Current and future predicted environmental exposure to engineered nanoparticles. Final client report by the Central Science Laboratory for Department of Environment Food and Rural Affairs (DEFRA)

Canas, J.E., Long, M.Q., Nations, S., Vadan, R., Dai, L., Luo, M.X., Ambikapathi, R., Lee, E.H. and Olszyk, D. 2008, "Effects of functionalised and nonfunctionalised single-walled carbon nanotubes on root elongation of select crop species", *Environmental Toxicology and Chemistry*, vol. 27, no. 9, pp. 1922-1931.

Choi, O. and Hu, Z.Q. 2008, "Size dependent and reactive oxygen species related nanosilver toxicity to nitrifying bacteria", *Environ.Sci.Technol.*, vol. 42, no. 12, pp. 4583-4588.

Federici, G., Shaw, B.J. and Handy, R.D. 2007, "Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): Gill injury, oxidative stress, and other physiological effects", *Aquatic Toxicology*, vol. 84, pp. 415-430.

Franklin, N.M., Rogers, N.J., Apte, S.C., Batley, G.E., Gadd, G.E. and Casey, P.S. 2007, "Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and ZnCl₂ to a freshwater microalga (*Pseudokirchneriella subcapitata*): The importance of particle solubility", *Environ.Sci.Technol.*, vol. 41, no. 24, pp. 8484-8490.

Griffitt, R.J., Weil, R., Hyndman, K.A., Denslow, N.D., Powers, K., Taylor, D. and Barber, D.S. 2007, "Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (*Danio rerio*)", *Environ.Sci.Technol.*, vol. 41, no. 23, pp. 8178-8186.

Griffitt, R.J., Luo, J., Gao, J., Bonzongo, J.C. and Barber, D.S. 2008, "Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms", *Environmental Toxicology and Chemistry*, vol. 27, no. 9, pp. 1972-1978.

Harper, S., Usenko, C., Hutchinson, J.E., Maddux, B.I.S., Tanguay, R.L. (2008). In vivo biodistribution and toxicity depends on nanomaterial composition, size, surface functionalisation and routes of exposure. *J. Experimental Nanoscience*, vol. 3, no. 3, 195-206.

Heinlaan, M., Ivask, A., Blinova, I., Dubourguier, H.C. and Kahru, A. 2008, "Toxicity of nanosized and bulk ZnO, CuO and TiO₂ to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*", *Chemosphere*, vol. 71, no. 7, pp. 1308-1316.

Henry, T.B., Menn, F.M., Fleming, J.T., Wilgus, J., Compton, R.N. and Saylor, G.S. 2007, "Attributing effects of aqueous C₆₀ nano-aggregates to tetrahydrofuran decomposition products in larval zebrafish by assessment of gene expression", *Environ.Health Perspect.*, vol. 115, no. 7, pp. 1059-1065.

Engineered Nanoparticles: Review of Health and Environmental Safety

Hund-Rinke, K. and Simon, M. 2006, "Ecotoxic effect of photocatalytic active nanoparticles (TiO₂) on algae and daphnids", *Environ.Sci.Pollut.Res.Int.*, vol. 13, no. 4, pp. 225-232.

Johansen, A., Pedersen, A., Karlson, U., Hansen, B.M., Scott-Fordsmand, J. and Winding, A. 2008, "Effects of C₆₀ fullerene nanoparticles on soil bacteria and protozoans", *Environ.Toxicol.Chem*, 1:1. pp. 1.

Lin, D.H. and Xing, B.S. 2007, "Phytotoxicity of nanoparticles: Inhibition of seed germination and root growth", *Environmental Pollution*, vol. 150, no. 2, pp. 243-250.

Linkov, I., Satterstrom, F.K., Steevens, J., Ferguson, E., Pleus, R.C. 2007. Multi-criteria decision analysis and environmental risk assessment for nanomaterials. *Journal of Nanoparticle Research*, 9 543-554

Lovern, S.B., Klaper, R. (2006). Daphnia magna mortality when exposed to titanium dioxide and fullerene (C₆₀) nanoparticles. *Environ. Toxicol. Chem.*, vol. 25, pp. 1132-1137.

Lyon, D.Y., Fortner, J.D., Sayes, C.M., Colvin, V.L. and Hughe, J.B. 2005, "Bacterial cell association and antimicrobial activity of a C₆₀ water suspension", *Environ.Toxicol.Chem.*, vol. 24, no. 11, pp. 2757-2762.

Mueller, N.C. and Nowack, B. 2008, "Exposure modeling of engineered nanoparticles in the environment" *Environ.Sci.Technol.*, vol. 42, no. 12, pp. 4447-4453.

Navarro E, Piccapietra F, Wagner B, Marconi F, Kaegi R, Odzak N, Sigg L and Behra R, 2008, Toxicity of Silver Nanoparticles to Chlamydomonas reinhardtii, *Environ. Sci. Technol.*, vol. 42, no. 23, pp. 8959–8964.

Oberdorster, E. 2004, "Manufactured nanomaterials (Fullerenes, C-60) induce oxidative stress in the brain of juvenile largemouth bass", *Environ.Health Perspect.*, vol. 112, no. 10, pp. 1058-1062.

Oberdorster, E., Zhu, S.Q., Blickley, T.M., McClellan-Green, P. and Haasch, M.L. 2006, "Ecotoxicology of carbon-based engineered nanoparticles: Effects of fullerene (C-60) on aquatic organisms", *Carbon*, vol. 44, no. 6, pp. 1112-1120.

Oughton, D.H., Hertel-Aas, T., Pellicer, E., Mendoza, E, Joner, E.J. (2008). Neutron activation of engineered nanoparticles as a tool for tracing their environmental fate and uptake in organisms. *Environ. Toxicol. Chem.*, vol. 27, no. 9, pp. 1883–1887.

Petersen, E.J., Huang, Q.G. and Weber, W.J. 2008a, "Bioaccumulation of radio-labeled carbon nanotubes by Eisenia foetida", *Environ.Sci.Technol.*, vol. 42, no. 8, pp. 3090-3095.

Petersen, E.J., Huang, Q.G. and Weber, W.J. 2008b, "Ecological uptake and depuration of carbon nanotubes by Lumbriculus variegatus", *Environ.Health Perspect.*, vol. 116, no. 4, pp. 496-500.

Powers, K.W., Palazuelos, M., Moudgil, B.M., Roberts, S.M. 2007, "Characterization of the Size, Shape, and State of Dispersion of Nanoparticles for Toxicological Studies", *Nanotoxicology*, vol. 1, no. 1, pp. 42-51.

Roberts, A.P., Mount, A.S., Seda, B., Souther, J., Qiao, R., Lin, S.J., Ke, P.C., Rao, A.M. and Klaine, S.J. 2007, "In vivo biomodification of lipid-coated carbon nanotubes by Daphnia magna", *Environ.Sci.Technol.*, vol. 41, no. 8, pp. 3025-3029.

SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks). 2009, *Risk assessment of products of nanotechnologies*, European Commission.

Scott-Fordsmand, J.J., Krogh, P.H., Schaefer, M. and Johansen, A. 2008, "The toxicity testing of double-walled nanotubes-contaminated food to Eisenia veneta earthworms", *Ecotoxicol.Environ.Saf.*, vol. 71, no. 3, pp. 616-619.

Engineered Nanoparticles: Review of Health and Environmental Safety

- Smith, C.J., Shaw, B.J. and Handy, R.D. 2007, "Toxicity of single-walled carbon nanotubes to rainbow trout, (*Oncorhynchus mykiss*): Respiratory toxicity, organ pathologies, and other physiological effects", *Aquatic Toxicology*, vol. 82, no. 2, pp. 94-109.
- Sorahan, T., Hamilton, L., van Tongeren, M., Gardiner, K. and Harrington J.M. 2001, "A cohort mortality study of U.K. carbon black workers, 1951-1996", *Am J Ind Med*, vol. 39, pp. 158-170.
- Sun, H.W., Zhang, X.Z., Niu, Q., Chen, Y.S. and Crittenden, J.C. 2007, "Enhanced accumulation of arsenate in carp in the presence of titanium dioxide nanoparticles", *Water Air and Soil Pollution*, vol. 178, no. 1-4, pp. 245-254.
- Van Hoecke, K., De Schamphelaere, K.A.C., Van der Meeren, P., Lucas, S. and Janssen, C.R. 2008, "Ecotoxicity of silica nanoparticles to the green alga *Pseudokirchneriella subcapitata*: Importance of surface area", *Environmental Toxicology and Chemistry*, vol. 27, no. 9, pp. 1948-1957.
- Vevers, W.F. and Jha, A.N. 2008, "Genotoxic and cytotoxic potential of titanium dioxide (TiO₂) nanoparticles on fish cells in vitro", *Ecotoxicology*, vol. 17, no. 5, pp. 410-420.
- Usenko, C.Y., Harper, S.L. and Tanguay, R.L. 2008, "Fullerene C-60 exposure elicits an oxidative stress response in embryonic zebrafish", *Toxicol.Appl.Pharmacol.*, vol. 229, no. 1, pp. 44-55.
- Wu Q *et al.* 2008, *The Behaviour of Aerosols Released to Ambient Air from Nanoparticle Manufacturing - A Pre-normative Study*, NANOTRANSPORT project publishable final activity report, EU project NMP4-CT-2006-033371. Accessed at: http://research.dnv.com/NANOTRANSPORT/NANOTRANSPORTdownload/A-NANOTRANSPORT-publisable_f.doc (16th October 2009)
- Yeo, M.K. and Kang, M. 2008, "Effects of nanometer sized silver materials on biological toxicity during zebrafish embryogenesis", *Bulletin of the Korean Chemical Society*, vol. 29, no. 6, pp. 1179-1184.
- Zhang, X., Sun, H., Zhang, Z., Niu, Q., Chen, Y., Crittenden, J.C. (2007). Enhanced bioaccumulation of cadmium in carp in the presence of titanium dioxide nanoparticles. *Chemosphere*, vol. 67, pp. 160-166.
- Zhu, X.S., Zhu, L., Duan, Z.H., Qi, R.Q., Li, Y. and Lang, Y.P. 2008a, "Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to Zebrafish (*Danio rerio*) early developmental stage", *Journal of Environmental Science and Health Part A-Toxic/hazardous Substances and Environmental Engineering*, vol. 43, no. 3, pp. 278-284.
- Zhu, X.S., Zhu, L., Lang, Y.P. and Chen, Y.S. 2008b, "Oxidative stress and growth inhibition in the freshwater fish *Carassius auratus* induced by chronic exposure to sublethal fullerene aggregates", *Environmental Toxicology and Chemistry*, vol. 27, no. 9, pp. 1979-1985.

APPENDICES

APPENDIX 1: REVIEW METHODOLOGY

The aim of the ENRHES review was to conduct a comprehensive and critical appraisal of the available health and environmental safety data of four types of nanomaterials: fullerenes, carbon nanotubes (CNT), metal and metal oxide nanoparticles.

A1.1 CO-ORDINATED INFORMATION MANAGEMENT

Information was collected from the peer-reviewed literature in the public domain that was relevant to the health and environmental safety of each of the four material types.

At the outset of the review, the project team agreed a set of search terms for each work package theme (physico-chemical characterisation; production, use and exposure; toxicology; epidemiology; ecotoxicology; and environmental fate and behaviour). These were derived from recognised standard terminology and nomenclature (e.g. documents from the BSI nano 9 collection, CEN ISO/TS 27687 and ASTM E2456-06) and the US National Library of Medicine's Medical Subject Headings. These terms were converted into a specific Boolean search strategy (using the agreed search terms in combination with logic operators e.g. AND, OR, NOT) for each theme. The structured literature searches for all WPs were completed by the end of December 2008. No structured Boolean literature searching was undertaken after this date. However, authors maintained a general awareness of the literature relating to their technical area and tasks throughout the writing stages and any newly published literature of high relevance was considered for inclusion based on meeting the following criteria: i) the content appeared to provide novel information within a particular area that was not previously encountered within the literature; or ii) the content was able to provide confirmation of findings of other investigators.

The preliminary Boolean searches for each theme were undertaken in web-based sources, namely PubMed and Web of Knowledge. All searches and search results (in terms of numbers of references obtained) were recorded using Microsoft Excel spreadsheets. In the majority of cases, the initial searches resulted in the collection of extremely high numbers of papers, including a high proportion of irrelevant literature. This was due to the relatively broad initial search strategy employed. The Boolean search strategy for each theme was therefore further refined through assessment of the references and consultation with the review task leads.

The refined Boolean searches were then carried out and the obtained references imported into the online reference management software 'RefWorks', categorised according to each theme. Through utilising the expertise of the report authors, the Refworks lists were further complemented with:

- i) additional literature that was known to be relevant to the area under investigation, but had not been obtained through the Boolean search strategy;
- ii) other papers of interest, brought to the attention of the authors through reading of the available literature contained in the RefWorks lists;
- iii) results of more specific PubMed/Web of Knowledge searches, required to more fully develop the understanding of particular aspects of the literature, or provide further confirmation of findings of other investigators.

Following removal of reference duplicates within the individual Refworks folders, the review task leaders then selected a final set of references after initial assessment of the title and/or abstracts. These were converted into final electronic reference lists within RefWorks for each of the review tasks. Links to these lists were included in a Wiki platform, creating a shared reference resource for the project that allowed authors to access references from any of the sourced literature as required.

Provided in Table A1.1 are the number of papers obtained through the initial Boolean searches for each review theme, followed by the number of papers obtained after refinement of the search terms and final selection by the review task leads.

Table A1.1: Summary of number of references obtained

Review Theme	No. of papers from initial search	No. of papers after search term refinement	No. of papers identified of possible value for review *
Nanoparticle Characterisation	~ 25, 000	1,815	240
Exposure	~ 1,500	779	36
Fate and Behaviour	~ 40, 000	3,776	294
Toxicity	~19,000	558	111
Epidemiology	~ 14, 000	5,329	62
Ecotoxicity	~ 80, 000	6,105	89

* Additional references may have been identified independently by chapter authors

The implemented information management strategy served to assist the component activities of the review by means of establishing and maintaining a shared reference resource for the review that:

- constituted a common resource accessible remotely and in real time by all partners;
- employed a standardised and efficient searching process;
- added value by combining the reference-sourcing capacity of all partners;
- allowed cross-referencing of resources appropriate to any of the multiple review tasks;
- reduced duplication of reference sourcing.

A1.2 REVIEW ACTIVITY

The objectives of the review were to appraise information on the production, use and exposure to the target engineered nanomaterials, persistence, bioaccumulation, toxicity (i.e. PBT) and interactions of the engineered nanoparticles in living and environmental systems.

A1.2.1 Review of Materials Production, Use and Exposure

To establish the context of the hazard data for the review, literature on the production, use and exposure routes for the four material types was reviewed. Specifically, the following information was identified and reviewed:

- the types of nanomaterials in common production and their applications, by conducting a focussed survey with the nanotechnology industry. This was carried out by the Institute of Nanotechnology, to provide an objective and non-biased contribution to the review, that minimises the risk of conflicts of interest (e.g. commercial confidentiality issues from individual company representation) and maximises the benefit from integrating with relevant initiatives and recognised expertise. A more detailed methodology for this task is outlined in Appendix 2;
- methodological aspects of nanoparticle characterisation;
- the types of scenarios which may lead to human exposure and the nature of these exposures and their measurement;
- the transport of nanomaterials in indoor and outdoor air;
- the potential for materials to leach from soils to ground waters;
- the potential movement from soils to surface waters;
- the transport mechanisms in water bodies.

A1.2.2 Review of Toxicity Data

Available information pertaining to particle, ultrafine and nanoparticle toxicology across the four material types was reviewed, specifically literature relating to:

- methodological aspects;
- toxicokinetics of uptake (ingestion, inhalation, dermal adsorption and injection), distribution, metabolism and excretion of manufactured unfixed nanoparticles and nanotubes in and by the body. The relationship between route of exposure and toxicokinetics as well as subsequent toxicity was also investigated;
- persistence and bioaccumulation potential of nanoparticles and nanotubes in the body;
- evidence of genotoxicity and reproductive toxicity;
- differences in toxicokinetics and any subsequent toxicity posed by variations in nanoparticle size, physical structure, chemical composition;
- mechanisms of interaction of nanoparticles with cells and their components, and partitioning within and between tissues in organisms;
- mechanisms of nanoparticle induced toxicity in relation to nanoparticle physico-chemical characteristics (e.g. size, surface area, surface charge, length etc.), in order to generate an improved understanding of the potential for nanoparticles to induce toxicity. Many of the studies published to date focus on acute endpoints related to inflammation, but chronic hazards have also been considered where there was available information.

Due to the paucity of data in relation to human exposure and toxicology, animal and *in vitro* studies have also been used and interpreted as appropriate. Information from older papers, in relation to model pollution particles (e.g. carbon black) and occupational dusts has been expanded to allow the understanding of the relatively small literature on engineered nanoparticles. As nanotubes are reported to have fibre-like morphology, a short summary of the major toxicological issue relating to fibres has also been included, in order to put into context the concerns relating to nanotube hazard.

Where possible, special attention was provided to papers conducting dose response relationships that might be relevant for risk assessment purposes.

A1.2.3 Review of Ecotoxicity Data

The review of literature relating to the ecotoxicity of manufactured nanomaterials across the four material types, specifically pertaining to:

- the persistence, bioaccumulation and toxicity of manufactured nanoparticles in aquatic and terrestrial species, including invertebrates, vertebrates and plants. Special emphasis has been put on aquatic base set organisms used in the risk assessment of chemicals (i.e. fish crustacean, algae);
- differences in ecotoxicity posed by variations in nanoparticles' physico-chemical characteristics (e.g. chemical composition, size, shape), test conditions (e.g. static, renewal or continuous exposure) and organisms (e.g. species, ages, feeding).

Again, where possible, special attention was provided to papers conducting dose response relationships that might be relevant for risk assessment purposes.

A1.2.4 Review of Epidemiology and Human Studies Data

In reviewing the epidemiology and human studies data, we have examined:

- methodological aspects;
- published results on the epidemiology studies on carbon black and titanium dioxide industries;

- published data on environmental and occupational exposure to diesel exhaust particulates;
- epidemiological studies on particles with a known nano size range such as metals will be examined, including any available information on exposure to emerging engineered nanoparticles.

In all cases, case-reports of exposure-health effect relationships have been identified for each study, where possible. The available human-studies tend to be short-term inhalation studies on human volunteers. Most importantly, we have also examined information on human dosimetry by considering existing studies of mathematical models of particle deposition, including those related to nanoparticles.

A1.3 RISK ASSESSMENT APPRAISAL

The objective of this stage of the review was to perform a coherent evaluation of the feasibility of conducting a (regulatory) risk assessment for each material type and perform basic risk assessments to the extent possible based on the information presented within the review chapters. More specifically, the exercise has evaluated the extent to which a traditional risk assessment/chemical safety assessment can be made based on available information and the specific nature of the nanomaterials.

The risk assessment appraisal has been based upon the information presented within the review chapters and it was outside the scope of the exercise to study again the original literature. Consequently, also no further literature searching has been conducted and thus the cut-off date of December 2008 also applies to this section.

The exercise aimed to conduct a regulatory risk/safety assessment as would apply in relation to chemicals policy. This means that in principle the entire substance life cycle should be addressed, including manufacturing, downstream and consumer use of the substance on its own, in preparations and in articles, as well as the final disposal. However, the exercise has been further scoped based on the data available for the different life cycle stages. Specific applications (e.g. for pharmaceuticals, cosmetics, pesticides, biocides) are not normally considered in a regulatory chemical legislation risk/safety assessment and have therefore not been addressed. As data for physico-chemical hazards (flammability and explosivity) was not included in the review chapters, the risk assessment appraisal has not addressed these types of hazards/risks. It was also not the purpose to attempt suggesting classification and labelling of the studied nanomaterials. The risk assessment exercise has been conducted for Fullerenes, CNT, and the most data rich substances from the metal and metal oxide nanoparticle categories.

The basic risk assessments carried out in this report are inspired by the REACH Guidance on Information Requirements and Chemicals Safety Assessment (http://guidance.echa.europa.eu/docs/guidance_document/information_requirements_en.htm?time=1252482386), accessed 16th October 2009), following its general methods and structure. However, given the availability of information (which is not complying with the REACH requirements both in terms of serious lack of knowledge of use and exposure, as well as data on inherent properties), the detailed assessments have been adapted to the available information and taking into account the overall structure of the ENRHES report. In a way the assessments therefore more resembles a risk assessment as carried out under the old chemicals legislation (for "existing substances") where the authorities did an assessment based on the available information.

A qualitative and/or quantitative risk characterisation was conducted to the extent data allowed. Uncertainties and possible gaps in data and methodology have been identified and described. Based on the outcomes of the appraisal, recommendations for focus and further research have been elaborated and presented.

APPENDIX 2: INDUSTRY SURVEY METHODOLOGY AND DATA

As part of the ENRHES review, the Institute of Nanotechnology (IoN), in co-operation with the Institute of Occupational Medicine (IOM), designed and carried out a web-based survey with the central aim of determining the quantities of various nanomaterials being produced and used within industry, as well as the type of products in which they are currently being used. Some basic information was also collected from each company regarding their knowledge of health and safety issues. This information was used to inform our risk assessment considerations as part of the scientific review.

A2.1 METHODOLOGY

The survey was open from 04/02/2009 – 06/03/2009 and announcements and invitations to participate were sent to industry associations including CEFIC, the CIA, the NIA, ENTA and the Institute of Nanotechnology's database and its media contacts. The survey was also announced on the IoN website, IOM's Safenano bulletin and other online forums such as (accessed 16th October 2009):

- <http://www.nanopaprika.eu/>
- <http://www.nanotechwire.com/>
- Nano electronics Forum
(http://www.nanotech.re.kr/newsletter/TNDnewsletter_20090216_Forum.htm)
- Anchor Science LLC (<http://www.anchorscience.com/nanovip-part-2.html>)
- <http://www.nanoforum.org/>
- UK Nanotechnology Knowledge Transfer Network

The questionnaire was security enabled and all of the obtained information treated in the strictest of confidence and used only as part of the ENRHES review. No information about individual companies will be made public.

An example invitation letter and blank questionnaire are provided below in sections A2.1.1 and A2.1.2, respectively.

A2.1.1 Example invitation to participate letter

Dear Colleague,

As part of a European Commission FP7 project to review the health and environmental safety of engineered nanoparticles, the Institute of Nanotechnology (ION) is carrying out a short survey on nanomaterials produced, used and incorporated into products.

We would like to invite you to participate in this short survey.

The survey is web based and will only take a few minutes to fill in. It would be extremely helpful to us if you would take the time to complete the survey.

The survey is security enabled and all information will be treated in strictest confidence. No information about individual companies will be made public.

To access this survey please visit:

https://www.surveymonkey.com/s.aspx?sm=2nQfGy6Y3bVUosqFJzFLNg_3d_3d

Please note the deadline for completing the survey is **Friday 6th of March 2009**.

If you have any questions about the survey or how the results will be used, please contact me, Del Stark at +44(0)141 303 8444 or del.stark@nano.org.uk.

Thank you in advance for your participation.

Kind regards,

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The Institute of Nanotechnology is a registered Scottish Charity, No. SC025709

A2.1.2 Blank questionnaire

Introduction

The European Commission have funded a Coordination and Support Action (CSA) to review current information on the health and safety of nanoparticles. The project, called "Engineered Nanoparticles: Review of Health and Environmental Safety (ENRHES)", involves a survey to gather information from manufacturers and uses of nanomaterials to provide context for the scientific review and inform the risk assessment considerations.

****PROJECT AIM****

The overall aim of the project is to perform a comprehensive and critical scientific review of the health and environmental safety of fullerenes, carbon nanotubes (CNT), metal and metal oxide nanomaterials. The review will consider sources, pathways of exposure, the health and environmental outcomes of concern, in the context illustrating the state-of-the-art in the field and informing the regulation of the potential risks of engineered nanoparticles.

****ABOUT THIS SURVEY****

As part of this project, ION is carrying out a survey to determine the quantities of various types of nanomaterials produced and used, as well as the type of products in which they are used. We are also collecting some basic information from each company regarding knowledge held about health and safety issues. This information is intended to be used to inform our risk assessment considerations as part of the scientific review. The questionnaire is security enabled and all information will be treated in strictest confidence. No information about individual companies will be made public. The information provided will only be used as part of the ENRHES review project.

The survey is a web based survey which will only take a few minutes to fill in. It would be extremely helpful to us if you would take the time to complete the survey.

****DEADLINE FOR RESPONSES****

Please note the deadline for completing the survey is Friday 6th of March 2009.

****WHY PARTICIPATE?****

Information from industry is an important component of the project to complement the research literature being reviewed. Participants in this survey will receive a complementary electronic copy of the ENRHES Review at the end of the project.

****IMPORTANT INFORMATION ABOUT THE SURVEY****

Please note: you can only choose one material at a time and materials must be chosen in ALPHABETICAL ORDER to prevent data loss.

The questionnaire is security enabled and all information will be treated in strictest confidence. No information about individual companies will be made public.

If you have any questions about the survey or how the results will be used, please contact me, Del Stark at +44(0)141 303 8444 or del.stark@nano.org.uk.

After completing the survey you will be directed to the project's website (<http://nmi.jrc.ec.europa.eu/project/ENRHES.htm>) where you can find more information.

Part 2: Company information - (Mandatory)

Please provide contact information about your company

Company:
Address:
Address 2:
City/Town:
State/Province:
ZIP/Postal Code:
Country:
Email Address:
Phone Number:

Part 3: Please describe your organisation:

An individual person
Governmental body
University/higher education
Commercial organisation more than 250 employees
Commercial organisation less than 250 employees
Association (e.g. trade association, trade union)

Part 4: What is your company's main area of business?

Part 5: Country or countries where your organisation manufactures/imports/uses nanomaterials:

Part 6: What material(s) are manufactured/imported or used by your company?

Aluminium	Iron	Tungsten oxide
Aluminum oxide	Iron oxide	Yttrium oxide
Antimony oxide	Lanthanum oxide	Zinc
Barium carbonate	Magnesium	Zinc oxide
Bismuth oxide	Magnesium oxide	Zirconium oxide
Boron oxide	Manganese oxide	
Calcium oxide	Molybdenum	
Cerium oxide	Molybdenum oxide	
Chromium	Multi Wall Carbon	
Chromium oxide	Nanotubes	
Cobalt	Neodymium oxide	
Cobalt oxide	Nickel	
Copper	Nickel oxide	
Copper oxide	Niobium	
Double Wall Carbon	Palladium	
Nanotubes	Praseodymium oxide	
Dysprosium oxide	Samarium oxide	
Erbium oxide	Silicon oxide	
Europium oxide	Silver	
Fullerenes (C60)	Single Wall Carbon	
Fullerenes (C70)	Nanotubes	
Fullerenes (C76)	Tantalum	
Fullerenes (C78)	Terbium oxide	
Fullerenes (C84)	Tin	
Gadolinium oxide	Tin oxide	
Germanium oxide	Titanium	
Gold	Titanium dioxide	
Hafnium oxide	Titanium oxide	
Indium	Titanium oxide (Anatase)	
Indium oxide	Tungsten	

Part 6: If a MANUFACTURER/IMPORTER, please indicate the manufactured or imported quantity per annum:

Not a manufacture or importer
< 1 kg
1 - 10 kg
10-100 kg/annum
100-1000 kg/annum
1-10 tonnes/annum
10-100 t/a tonnes/annum
100-1000 t/a tonnes/annum
> 1000 t/a tonnes/annum
Other (please specify)

OR

If a USER (i.e. a person or entity who employs, applies, utilises or examines nanomaterials or undertakes similar activities involving nanomaterials as supplied), please indicate the used quantity per annum:

Not a user
< 1 kg
1 - 10 kg
10-100 kg/annum
100-1000 kg/annum
1-10 tonnes/annum
10-100 t/a tonnes/annum
100-1000 t/a tonnes/annum
> 1000 t/a tonnes/annum
Other (please specify)

Part 7: Please indicate the form under which the nanomaterial is used and/or put on the market:

(Preparation means a mixture or solution of two or more substances; article means an object which during production is give a specific shape, surface or design its function to a greater degree than does its chemical composition)

As a pure substance
In a preparation/mixture
In an article

10.10.1 Part 8: Also please indicate the nature of the substance/preparation/mixture:

powder
granulate
paste
solution
dispersion
Other (please specify)

Part 9: Please indicate:

the Sector of Use(s) (SU) for this substance:
the Process Categories(s) (PROC) for this substance:
the Product Category(s) (PC) for this substance:
the Article Category(s) (AC) for this substance:

Part 10: Does your company have an internal nanotechnology risk assessment programme?

Yes
Currently Under Development
No
Don't Know
Please describe, where information is available

Part 11: Is your company using any good practice guidelines?

Yes (examples in next question)
No
Under Consideration
Don't Know

Part 12: Which of the following guidelines does your company use?

European Commission Recommendation of 07/02/2008 on a Code of Conduct for Responsible Nanosciences and Nanotechnologies Research

ISO/TR 12885 Nanotechnologies — Health and safety practices in occupational settings relevant to nanotechnologies

BSI PD 6699-2:2007:-Nanotechnologies – Part 2: Guide to safe handling and disposal of manufactured nanomaterials

DuPont Nano Risk Framework

ASTM E2535 Standard Guide for Handling Unbound Engineered Nanoscale Particles in Occupational Settings

None

Other (please specify)

10.10.2 Part 13: Does your company/organisation gather or possess any exposure data to inform risk assessment?

Yes
Don't know
No

10.10.3 Part 14: If you answered “yes” or “don't know”, to question 13 above, would you mind if a member of the ENHRES team contacts you about potentially using exposure data in an anonymised way for our risk assessment consideration?

Yes
No

10.10.4 Final Questions:

Engineered Nanoparticles: Review of Health and Environmental Safety

Are there specific environmental, health and safety issues related to the nanomaterials produced by your company that you have considered?

Are there specific social, ethical or legal issues related to the nanomaterials produced by your company that you have considered?

Are there any other specific issues concerning the nanomaterials produced by your company that you would like to mention?

A2.2 SURVEY DATA

The following section provides further analysis of the survey data as described in the Materials Productions and Use Chapter of the report. The survey data is incomplete with CNT seeming to dominate, which does not relate to volume of product in the market at present or for the foreseeable future i.e. silica and carbon black products.

There is therefore concern that at least some of the information requested was company confidential and so what was received was limited and based on product of limited commercial quantity and value. It can be concluded therefore that the survey is not representative of manufacture and use of nanomaterials in the UK, EU or the US.

Since the survey did not yield a vast number of responses and therefore no statistical analysis could be obtained it was determined to use a simple tabular analysis of the survey data.

Table A2.1 provides a summary of the responses by organisation, business area and country. The term 'count' refers to the total number of responses for each field. Similarly, responses for each material were individually reported and have been summarised in Table A2.2.

Subsequently, the reported information for each specific material have been collated into a series of tables.

Table A2.1: Summary of responses by organisation, business area and country

Field	Option	Count
Organisation Type	Commercial organisation <250	7
	Commercial organisation > 250	3
	Association	1
	University	2
Business Area	Micro fabrication	1
	Standardisation	1
	R & D regarding fluid dynamics	1
	Research and education	2
	Thin film coatings	1
	Power cables and flexible pipes	1
	Nano medicine	1
	Additives of lubricants and fuels	1
	R & D and manufacture of powders for forensic applications	1
	Chemistry	1
	Specialty chemicals	1
	Nanotube and applications	1
	Chemicals for the pulp and paper industry	1
Manufacturing Country	Germany	1
	UK	2
	France	1
	USA	2
	Greece	1
	Sweden	1
Importing Country	UK	1
	Denmark	1
	Italy	1
	USA	1
	France	1
	Japan	1
Using Country	Belgium	1
	UK	2
	Romania	2
	Denmark	1
	India	1
	France	1
	USA	1
	Singapore	1

Table A2.2: Summary of responses by material

	MATERIAL TYPE												
	Nickel	Titanium dioxide	Iron oxide	Double wall carbon nanotubes	Silicon Oxide	Silicon dioxide	Multi Wall Carbon Nanotubes	Single Wall Carbon Nanotubes	Yttrium oxide	Zirconium oxide	Nanoclay (aluminium silicon oxide)	Tungsten disulphide	Magnetic materials
ORGANISATION TYPE													
Commercial organisation <250	1	1	1		1	1	3	2	1	1	1		
Commercial organisation > 250			1			1	1					1	
Association		1											
University				1									1
BUSINESS AREA													
Micro fabrication	1												
Standardisation		1											
R&D regarding fluid dynamics			1								1		
Research and education				1									1
Thin film coatings												1	
Power cables and flexible pipes					1								
Nano medicine		1					1	1	1	1			
Additives of lubricants and fuels			1										
R&D and manufacture of powders for forensic applications						1							
Chemistry							1						
Specialty chemicals							1	1					
Nanotube and applications							1						
Chemicals for the pulp and paper industry						1							

Nickel

Field	Option	Response	Count
Quantity	Manufactured or Imported	Tonnage options	
		No response	
	User	10-100 kg	1
Form	Pure substance		
	Prep/mixture		
	Article		1
Nature	Powder		
	Granulate		1
	Paste		
	Solution		
	Dispersion		
Sector of use (SU)	SU 12 – Manufacture of plastic products, including compounding and conversion		1
Process Categories (PROC)	PROC 3 – Used in closed batch process (synthesis or formulation)		1
Product category	PC 14 – Metal surface treatment products		1
Article category	AC 0 – Other	Polymer chips	1

Engineered Nanoparticles: Review of Health and Environmental Safety

Field	Response	Count	
Risk Assessment Programme	Yes		
	No	1	
	Currently under development		
	Don't know		
	No response to question		
Good practice guidelines	Yes		
	No		
	Under consideration	1	
	Don't know		
	No response to question		
Guidelines followed	EC		
	ISO		
	BSI		
	Dupont		
	ASTM		
	None	1	
	Other (specify)		
	No response		
Exposure data gathered	Yes		
	No	1	
	Don't know		
	No response to question		
EHS issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question	1	
Social, ethical or legal issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question	1	
Other specific issues	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question	1	

Titanium dioxide

Field	Option	Response	Count
Quantity	Manufactured or Imported	Tonnage options	
		No response	
	User	No response	2
Form	Pure substance		
	Prep/mixture		1
	Article		1
Nature	Powder		
	Granulate		
	Paste		1
	Solution		
	Dispersion		1
Sector of use (SU)	SU 01 – Other activity related to manufacturing of chemical products	No Details	2
	SU 20 – Health services		1
	SU 21 – Private households (general public , consumers)		1
	SU 22 – Public domain (administration, education, entertainment, services, craftsmen)		1
	SU 23 - Recycling		1
Process Categories (PROC)	PROC 0 – Other	No detail	2
Product category	PC 0 - Other	No detail	2
Article category	AC 0 – Other articles	No details	1
	AC 12.2 Constructional articles and building materials for outdoor use		1

Engineered Nanoparticles: Review of Health and Environmental Safety

Field	Response		Count
Risk Assessment Programme	Yes		
	No		
	Currently under development		2
	Don't know		
	No response to question		
Good practice guidelines	Yes		2
	No		
	Under consideration		
	Don't know		
	No response to question		
Guidelines followed	EC		2
	ISO		1
	BSI		1
	Dupont		
	ASTM		
	None		
	Other (specify)		
	No response		
Exposure data gathered	Yes		
	No		
	Don't know		2
	No response to question		
EHS issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		2
Social, ethical or legal issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		2
Other specific issues	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		2

Iron oxide

Field	Option	Response	Count
Quantity	Manufactured or Imported		
	User	1-10 kg	1
	User	> 1000 t	1
Form	Pure substance		1
	Prep/mixture		2
	Article		
Nature	Powder		1
	Granulate		
	Paste		
	Solution		1
	Dispersion		2
Sector of use (SU)	SU 01 – Other activity related to manufacturing of chemical products	No detail	1
	SU 8 – Manufacture of bulk, large scale chemicals		1
Process Categories (PROC)	PROC 0 – Other Process	No detail	1
	PROC 17 – Lubrication at high energy conditions in partly open processes		1
	PROC 16 - Using materials as fuel sources, limited exposure to unburned product to be expected		1
Product category	PC 0 – Other Products	No detail	1
	PC 24 – Lubricants, greases, and release products		1
	PC 13 – Fuels		1
Article category	AC 0 - Other Articles	No detail	1
	AC 1-2 Other vehicles		1
	AC 1-1 Passenger cars and motor cycles		1

Engineered Nanoparticles: Review of Health and Environmental Safety

Field	Response		Count
Risk Assessment Programme	Yes		1
	No		
	Currently under development		
	Don't know		
	No response to question		1
Good practice guidelines	Yes		1
	No		
	Under consideration		
	Don't know		
	No response to question		1
Guidelines followed	EC		
	ISO		1
	BSI		1
	Dupont		
	ASTM		
	None		
	Other (specify)		
	No response		1
Exposure data gathered	Yes		
	No		1
	Don't know		
	No response to question		1
EHS issues considered?	Yes	Action taken	1
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		1
Social, ethical or legal issues considered?	Yes	Action taken	1
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		1
Other specific issues	Yes	Action taken	
		No issues identified	1
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		1

Double wall carbon nanotubes

Field	Option	Response	Count
Quantity	Manufactured or Imported		
	User	< 1 kg	1
Form	Pure substance		
	Prep/mixture		1
	Article		1
Nature	Powder		1
	Granulate		
	Paste		1
	Solution		
	Dispersion		1
Sector of use (SU)	SU 12 – Manufacture of plastic products, including compounding and conversion		1
Process Categories (PROC)	PROC 15 – Use as laboratory reagent		1
Product category	PC 32 – Polymer preparations and compounds		1
Article category	AC 3-2 – Electrical batteries and accumulators		1

Engineered Nanoparticles: Review of Health and Environmental Safety

Field	Response	Count	
Risk Assessment Programme	Yes		
	No		
	Currently under development		
	Don't know		
	No response to question	1	
Good practice guidelines	Yes		
	No		
	Under consideration		
	Don't know		
	No response to question	1	
Guidelines followed	EC		
	ISO		
	BSI		
	Dupont		
	ASTM		
	None		
	Other (specify)		
	No response	1	
Exposure data gathered	Yes		
	No		
	Don't know		
	No response to question	1	
EHS issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		1
Social, ethical or legal issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		1
Other specific issues	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		1

Silicon Oxide

Field	Option	Response	Count
Quantity	Manufactured or Imported		
	User	10 – 100 kg	
Form	Pure substance		
	Prep/mixture		
	Article		1
Nature	Powder		1
	Granulate		
	Paste		
	Solution		
	Dispersion		
	Other	Glass Tube	1
Sector of use (SU)	SU 3 – Industrial manufacturing (all)		1
Process Categories (PROC)	PROC 23– Open processing and transfer operations at elevated temperatures		1
Product category	PC 0 – Other products	Optical Fibers	1
Article category	AC 3-1 – Electrical and electronic products		1

Engineered Nanoparticles: Review of Health and Environmental Safety

Field	Response		Count
Risk Assessment Programme	Yes		
	No		1
	Currently under development		
	Don't know		
	No response to question		
Good practice guidelines	Yes		
	No		1
	Under consideration		
	Don't know		
	No response to question		
Guidelines followed	EC		
	ISO		
	BSI		
	Dupont		
	ASTM		
	None		1
	Other (specify)		
	No response		
Exposure data gathered	Yes		
	No		1
	Don't know		
	No response to question		
EHS issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	1
	No	No issues identified	
		No detail specified	
	No response to question		
Social, ethical or legal issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	1
	No response to question		
Other specific issues	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	1
	No response to question		

Silicon dioxide

Field	Option	Response	Count
Quantity	Manufactured or Imported	> 1000 t	1
	User	10 – 100 kg	
Form	Pure substance		1
	Prep/mixture		2
	Article		
Nature	Powder		1
	Granulate		
	Paste		
	Solution		
	Dispersion		2
	Other		
Sector of use (SU)	SU 10 – Chemical formulation and/or re-packaging		1
	SU 6 – Manufacture of pulp, paper and paper products		1
	SU 16 – Manufacture of computer, electronic and optical products, electrical equipment		1
	SU 19 – Building and construction work.		1
	SU 21 – Private households (general public = consumers)		1
Process Categories (PROC)	PROC 3 – Using in closed batch processes		1
	PROC 5 – Mixing or blending in batch processes for formulation of preparation and articles		1
	PROC 9 – Transfer of substance or preparation into small containers		1
	PROC 4 – Use in batch and other processes where opportunity for exposure arises		1
	PROC 10 – Roller application or bursting of adhesive and other coating		1
	PROC 11 – Spraying outside industrial settings and/or applications		1
Product category	PC 0 – Other products	Forensics	1
	PC 9 – Coatings, paints, fillers, putties, thinners		1
	PC 10 – Building and construction preparations not covered elsewhere		1
	PC 20 – Products such ph-regulators, flocculants, precipitants, neutralisation agents, other unspecific		1
	PC 15 – Non-metal-surface treatment products		1
Article category	AC 0 – Other articles	Fingerprint development powders	1
	AC 8-2 Paper products: newspaper and packaging		1

Engineered Nanoparticles: Review of Health and Environmental Safety

Field	Response		Count
Risk Assessment Programme	Yes		
	No		
	Currently under development		2
	Don't know		
	No response to question		
Good practice guidelines	Yes		1
	No		
	Under consideration		1
	Don't know		
	No response to question		
Guidelines followed	EC		
	ISO		
	BSI		1
	Dupont		
	ASTM		
	None		1
	Other (specify)		
	No response		
Exposure data gathered	Yes		1
	No		1
	Don't know		
	No response to question		
EHS issues considered?	Yes	Action taken	1
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	1
	No response to question		
Social, ethical or legal issues considered?	Yes	Action taken	1
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	1
	No response to question		1
Other specific issues	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	1
	No response to question		

Multi Wall Carbon Nanotubes

Field	Option	Response	Count
Quantity	Manufactured or Imported	1-10 tonnes	1
		1-10 kg	1
		100 – 1000 kg	1
	User	Did not respond	1
Form	Pure substance		3
	Prep/mixture		3
	Article		1
Nature	Powder		3
	Granulate		1
	Paste		2
	Solution		1
	Dispersion		2
	Other		
Sector of use (SU)	SU 20 – Health Services		1
	SU 12 – Manufacture of plastics products, including compounding and conversion		2
	SU 11 – Manufacture of rubber products		1
	SU 16 – Manufacture of computer, electronic and optical products, electrical equipment		1
	SU 3 – Industrial manufacturing (all)		1
	SU 0 – 1 Other activity related to manufacturing chemical products	Research and Development	1
	SU 16 – Manufacture of computer, electronic and optical products, electrical equipment		1
	SU 5 – Manufacture of textiles, leather, fur		1
Process Categories (PROC)	PROC 0 – Other	No details	1
	PROC 1 – Use in closed process, no likelihood of exposure		1
	PROC 3 – Use in closed batch process		2
	PROC 2 – Use in closed systems, continuous process with occasional controlled exposure		1
	PROC 4 – Use in batch and other processes		1
	PROC 5 – Mixing and blending in batch processes for formulation of preparations and articles		1
	PROC 9 – Transfer of substances or preparation into small containers		1
	PROC 22 – Potentially closed processing operations at elevated temperature		1
Product category	PC 29 – Pharmaceuticals		1
	PC 32 – Polymer Preparations and Compounds		2
	PC 33 – Semiconductor		1
	PC 21 – Laboratory Chemicals		1
	PC 0 – Other products	RandD	1
Article category	AC 8-1 Paper Products		1
	AC 1-2 Other vehicles		2
	AC 3-2 Electrical batteries and accumulators		1
	AC 1-1 Passenger cars and motorcycles		2
	AC 3-1 Electrical and Electronic products		1
	AC 3-3 Electrical and electronic products : household appliances		1

Engineered Nanoparticles: Review of Health and Environmental Safety

Field	Response		Count
Risk Assessment Programme	Yes		
	No		
	Currently under development		2
	Don't know		
	No response to question		2
Good practice guidelines	Yes		1
	No		
	Under consideration		1
	Don't know		
	No response to question		2
Guidelines followed	EC		2
	ISO		
	BSI		
	Dupont		
	ASTM		
	None		
	Other (specify)		
	No response		2
Exposure data gathered	Yes		
	No		1
	Don't know		1
	No response to question		2
EHS issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		4
Social, ethical or legal issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		4
Other specific issues	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		4

Single Wall Carbon Nanotubes

Field	Option	Response	Count
Quantity	Manufactured or Imported	1 -10 kg	1
		10-100 kg	1
	User	Did not respond	1
Form	Pure substance		2
	Prep/mixture		2
	Article		
Nature	Powder		2
	Granulate		
	Paste		2
	Solution		
	Dispersion		
	Other		
Sector of use (SU)	SU 01 – Other activity related to manufacturing of chemical products	No details	1
	SU 01 – Other activity related to manufacturing of chemical products	RandD	1
	SU 16 – Manufacture of computer, electronic and optical products, electrical equipment		1
Process Categories (PROC)	PROC 0 – Other	No details	1
	PROC3 – Use in closed batch process		2
	PROC 5 – Mixing and blending in batch processes for formulation of preparations and		1
	PROC 22 – Potentially closed processing operations at elevated temperature		1
Product category	PC 0 – Other products	No Details	1
	PC 32 – Polymer preparations and compounds		1
	PC 21 – Laboratory Chemicals		1
	PC 33 – Semiconductor		1
Article category	AC 8-1 Paper Products		1
	AC 0 – Other activities	RandD	1
	AC 3-2 Electrical batteries and accumulators		1

Engineered Nanoparticles: Review of Health and Environmental Safety

Field	Response		Count
Risk Assessment Programme	Yes		
	No		
	Currently under development		2
	Don't know		
	No response to question		1
Good practice guidelines	Yes		1
	No		
	Under consideration		1
	Don't know		
	No response to question		1
Guidelines followed	EC		2
	ISO		
	BSI		
	Dupont		
	ASTM		
	None		
	Other (specify)		
	No response		1
Exposure data gathered	Yes		
	No		1
	Don't know		1
	No response to question		1
EHS issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	1
	No	No issues identified	
		No detail specified	
	No response to question		3
Social, ethical or legal issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		3
Other specific issues	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		3

Yttrium oxide

Field	Option	Response	Count
Quantity	Manufactured or Imported		
	User	Did not respond	1
Form	Pure substance		
	Prep/mixture		1
	Article		
Nature	Powder		
	Granulate		
	Paste		1
	Solution		
	Dispersion		1
	Other		
Sector of use (SU)	SU 01 – Other activity related to manufacturing of chemical products	No details	1
Process Categories (PROC)	PROC 0 – Other	No details	1
Product category	PC 0 – Other products	No Details	1
Article category	AC 0 – Other articles	No details	1

Engineered Nanoparticles: Review of Health and Environmental Safety

Field	Response	Count	
Risk Assessment Programme	Yes		
	No		
	Currently under development	1	
	Don't know		
	No response to question		
Good practice guidelines	Yes	1	
	No		
	Under consideration		
	Don't know		
	No response to question		
Guidelines followed	EC	1	
	ISO		
	BSI		
	Dupont		
	ASTM		
	None		
	Other (specify)		
	No response		
Exposure data gathered	Yes		
	No		
	Don't know	1	
	No response to question		
EHS issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
		No response to question	1
Social, ethical or legal issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
		No response to question	1
Other specific issues	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
		No response to question	1

Zirconium oxide

Field	Option	Response	Count
Quantity	Manufactured or Imported		
	User	Did not respond	1
Form	Pure substance		
	Prep/mixture		1
	Article		
Nature	Powder		
	Granulate		
	Paste		1
	Solution		
	Dispersion		
	Other		
Sector of use (SU)	SU 01 – Other activity related to manufacturing of chemical products	No details	1
Process Categories (PROC)	PROC 0 – Other	No details	1
Product category	PC 0 – Other products	No Details	1
Article category	AC 0 – Other articles	No details	1

Engineered Nanoparticles: Review of Health and Environmental Safety

Field	Response	Count	
Risk Assessment Programme	Yes		
	No		
	Currently under development	1	
	Don't know		
	No response to question		
Good practice guidelines	Yes	1	
	No		
	Under consideration		
	Don't know		
	No response to question		
Guidelines followed	EC	1	
	ISO		
	BSI		
	Dupont		
	ASTM		
	None		
	Other (specify)		
	No response		
Exposure data gathered	Yes		
	No		
	Don't know	1	
	No response to question		
EHS issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
		No response to question	1
Social, ethical or legal issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
		No response to question	1
Other specific issues	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
		No response to question	1

Other (free text specification)

Nanoclay (aluminium silicon oxide)

Field	Option	Response	Count
Quantity	Manufactured or Imported		
	User	1-10 kg	1
Form	Pure substance		1
	Prep/mixture		1
	Article		
Nature	Powder		1
	Granulate		
	Paste		1
	Solution		1
	Dispersion		1
Sector of use (SU)	SU 01 – Other activity related to manufacturing of chemical products	RandD samples for developing processes	1
	SU 9 – Manufacture of fine chemicals		1
Process Categories (PROC)	PROC 3 – Use in closed batch processes		1
Product category	PC 0 – Other Products	All products are applicable	1
Article category	AC 0 - Other Articles	All articles may be applicable	1

Engineered Nanoparticles: Review of Health and Environmental Safety

Field	Response		Count
Risk Assessment Programme	Yes		
	No		
	Currently under development		
	Don't know		
	No response to question		1
Good practice guidelines	Yes		
	No		
	Under consideration		
	Don't know		
	No response to question		1
Guidelines followed	EC		
	ISO		
	BSI		
	Dupont		
	ASTM		
	None		
	Other (specify)		
	No response		1
Exposure data gathered	Yes		
	No		
	Don't know		
	No response to question		1
EHS issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		1
Social, ethical or legal issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		1
Other specific issues	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		1

Tungsten disulphide

Field	Option	Response	Count
Quantity	Manufactured or Imported		
	User	< 1 kg	
Form	Pure substance		1
	Prep/mixture		1
	Article		
Nature	Powder		1
	Granulate		
	Paste		
	Solution		
	Dispersion		
Sector of use (SU)	SU 3 - Industrial Manufacturing (all)		1
Process Categories (PROC)	PROC 4 – Use in batch and other processes (synthesis) where opportunity for exposure arises		1
Product category	PC 14 – Metal surface treatment products, including galvanic and electroplating products		1
Article category	AC 1-1 Passenger cars and motor cycles		1
	AC 1-2 Other vehicles		1
	AC 2 Machinery and mechanical applications thereof		1

Engineered Nanoparticles: Review of Health and Environmental Safety

Field	Response		Count
Risk Assessment Programme	Yes		
	No		
	Currently under development		
	Don't know		
	No response to question		1
Good practice guidelines	Yes		
	No		
	Under consideration		
	Don't know		
	No response to question		1
Guidelines followed	EC		
	ISO		
	BSI		
	Dupont		
	ASTM		
	None		
	Other (specify)		
	No response		1
Exposure data gathered	Yes		
	No		
	Don't know		
	No response to question		1
EHS issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		1
Social, ethical or legal issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		1
Other specific issues	Yes	Action taken	
		No issues identified	
		No detail specified	
	No	No issues identified	
		No detail specified	
	No response to question		1

Magnetic materials

Field	Option	Response	Count
Quantity	Manufactured or Imported		
	User	10 – 100 kg	
Form	Pure substance		1
	Prep/mixture		1
	Article		
Nature	Powder		1
	Granulate		1
	Paste		
	Solution		1
	Dispersion		1
Sector of use (SU)	SU 17 - General Manufacturing		1
	SU 3 - Industrial Manufacturing (all)		1
	SU 7 - Printing and reproduction of recorded material		1
	SU 9 – Manufacture of fine chemicals		1
Process Categories (PROC)	PROC 4 – Use in batch and other processes (synthesis) where opportunity for exposure arises		1
	PROC 5 – Mixing or blending in batch processes for formulation of preparations and articles		1
	PROC 24 – High (mechanical) energy work-up of substances bound in materials and/or articles		1
	PROC 20 – Heat and pressure transfer fluids in dispersive use but closed systems		1
Product category	PC 1 – Adhesives , Sealants		1
	PC 7 - Base metals and alloys		1
	PC 16 – Heat Transfer Fluids		1
	PC 25 – Metal working fluids		1
	PC 33 – Semiconductor		1
Article category	AC 3-1 Electrical and electronic products		1
	AC 7-1 Metal Products		1

Engineered Nanoparticles: Review of Health and Environmental Safety

Field	Response	Count	
Risk Assessment Programme	Yes		
	No		
	Currently under development		
	Don't know	1	
	No response to question		
Good practice guidelines	Yes		
	No		
	Under consideration		
	Don't know	1	
	No response to question		
Guidelines followed	EC		
	ISO		
	BSI		
	Dupont		
	ASTM		
	None	1	
	Other (specify)		
	No response		
Exposure data gathered	Yes		
	No	1	
	Don't know		
	No response to question		
EHS issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	1
	No	No issues identified	
		No detail specified	
		No response to question	
Social, ethical or legal issues considered?	Yes	Action taken	
		No issues identified	
		No detail specified	1
	No	No issues identified	
		No detail specified	
		No response to question	
Other specific issues	Yes	Action taken	
		No issues identified	
		No detail specified	1
	No	No issues identified	
		No detail specified	
		No response to question	