Scientific Committee on Emerging and Newly Identified Health Risks

SCENIHR

Risk Assessment of Products of Nanotechnologies

Scientific Committees

- on consumer products
- on emerging and newly identified health risks
- on health and environmental risks

The SCENIHR adopted this opinion at its 28th plenary on 19 January 2009

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.

They are: the Scientific Committee on Consumer Products (SCCP), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Evaluation Agency (EMEA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCENIHR

Questions concerning emerging or newly-identified risks and on broad, complex or multi-disciplinary issues requiring a comprehensive assessment of risks to consumer safety or public health and related issues not covered by other Community risk-assessment bodies.

In particular, the Committee addresses questions related to potential risks associated with interaction of risk factors, synergic effects, cumulative effects, antimicrobial resistance, new technologies such as nanotechnologies, medical devices, tissue engineering, blood products, fertility reduction, cancer of endocrine organs, physical hazards such as noise and electromagnetic fields and methodologies for assessing new risks.

Scientific Committee members
Anders Ahlbom, James Bridges, Wim De Jong, Philippe Hartemann, Thomas Jung, Mats-Olof Mattsson, Jean-Marie Pagès, Konrad Rydzynski, Dorothea Stahl, Mogens Thomsen

Contact:

European Commission
Health & Consumers DG
Directorate C: Public Health and Risk Assessment
Unit C7 - Risk Assessment
Office: B232   B-1049 Brussels

Sanco-Sc1-Secretariat@ec.europa.eu

http://ec.europa.eu/health/ph_risk/risk_en.htm

ACKNOWLEDGMENTS

Members of the working group are acknowledged for their valuable contribution to this Opinion. The members of the working group are:

The SCENIHR members:

Prof. Jim Bridges

Dr. Wim De Jong (Chair and Rapporteur)

Dr. Thomas Jung

Prof. Konrad Rydzynski

External experts:

Dr. Jukka Ahtiainen[1], Finnish Environment Institute, Finland

Prof. Ken Donaldson, University of Edinburgh, United Kingdom

Dr. Teresa Fernandes, Napier University, United Kingdom

Prof. Jorma Jokiniemi, Technical Research Centre and University of Kuopio, Finland

Dr. Wolfgang Kreyling, GSF-Research Centre for Environment and Health, Germany

Prof. Francelyne Marano, Université Paris Diderot – Paris 7, France

Prof. Dik van de Meent, University of Nijmegen, The Netherlands

The additional contribution from the following expert is gratefully acknowledged:

Dr Thomas Kuhlbusch, Institut für Energie- und Umwelttechnik IUTA e.V., Duisburg, Germany

---

[1] Declared Interest (see minutes of the 26th SCENIHR plenary meeting of 23 September 2008): http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_mi_026.pdf

ABSTRACT

This Opinion deals with the recent developments in the risk assessment of nanomaterials for both man and the environment. The in-depth characterisation of a manufactured nanomaterial on the basis of its physical-chemical characteristics is essential. Due to the size and material specific temporal evolution of some nanomaterials, potentially hazardous nanomaterials need to be characterised both 'as manufactured' and in the various possible forms 'as delivered' in biological systems, or to a human in a specific application, or to a particular ecosystem of concern. The characterisation 'as manufactured' provides information for the material safety data sheet of the product itself. The characterisation 'as used' in biological systems is needed as properties of nanomaterials may change considerably, notably the size distribution due to agglomeration/aggregation of the particles. An issue of specific importance is the properties of the nanomaterial as it is actually used in products and to which consumers may be exposed. For the risk assessment the latter characterisation is of highest relevance.

Some specific hazards, discussed in the context of risk for human health, have been identified. These include the possibility of some nanoparticles to induce protein fibrillation, the possible pathological effects caused by specific types of carbon nanotubes, the induction of genotoxicity, and size effects in terms of biodistribution. Knowledge is gradually becoming available on the behaviour of manufactured nanoparticles in the environment in terms of the development of possible fate scenarios. For some nanomaterials, toxic effects on environmental organisms have been demonstrated, as well as the potential to transfer across environmental species, indicating a potential for bioaccumulation in species at the end of that part of the food chain. Although for some manufactured nanomaterials adverse effects were observed. they should not be extrapolated to other manufactured nanomaterials. These observations indicate potential hazards which should be taken into consideration in the safety evaluation of manufactured nanomaterials. As there is not yet a generally applicable paradigm for nanomaterial hazard identification, a case by case approach for the risk assessment of nanomaterials is warranted.

One of the main limitations in the risk assessment of nanomaterials is the general lack of high quality exposure data both for humans and the environment. A differentiation between background and incidental exposure is generally difficult in real life situations as the methods employed mainly measure the presence of (nano)particles and do not generally discriminate between the different types of particles (manufactured or naturally occurring) that may be present. Currently, the risk assessment procedure for the evaluation of potential risks of nanomaterials is still under development. It can be expected that this will remain so until there is sufficient scientific information available to characterise the possible harmful effects on humans and the environment. Therefore the knowledge on the methodology for both exposure estimations and hazard identification needs to be further developed, validated and standardised.

TABLE OF CONTENTS

EXECUTIVE SUMMARY

Currently, the procedure for assessing the potential risks of manufactured nanomaterials is still under development. It can be expected that this will remain so until there is sufficient scientific information available to characterise the possible harmful effects on humans and the environment. Therefore the knowledge on the methodology for both exposure estimations and hazard identification needs to be further developed, validated and standardised. As already detailed in previous SCENIHR opinions (SCENIHR 2006, SCENIHR 2007a), free and low solubility nanoparticles (nanomaterials) are a priority concern in the context of human and environmental risk. It should be realised that (especially for inhalation exposures) exposure to particulate matter may be due to natural and accidentally induced (i.e. combustion processes) nanoparticles.

For the characterisation of manufactured nanomaterials several issues are important. The nanomaterial should be characterised as it is produced by a manufacturer, resulting in information that may be used for safety evaluation and the material safety data sheet (MSDS) of the nanomaterial (nanoparticle) itself. In addition, the nanomaterial should be characterised as it is used in biological systems for safety evaluation. When nanomaterials come into contact with a biological fluid they may become coated with proteins and other biomolecules. The preparation of nanomaterials for use in biological systems may considerably change nanomaterial properties, notably the size distribution due to agglomeration/aggregation of the particles. Another issue is the characteristics of the nanomaterial as it is actually used in products, and to which consumers may be exposed. For the risk assessment the latter characterisation is of highest relevance. A consensus is now emerging regarding the physical-chemical properties that need to be determined in the characterisation of nanomaterials and which properties may be important in the risk assessment of nanomaterials. For (partially) soluble nanomaterials the toxicity may be governed at least in part by the soluble species released from the nanomaterial. For low solubility or a slow release, the particulate nature of the substance maybe relevant with regard to tissue distribution and local release of toxic species which should then be considered in the risk assessment of such nanomaterials.

There is a need for reference nanomaterials since this would allow the assessment of fate and behaviour as well as effects, which could then be related to the material's properties and characteristics. It would also allow comparisons between different nanomaterials. Some reference nanomaterials are available, but they are spherical model materials which are certified primarily for size and are used mainly to calibrate instruments which measure particle size. The absence of well-defined parameters to measure and standardise test protocols is identified as a major obstacle for reference material production.

Currently the definition of what is "nano" is still under debate. Generally nanomaterials are defined as being smaller than about 100 nm in at least one dimension. The currently proposed definitions use the size of the primary particle/structure as a starting point. However, when a nanomaterial is in particulate form, the particles may be present as single particles but might also be present as agglomerates/aggregates. Depending on the nanomaterial, the majority of the particulates may actually be agglomerates/aggregates. This may lead to the misinterpretation that agglomerates/aggregates of nanoparticles that have dimensions well beyond the 100 nm size are not considered nanomaterials. Yet they retain specific physicochemical properties which are characteristic for nanomaterials, most likely due to their relative large specific surface area (SSA). Therefore, when describing a nanomaterial it is important to describe not only the mean particle size but also the size of the primary particles. In addition, information on the presence of agglomerates/aggregates should be presented. When the mean particle size deviates (i.e. is larger) from the primary particle size this would indicate the presence of agglomerates/aggregates. In addition to size the specific surface area as determined by BET method is a good metric to describe particulates as this metric is independent of the primary versus the agglomerated state. Hence, extending the current definition based on physical size by the addition of a limit of the specific surface area to be above 60 m²/g of

material volume (the value of 60 m²/g corresponds to the specific surface area of 100 nm solid spheres of unit density) should be considered.

One of the main limitations in the risk assessment of nanomaterials is the general lack of high quality exposure and dosimetry data both for humans and the environment. One of the issues is the difficulty in determining the presence of nanomaterials, and properly measuring them on a routine basis in various substrates. In contrast to the situation for other exposure routes, for air-borne nanomaterials, analytical instruments are generally available to determine exposure (size distribution of mass and number). This is particularly true in the context of test atmospheres. However, differentiation between background and incidental exposure is generally not possible in real life situations as the methods employed mainly measure the presence of (ultrafine) particles and do not discriminate between the different types of particles that may be present. There is a need to further establish reliable and standardised measurement techniques, to develop measurement strategies, and to further implement screening/monitoring of nanoscale particles in sensitive work areas. Challenges are currently seen, especially in the detection and assessment of manufactured nanoparticles in the environment. Similarly, exposure estimates for consumers from food and consumer products remains difficult. Information on the presence of manufactured nanomaterials solely relies on information (claims) provided by manufacturers. In addition, exposure estimation is also hampered by lack of information on product use and use of multiple products containing manufactured nanomaterials. In a similar fashion to air measurements, determination of manufactured nanomaterials in consumer products suffers from the difficulty in discriminating between background and intentionally added manufactured nanomaterials. Coordinated efforts and research strategies for a comprehensive exposure assessment of manufactured nanomaterials still have to be defined.

When nanomaterials contact a biological fluid they may become coated with proteins and other biomolecules. As the protein coating may affect the nanoparticle behaviour including its biological effect, the nanoparticle may also have an effect on protein behaviour. Nanoparticles were found to have the potential to promote and to retard protein assembly into amyloid fibrils *in vitro.* These experiments were performed using an incubation of nanoparticles with certain purified proteins. Whether the observed nucleation process also occurs in an *in vivo* situation or in more complex biological fluids where competitive binding may take place remains to be determined.

It should be noted that from the lung and gastrointestinal tract only minimal amounts (approximately 1% or less of the administered dose) enter the systemic circulation. However, although minimal in percentage, this may result in a systemic availability of a considerable number of nanoparticles. The liver and the spleen are the two major organs for distribution. For certain nanoparticles all organs may be at risk as, for all organs investigated so far, either the chemical component of the nanoparticles or the nanoparticles themselves could be detected, indicating potential nanoparticle distribution to these organs. These organs include the brain and the reproductive system (i.e. testis). For distribution to the foetus *in utero* contradictory results were observed. The knowledge on toxicokinetics has been increased showing that especially the smaller nanoparticles do have a much wider organ distribution than the larger nanoparticles. There are indications that after deposition at the olfactory mucosa of the nose nanoparticles may translocate into the brain. This may offer a potential route of entry for medicinal products into the brain. On the other hand this observation may also raise some concern in view of the amyloid diseases of the brain in the context of the potential of nanoparticles to cause protein fibrillation *in vitro*. This is certainly an area for which additional research is urgently needed.

Based on the observations on the effects of particulate matter present in air pollution, some concern exists about the possible effect of manufactured nanoparticles on the cardiovascular system. However, this has not been clearly demonstrated to be the case for manufactured nanoparticles so far. Overall the information on the possible hazard of nanoparticles for cardiovascular effects is rather limited and needs expansion.

When nanotubes were found to have similar characteristics to some types of hazardous asbestos, it was demonstrated that similar inflammatory reactions can be induced by these specific nanotubes as induced by asbestos. The main characteristics of substances which induce these reactions are long thin fibrous forms (length >20 micrometer), rigidity, and no degradability (biopersistence). Whether such nanotubes would pose a risk for humans is unknown, as in addition to these specific nanomaterial characteristics, inhalation exposure to such structures would be essential. The main conclusion of the studies on these specific carbon nanotubes relating to a risk for mesothelioma is that such a risk cannot be excluded. So, when manufacturing nanotubes (possibly of any chemical composition) one should be aware that certain characteristics (e.g. length, rigidity, biopersistence) may pose a risk. The possibility for chronic inflammation and mesothelioma induction should therefore be considered in the safety evaluation of that particular manufactured nanomaterial.

The genotoxic effects of conventional particles are driven by two mechanisms – direct genotoxicity and indirect (inflammation-mediated) genotoxicity. Nanoparticles may act via either of these pathways since they cause inflammation and can also enter cells and cause oxidative stress. There is some evidence that the small size allows nanoparticles to penetrate into sub-cellular compartments like the mitochondria and the nucleus. The presence of nanomaterials in mitochondria and the nucleus opens the possibility for oxidative stress mediated genotoxicity, and direct interaction with DNA, respectively. For some manufactured nanomaterials genotoxic activity has been reported, mainly associated to ROS generation, while for others contradictory results were obtained.

In view of the increasing production, use and disposal of manufactured nanomaterials, there will be an increase in environmental exposure of these materials. As in the case of human health risks, understanding the fate and behaviour of the manufactured nanomaterials in the environment is crucial for predicting the potential ecotoxic effects in various environmental species. Of major importance is the estimation of nanoparticle release and fate, and exposures within the environment. For the environmental risk assessment the estimation of water concentrations is essential. The assessment of exposure concentrations of dispersed nanomaterials requires detailed insight into the processes that act on the particles in the environment. However, currently available knowledge of these processes is insufficient to allow quantitative predictions of the environmental fate of nanomaterials.

The solubility of the nanomaterials is an important property that needs to be addressed. Knowledge of the extent to which nanomaterials dissolve and the rate at which this takes place is essential in two respects: (i) it is a direct control of the concentrations of nanomaterials in the environment and of the time that the nanomaterials reside in the environment and in organisms, and (ii) it determines the concentrations of dissolved species that originate from the nanomaterials. It is doubtful whether currently available standard methods for measuring the (rate of) dissolution can adequately deliver this knowledge.

Unlike in the assessment of exposure concentrations of conventional (dissolved) chemical substances, the octanol-water partition coefficient K_{ow} is likely to have a limited role in predicting water-solids partitioning. An alternative theory to predict the exposure levels of nanomaterials in water is yet to be developed. Based on well-established knowledge of colloid science, it is expected that pH, ionic strength and presence of natural organic matter in the water compartment (freshwater versus marine environments) are important factors influencing the residual levels of nanomaterials in suspension. Depending on these factors and the chemistry of the manufactured nanomaterial, increased aggregation and thus sedimentation or in contrast enhanced dispersion may occur.

In addition, for many manufactured nanomaterials the currently used methods (carbon dioxide production, integration into biomass) for determining biological degradation will not be applicable.

For some nanomaterials (i.e. quantum dots) the transfer across environmental species was demonstrated indicating the potential for bioaccumulation via the food chain. For simple organic chemicals, there is an established relationship between octanol water partition coefficient (K_{ow}) and bioaccumulation or bioconcentration factor (BCF). However, it is not known whether this relationship may be applicable for nanomaterials, as insufficient data are available to evaluate this and more data are needed.

Ecotoxic effects on environmental species have been demonstrated, especially using aquatic species. One of the major problems in ecotoxicological fate and effects testing is the absence of consistent and broadly-applicable information on how nanomaterials are to be suspended or dispersed in various exposure media commonly used in ecotoxicological testing. Mixing of nanomaterials with sediments/soils, as well as characterisation over time, are areas which are still at a very early stage of development. The common endpoints used in ecotoxicology such as mortality, growth, feeding, and reproduction can also be used for the evaluation of ecotoxicity by nanomaterials. In addition, specific biomarkers similar to mammalian toxicity including oxidative stress, genetic damage and gene expression may provide some insight in toxic mechanisms of nanomaterials.

The health and environmental hazards were demonstrated for a variety of manufactured nanomaterials. The identified hazards indicate potential toxic effects of nanomaterials for man and environment. However, it should be noted that not all nanomaterials induce toxic effects. Arguably, some manufactured nanomaterials have been in use for a long time (carbon black, TiO_2) and show low toxicity. The hypothesis that smaller means more reactive and thus more toxic cannot be substantiated by the published data. In this respect nanomaterials are similar to normal substances in that some may be toxic and some may not. As there is not yet a generally applicable paradigm for nanomaterial hazard identification, a case by case approach for the risk assessment of nanomaterials is recommended.

1. BACKGROUND

Products of nanotechnologies are considered to bring benefits to everyday life of citizens and to offer challenges for better optimisation of use of natural resources and protection of the environment. They are already being marketed in sectors such as healthcare (targeted drug delivery, regenerative medicine, and diagnostics – as indicated by patent analysis[2]), electronics, cosmetics, textiles, information technology, and environmental protection. With the rapidly evolving process technologies, mass productions of nanomaterials will take place implying also potential wide scale exposure of workers and consumers as well as the environment.

The European Union has in its Strategy and Action Plan for nanosciences and nanotechnologies provided for developing the means to benefit from the potential of nanotechnologies, but also to do this in a "safe, integrated, and responsible" way. A review of the Community legislation in relation to nanomaterials was published. The objective of safe, integrated and responsible development of nanotechnologies is also pursued in the 7th Framework Programme for Research and Technological development for 2007-2013, activities of the Joint Research Centres, national research programmes, in the European Technology Platforms (ETPs) and research by industry and other stakeholders. Internationally, the co-operation for the safety of nanotechnologies is also taking place, especially with respect to activities in OECD, standardisation in ISO/CEN, pharmaceutical products in Trans-Atlantic co-operation and for medical devices in the Global Harmonisation Task Force.

In its opinion of 2006, the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) concluded that nanomaterials may have different toxicological and (eco)toxicological properties than the substances in bulk[3] form. Therefore their risks need to be assessed on a case-by-case basis and the risk assessment methods and instruments may require further development.

A second SCENIHR opinion, adopted on 21-22 June 2007, on the nanomaterials in Technical Guidance Documents (TGDs) of chemicals legislation concluded that the current methodologies described in the TGDs are generally likely to be able to identify the hazards, but modifications are required for the guidance on the assessment of risks to human health and the environment. Furthermore, the opinion highlights the need to determine the appropriateness of current test procedures for the prediction of human health hazards and estimation of risks for all types of nanoparticles/materials. Depending on the regulatory environment, the roles and involvement of different parties and stakeholders, the scope and responsibilities for development and implementation of risk assessment of nanomaterials vary across sectors/areas. It is therefore useful to substantially contribute to a thorough exchange of scientific information across sectors/areas. Consequently, it is envisaged to either make use of existing, or organising on a case-by-case basis, events or other suitable exchange mechanisms with all interested parties to enhance exchange of the evolving scientific information from various sources in the area of risk assessment of nanomaterials.

Hence, the Commission considers it important that this process benefits from, and is supported by, the expertise that the Scientific Committees have built up in their opinions over recent years. Therefore the SCENIHR is expected to update and provide scientific advice on the risk assessment of nanomaterials in the light of new and upcoming

---

[2] See recent OECD-Document entitled "*New patent analysis captures nanotechnology's current state of development 15-Jun-2007*". This new STI Working Paper (2007/4) aims to capture current inventive activities in nanotechnologies based on the analysis of patent applications to the European Patent Office (EPO) - http://www.oecd.org/dataoecd/6/9/38780655.pdf.

[3] In particle toxicology, the term "bulk" is often used to distinguish nanoparticles to larger particles of the same chemical substance. Equally relevant is the comparison of the nanoparticulate form of a chemical with the free (atomic, ionic, molecular) gaseous or dissolved species. All possible species (gaseous/dissolved, nanoform, aggregates/agglomerates and conglomerates with other materials) may play a role in the way nanomaterials affect organisms. In this text, the term "bulk" is used to refer to all non-nano species of a nanomaterial.

scientific information, including the outputs of various events on the Safety of Nanomaterials and the opinions of other Community Scientific Committees and groups, including the European Group on Ethics (here especially: Opinion on Ethics of Nanomedicine), on substances by the European Chemicals Agency (ECHA), on food and feed by the European Food Safety Authority (EFSA) and on pharmaceuticals by the European Medicines Agency (EMEA). The scientific opinions will also provide inputs to various Commission activities. Based on these Commission activities a further contribution to various activities at European and international level (i.e. in OECD, ISO/CEN, and the EU-US Partnership activities) in the area of risk assessment of nanomaterials is envisaged.

2. TERMS OF REFERENCE

The SCENIHR is asked:

To identify and assess new information and update the opinions of the SCENIHR on potential risks of products of nanotechnologies, in particular, with respect to characterisation, eco-toxicology and toxicology as well as exposure assessments. This update should be done in a step-wise manner taking into account the upcoming risk assessment demands related to specific nanomaterials and the evolving scientific information from various sources, including results from scientific research projects and activities of the European Technology Platforms related to the safety of nanomaterials. The update should:

i)   *Provide, on the basis of the results obtained, recommendations on:*

➢ *improvements of existing test methods and/or on the development of new ones, including in vitro and in vivo methods, to address aspects specific to nano in characterisation and hazard assessment;*

➢ *improvements in exposure assessment (including, amongst others, also relevant information on sampling, detection tests, instrumentation, modelling) to address aspects specific to nano and provide a list of specific nanomaterials/particles with possible substantial exposure noting current activities within the OECD Working Party on Manufactured Nanomaterials;*

➢ *improvements in risk assessment in general including specifically information linked to mechanistic information to address aspects specific to nano.*

ii) *Recommend further prioritised needs for short, medium and long-term research in areas related to the possible risks of products of nanotechnologies based on a knowledge gap closure analysis.*

iii) *Identify, as much as possible scientific evidence permits, direct or indirect health risks with regard to current and foreseeable applications of nanomaterials based on information related to volume of production in different sectors. For the sector of cosmetics and medical devices indications from patents[4] should also specifically be taken into account. Risks and specificities of different nanomaterials serving the same purpose shall, in as much as possible, be compared.*

It should be noted that the Commission may ask the SCENIHR and the SCCP to prepare ad hoc opinions on specific applications of nanomaterials in the field of cosmetics and medical devices and handle these as a matter of priority.

---

[4] See recent OECD-Document entitled "New patent analysis captures nanotechnology's current state of development 15-Jun-2007". This new STI Working Paper (2007/4) aims to capture current inventive activities in nanotechnologies based on the analysis of patent applications to the European Patent Office (EPO) - http://www.oecd.org/dataoecd/6/9/38780655.pdf.

3. Scientific Rationale

3.1. Introduction

Over the last few years, there has been an increase in awareness of the potential risks associated with manufactured nanomaterials. Legally, manufactured nanomaterials are covered by the definition of substances[5] as mentioned in the REACH legislation (Regulation (EC) No 1907/2006) (European Commission 2006). Risks associated with substances have to be evaluated according to various EU regulations depending on product category and production volume. A review of the European Community legislation in relation to nanomaterials has recently been finalised (COM/2008/0366 final) (European Commission 2008). The main conclusion was that the current legislation does cover in principle the potential health, safety and environmental risks in relation to nanomaterials. The protection of health, safety and the environment needs mostly to be enhanced by improving implementation of current legislation. In addition, it was concluded that the knowledge on essential questions such as characterisation, hazards, exposure, risk assessment and the risk management of nanomaterials needs to be improved (European Commission 2008).

To date, the SCENIHR has published three Opinions dealing with various aspects of the possible risks of the use of nanotechnology in all aspects of society. The first Opinion dealt with the risk assessment methodologies available for evaluating the possible adverse health and environmental effects of nanotechnology products (SCENIHR 2006), while the second and third described more technical aspects on how to properly investigate the safety of nanomaterials when using the Technical Guidance Documents for the evaluation of dossiers of chemical substances (SCENIHR 2007a), and what definitions within the nanotechnology area can be used for risk assessment (SCENIHR 2007b). It must be noted that nanotechnology has introduced new nanoparticulate forms of chemicals, of which properties, behavior and effects are largely unknown, and, hence, of concern. Although only two years have passed since the first evaluation of possible risks of nanotechnologies, there has been substantial activity in the evaluation of the harmful effects of nanomaterials, notably in the evaluation of potential toxic effects of nanomaterials by *in vitro* assays. Currently, *in vitro* assays are useful for screening purposes and may provide valuable insights into the underlying mechanisms of adverse effects. However, *in vitro* assays have their limitations, especially in relation to evaluation of a possible risk for humans and the environment. Therefore, at present, *in vivo* assays are still needed for risk assessment.

An important issue of appropriate safety evaluation is the choice of an exposure dose in the test system that is relevant for human or environmental nanomaterial exposure. In addition, there are still some uncertainties about the best dose metric to be used in safety evaluations and the risk assessment of manufactured nanomaterials.

Another lack of current safety evaluation of nanomaterials is the fact that most *in vitro* and *in vivo* studies are only short-term while impacts on human health and the environment are more likely to occur during and after long-term exposure. Consequently, there is an urgent need for long-term exposure studies.

There are indications that there is a steady increase in products produced by nanotechnology or containing nanomaterials that are available on the market. The inventory of the Woodrow Wilson International Centre for Scholars now contains almost 800 consumer products with a nanotechnology claim (WWICS 2008). A major drawback of this registration is that it is based on voluntary information and claims from the manufacturers, which in many cases cannot be verified.

---

[5] Substance: a chemical element and its compounds in the natural state or obtained by any manufacturing process, including any additive necessary to preserve its stability and any impurity deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition.

As a matter of example, one of the nanomaterials that has been increasingly applied is nanosilver, reported to be present in a great variety of products such as washing machines, socks, food contact material, wound dressings and food supplements (Wijnhoven et al. 2009, WWICS 2009). The possible use of nanoformulations for food supplements must be looked at carefully as it may be regarded either as potentially hazardous (EFSA 2008) or as potentially beneficial depending on the specific case. Increased bioavailability due to the nanoformulation of the supplement may be beneficial for some applications but may create the possibility of overdosing.

In fact, when nanomaterials are firmly embedded in large structures, for example in electronic circuits, they are less likely to escape this structure and no human or environmental exposure is likely to occur. However, while this may be true during production and appropriate use of nanomaterial-containing products, exposure may occur during abuse, waste and recycling. In other words without any exposure there is no risk. Hence the estimation of exposure scenarios in terms of their frequency, their quantity and quality, and their targets (individuals, populations, etc.) are absolutely mandatory for a rational risk assessment. It should be realised that (especially for inhalation exposures) exposure to particulate matter may be due to natural and accidentally induced nanoparticles (i.e. particulate air pollution by combustion processes).

Based on discussions in OECD and ISO working groups, a consensus is now emerging on the physical-chemical properties of nanoparticles that need to be addressed in the risk assessment process of nanomaterials (OECD 2008a). It should be noted that these properties should also be determined for the nanomaterials as used in the testing for safety evaluation, and not only on the nanomaterials as provided by the manufacturer. For most nanomaterials, a full evaluation of potential hazards has not yet been performed. Recently, the OECD has started a sponsorship programme in which, for 14 of the most used nanomaterials, a dossier on hazard identification will be produced (OECD 2008a). The programme contains an extensive list of endpoints to be determined including those for nanomaterial information/identification, physical-chemical properties, as well as material characterisation, environmental fate, environmental toxicology, mammalian toxicology, and material safety (OECD 2008a). For this evaluation, current OECD guidelines and other tests will be used. One of the outcomes of this programme will be insights into the suitability of the current OECD guidelines for hazard identification and where adaptations of these guidelines will be needed specifically for manufactured nanomaterials. This will contribute to the design of a testing strategy.

This Opinion deals with the recent developments in the area of risk assessment of nanomaterials. Some specific hazards have been identified which will be discussed in the context of risk for human health. These include developments in the understanding of toxicokinetics of nanomaterials, the possibility of nanoparticles to induce protein fibrillation, the possible pathological effects of specific types of carbon nanotubes, genotoxicity and size effects. Knowledge is becoming available on the behaviour of nanoparticles in the environment in terms of the development of possible fate scenarios. In addition, effects on environmental organisms have been demonstrated. The staged approach to the risk assessment of human and environmental risks as presented in a previous Opinion of SCENIHR (SCENIHR 2007a) will be elaborated on further.

3.2. Physical-chemical characterization and analysis

There is a general need for harmonisation of the methodologies used for the characterisation of nanomaterials. As a starting point, the detailed description of the nanomaterials is very important to assess the physical-chemical properties of the nanomaterials with regard to their potential adverse effects. This should include a description of the possible impurities or contaminants. Knowledge of the properties of the nanomaterials used is also necessary to be able to compare various studies. A consensus

is now emerging on which nanoparticle properties are important in the risk assessment of nanomaterials (OECD 2008a). It should be noted that these properties should be determined for the nanomaterials both as used in the testing for safety evaluation and as provided by the manufacturer.

Although nanomaterials themselves are covered by the definition of substance within the REACH legislation (Regulation (EC) No 1907/2006) (European Commission 2006), currently the definition of what is "nano" is still under debate. Various organisations have proposed definitions of nanoscale using an upper limit of about 100 nm. It should be noted that most currently proposed definitions use the size of the primary particle/structure as a starting point. However, when a nanomaterial is in particulate form, the particles may be present either as single particles or as agglomerates or aggregates. Depending on the nanomaterial, the majority of the particles may even be agglomerates or aggregates. This may lead to the misinterpretation that agglomerates or aggregates of nanoparticles that have external dimensions well beyond 100 nm are not considered nanomaterials. Yet they retain specific physicochemical properties characteristic of nanomaterials, most likely due to their large specific surface area (SSA). The uncertainty regarding the presence of nanomaterials (either determined by size, <100 nm, or SSA >60 m^2/g when calculated for <100 nm unit density spheres) in products becomes of major importance when the only information on the presence of a nanomaterial relies solely on the information provided by the manufacturer. Currently, it is frequently not possible to evaluate the nanomaterial contents of these products when the nanomaterial in question is mixed into a complex matrix of the finished product. This unresolved issue occurs in consumer products, particularly cosmetics and health care products, and also in food and feed products. All of these products contribute to the current exposure of the European population.

When describing a nanomaterial it is therefore important to describe not only the mean particle size but also the size of the primary particles. In addition, information on the presence of agglomerates and/or aggregates should be presented. When the mean particle size deviates (i.e. is larger) from the primary particle size this would indicate the presence of agglomerates/aggregates. This information should be included in the description of the nanomaterial and/or the product containing the nanomaterial. In addition to size, the specific surface area is a good metric to describe particulates. The specific surface area as determined by the BET method (Brunauer et al. 1938) has the advantage of being independent of the primary versus the agglomerated state.

Scientific toxicological data suggest that the total surface area of nanoparticles is a reasonable metric to describe toxicological responses in biological systems. The total surface area should not be confused with the specific surface area (SSA) where smaller particles have a larger SSA independent of whether they are present as primary, agglomerated or aggregated particles.

3.2.1. Characterisation of physical-chemical properties

Discussions are currently ongoing, both at OECD and ISO level, concerning the various characteristics of nanoparticles which need to be measured (OECD 2008a). The main parameters of interest with respect to nanoparticle safety are:

Physical properties
- Size, shape, specific surface area, aspect ratio
- Agglomeration/aggregation state
- Size distribution
- Surface morphology/topography
- Structure, including crystallinity and defect structure
- Solubility

Chemical properties

- Structural formula/molecular structure
- Composition of nanomaterial (including degree of purity, known impurities or additives)
- Phase identity
- Surface chemistry (composition, charge, tension, reactive sites, physical structure, photocatalytic properties, zeta potential)
- Hydrophilicity/lipophilicity

When nanomaterials are used in test systems, one has to be aware that some of the properties which need to be determined are largely dependent on the surrounding media and the temporal evolution of the nanomaterials. Thus, a primary focus should be to assess the nanomaterials in exactly the form/composition they have as manufactured, and in the formulation delivered to the end-user or the environment if the formulation contains free nanoparticles. Nanomaterials can exist as nanopowders; suspended in air (ultrafine particles, nanoparticles, aerosols), suspended in liquid (colloids) and incorporated in solids. For biological safety evaluation, manufactured nanomaterials need to be dispersed in an appropriate media. The interaction between these media and the nanomaterials can have a profound influence on the behaviour of the suspension.

With the increasing number of newly emerging manufactured nanomaterials the importance of the potential dissolution kinetics needs to be emphasised. Since dissolution kinetics is frequently proportional to the surface area, nanomaterials are likely to dissolve much more rapidly than larger sized materials. This applies e.g. to silver nanoparticles which are increasingly used for their release of silver ions as anti-bactericidal agents. Yet the dissolution kinetics is not properly studied. The example of silver nanoparticles highlights the complexity of risk estimates of nanomaterials since adverse interactions of the silver nanoparticles with biological systems need to be distinguished from those interactions of the ionic silver.

It should be emphasised that not all properties can be determined in every situation, nor is it necessary to do so.

3.2.2. Detection and analysis

Methods for the assessment of nanoparticles in the air (aerosols) and suspended in liquids or fluids have been further developed, and new methods have become available. Notably, similar to most advanced chemical analysis many of these methods involve research grade instruments requiring trained operating personnel and are not always straightforward to use in typical 'public health' settings. On the other hand, mobile and portable/handheld equipment is also becoming available, and an increasing number of studies have been performed and published in recent years (Mordas et al. 2008, Smith 2004). However, the wealth of these studies relates to the background of atmospheric nanoparticles, and little work in the context of manufactured nanoparticles has actually appeared. Furthermore, there is still a deficiency of comparable, reproducible and repeatable harmonised protocols for measuring and characterising nanomaterials (SCENIHR 2006). The ability to provide more routinely operable instruments, together with optimised protocols is important for providing meaningful and valid data that are comparable, reproducible, and repeatable, and which can produce a system of reliable risk identification, assessment and management. This requires defining the metrics most appropriate for hazard characterisation and exposure, including the methodology to perform the measurements. For a broader overview on the full portfolio of available methods for nanoparticle detection and analysis, the reader is refered to SCENIHR (2006).

For the measurement of particles in air, a number of methods are available. They have been developped since the 1980's, are very reliable and often highly sensitive, but sometimes costly. Depending on the physicochemical parameters of a nanomaterial and including off-line analyses, many companies provide instrumentation able to characterise airborne particles down to the nanometer range. Experience is also available in the field of electron microscopy and microanalysis of nanoparticles in tissue sections and precipitated on substrates (e.g. Geiser et al. 2005). Other measurement methods, in particular optical techniques such as light scattering (Lindfors et al. 2004), can be applied to suspensions in various gaseous and liquid media and to solid matrices. The particle dynamics in suspension depend strongly on the medium of suspension.

Absorption and scattering microscopy of single metal nanoparticles allow for the tracking of nanoparticles suspended in the liquid phase (Van Dijk et al. 2006). This technology resulted in new equipment with the capability of optical tracking and identification of metal nanoparticles in fluids. The use of Condensation Nucleus Counters, well established in aerosol science, can now be routinely used to obtain information on nanoparticles, but is still unable to discriminate the particles from the background.

In an analytical sense, the most powerful method, real-time single particle mass spectrometry, has been further developed to provide a reliable method for the assessment of nanoparticles suspended in gases and liquids (by Electrospray Ionisation) with potential applicability to other fluids (Kane et al. 2001, Noble and Prather 2000). Here, a mass spectrum suitable for chemical analysis of the components of individual nanoparticles including the surface layer can be sampled and analysed. At least two commercial set-ups are currently available. All these analytical techniques have their specific reliability and sensitivity profiles and typically need to be combined to obtain reliable and specific assessments. Therefore, special consideration needs to be given to each methodology to verify the characterisation of the nanomaterials in the various phases. Typically for high performance analytical techniques, a number of generic issues need to be considered in the application of these methods for a specific case (e.g. accuracy, specimen preparation, role of substrate and presence of contamination).

3.2.3. Nanomaterial preparations for biological testing

3.2.3.1. The importance of dispersion

When manufactured nanoparticles are analysed in a clean sample which does not contain any other material, their physical-chemical properties can be studied (using the many instruments which are commercially available) with the level of precision required for their targeted production and testing. However, if nanoparticles are mixed within a matrix of different materials, as is the case for scientific and technological applications, consumer products and in toxicological and ecotoxicological samples, then it becomes exceedingly difficult to identify those nanoparticles since they may occur only in parts per 10^6 to 10^{12} of the surrounding matrix. In effect, the nanoparticles become "needles in the hay stack" which are extremely laboursome to find, identify and characterise.

It is well known from colloid science that nanoparticles can form agglomerates or aggregates, especially when they are kept as powder under dry conditions. This tendency to aggregate can create difficulties when testing the toxicity of nanoparticles. However, despite their tendency to aggregate, nanoparticles do not usually change their specific surface area. The total surface area is an important parameter for interactions with biological systems. Usually, a dry powder or a suspension in a water-based medium or some other fluid is used to administer the nanoparticle into the biological system. Several studies have made suggestions as to how best disperse the nanoparticles (Bihari et al. 2008, Buford et al. 2007, Sager et al. 2007). Best protocols may vary between the different nanomaterials. It seems obvious that there should be a best attempt to render the nanoparticle in a size that is relevant to the expected consumer/population exposure.

Dispersal methodologies suggested for particles using rational approaches include the use of albumin, a fairly bland and ubiquitous globular protein (Bihari et al. 2008), and lung lining fluid phospholipids (Wallace et al. 2007). Researchers must be aware that these coatings may alter the properties of the nanomaterial being tested and, therefore, the biological activity under consideration.

Synthetic detergents such as polyoxyethylene sorbitan monooleate (Wick et al. 2007) and Tween (Warheit et al. 2003) have been used to disperse nanoparticles for experimental purposes. Researchers must be aware that these additions may be toxic by themselves or act as an antioxidant (e.g. Tween). These additions should be taken into consideration when characterising the nanomaterials prepared for testing.

3.2.3.2. Reference nanomaterials, characterisation and test item preparation

"Reference material" (RM) is the generic name for the materials having a proven and sufficient homogeneity and stability in terms of a defined intended use. "Reference substance" or "reference chemical" are terms used in toxicology for materials that need to meet similar conceptual requirements but that are used for hazard identification, usually under GLP. Reference materials (RMs) need to be produced and used applying the conditions and terms standardised and described in ISO guides 30 to 35. When used in toxicology as test items, principles of OECD GD 34 and GLP apply *mutatis mutandis* (OECD 2005).

RMs can be used for different purposes, such as calibration, assessment of laboratory proficiency or test method performance. In toxicological assays for hazard identification reference substances/materials may be used for comparison with both positive (toxic) and negative responses. Currently, a small number of reference materials already exist in the field of nanomaterials and manufactured nanoparticles (e.g. gold nanoparticles from the National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA and silica from the European Commission, Institute for Reference Materials and Measurements (IRMM), Joint Research Centre (JRC), Geel, Belgium). They are spherical model materials which are certified primarily for size and are used mainly to calibrate instruments which measure particle size. The absence of well-defined parameters to measure ("measurands") and of standardised test protocols is identified as a major obstacle for reference material production, because agreed and harmonised methods are needed.

A typical issue of information generated by measurements or studies is to combine a metrological part with knowledge about a reference material with an intended use in toxicological test systems under GLP. Toxicological test systems mimic routes (and scenarios) of exposure and typically require information about dosage. A study, examination or test, when successfully performed, generates a prediction of the effect of interest. In toxicological tests, reliability AND relevance both contribute to the overall predictive capacity and to the validity of a test for its purpose.

In practice, and in agreement with the requirements mentioned above, characterisation results should be obtained and used in their appropriate context scenarios. The information should be used for description of intrinsic and extrinsic properties. The metrological principles of the reference nanomaterials available so far cannot be used or extrapolated to toxicological tests and related results, but the information on the properties provides a reliable basis as starting point for the test and reference items used in such studies. The preparation and use of a reference material comprises two stages.

Stage 1 is the characterisation of the intrinsic properties of a reference nanomaterial, its stability and homogeneity. Physicochemical properties need to be determined. The physical state and preparation form of the material examined should thereby be relevant for production and use. Sample preparation steps corresponding to analytical sample preparation should be critically assessed with regard to being a determinant of the measurement result itself.

When a reference material is prepared for use in test systems for toxicological evaluation or environmental fate analysis, it will be brought into a matrix/media/vehicle depending on the type of test assays used. This comprises conditioning and choice of matrix components. The prepared test sample should thereby correspond to the requirements of the test method and preferably be representative for the identified exposure situation.

Stage 2 comprises the characterisation of properties following test sample preparation. Results depend on the protocol used and matrix components, which may be essential for a certain test system and form part of that test system. Several results may be gained for the same reference nanomaterial and its properties depending on the conditioning and matrix used.

Indeed, shape, size and surface area affect the hazards associated with nanomaterials, at least because these parameters affect the transport properties of the particles (absorption, distribution, and excretion). Reference nanomaterials have to be seen in their context of intended use. They are tools of Quality Assurance and method validation. They serve method harmonisation and standardisation, and performance assessment.

3.3. Developments in methodology to measure exposure

One of the major routes of exposure for humans is considered to be inhalation, for which a lot of information is available including exposure measurements. Exposure data are available for non-manufactured nanoparticles (often referred to as ultrafines) from combustion processes but these data are not specific to manufactured nanoparticles. Nevertheless, the knowledge gained from studies of combustion products may make extrapolation possible and allow tentative conclusions to be drawn for nanoparticle atmospheric transport and exposure in humans. In contrast to the situation for other exposure routes, for airborne nanomaterials, analytical instruments are generally available to determine exposure (size distribution of mass and number). This is particularly true in the context of test atmospheres. However, it remains difficult to differentiate background from incidental exposure in real life situations as these methods mainly measure the presence of (ultrafine) particles and do not discriminate between the different types of particles.

Exposure of humans and ecosystems may occur via the gas-, water-, and solid phases. The latter may include food and consumer products such as cosmetics. The uptake route, dose, and group of humans exposed must be differentiated in addition to the exposure matrix. For exposure measurements, three different groups are generally distinguished, namely workers, consumers, and the general public. In the case of workers, inhalation is generally the main route of exposure. In addition, consumers and the general public are increasingly exposed to nanomaterials in various consumer products via oral and dermal routes

It should be noted that part of the nanomaterials taken up by inhalation will result in a gastro-intestinal uptake due to the mucociliary mechanism present in the lung for particle removal. One key point, currently very often neglected in exposure and health effects studies, is the determination of the dose which can vary significantly. Taking airborne exposure as an example, exposure to manufactured particles with a median diameter of 90 nm leads to an overall internal dose of about 30-40% of the exposure value while the same value for 20 nm particles increases to 70-80% (according to the ICRP-model for a healthy worker) (ICRP 1994).

Figure 1 summarises the different measurement techniques and approaches possible for the assessment of exposure. It also gives the outline to measurement strategies since it differentiates personal and spatial (fixed point monitors) as well as continuous and discontinuous measurements. The limitations of measurement techniques directly influence measurement strategies. Generally, quite a few measurement techniques are

available to assess nanoparticles exposure including mass and number based techniques, single particle chemical analysis online/offline techniques etc. (Kuhlbusch et al. 2008a). The main handicap for making good exposure assessments is the lack of instrumentation to determine personal exposure that can continuously analyse single particles or their agglomerates/aggregates for chemical and physical properties relevant for health.
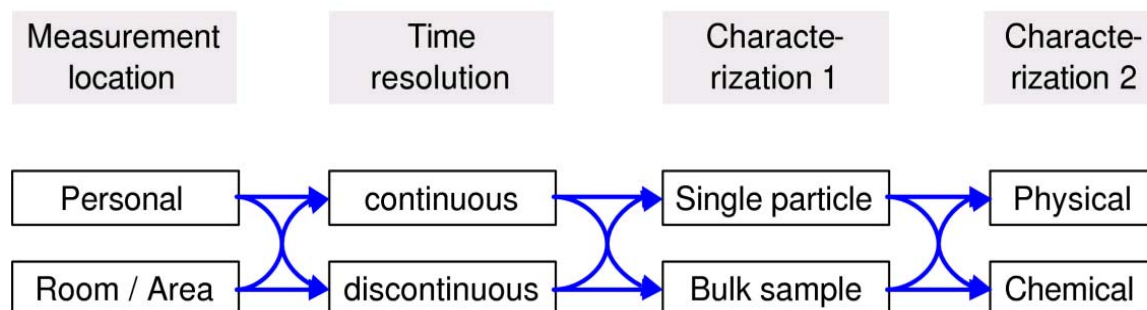
Measurement location	Time resolution	Characterization 1	Characterization 2
Personal	continuous	Single particle	Physical
Room / Area	discontinuous	Bulk sample	Chemical

Figure 1: Exposure related measurements (adapted from Borm et al. 2006)

Only a few papers have been published on measurement strategies which are currently necessary to allow first exposure assessments towards manufactured nanomaterials (Brouwer et al. 2004, Kuhlbusch et al. 2008b).

Currently most research and measurements have been conducted to assess the exposure of workers via inhalation. Data on airborne exposure are still scarce and do not always clearly differentiate ambient from manufactured particles (Fujitani et al. 2008,; Kuhlbusch et al. 2004, Kuhlbusch and Fissan, 2006, Kuhlbusch et al. 2008a, Kuhlbusch et al. 2008b, Maynard et al. 2004,Tsai et al. 2008, Wake et al. 2002, Yeganeh et al. 2008). In most cases it was seen that agglomerates of nanoparticles with diameters >400 nm are released during handling. In one case (Yeganeh et al. 2008), significant increases of sub-100 nm particle number concentrations during the handling of carbonaceous nanomaterials were reported. The latter indicates that coordinated measurement campaigns in various work areas are still needed to derive a comprehensive overview.

No quantitative or qualitative measurements of manufactured nanomaterials in ambient air outside of workplaces are known. Investigations by Murr (Murr et al. 2004, Murr and Guerrero 2006) revealed that carbon nanotubes may originate from general combustion processes and can be found in ambient locations. This illustrates the difficulty of identifying airborne manufactured nanomaterials.

Overall, the information base for exposure assessment in workplaces is currently built on a limited database which has to be improved in volume, comparability and reproducibility. This can be achieved by working on the feasibility of routine assessments, developing reliable measurement techniques, standardising measurement techniques, developing measurement strategies and implementing the screening and monitoring of nanoscale particles in sensitive work areas. Challenges are currently seen especially in the detection and assessment of product nanoparticles in the environment.

In addition to particle size and number, other metrics can be determined to express exposure. These include particle surface area, surface charge (zeta potential), surface area reactivity (radical formation, photo-catalysis, oxidation/reduction) etc. The choice of dose metrics depends on the endpoint of interest.

Exposure estimates from food and consumer products remains difficult. Information on the presence of manufactured nanomaterials solely relies on information (claims) provided by manufacturers. In addition, exposure estimation is also hampered by lack of information on product use and use of multiple products containing manufactured nanomaterials. In a similar fashion to air measurements, determination of manufactured

nanomaterials in consumer products suffers from the difficulty of discrimination between background and intentionally added manufactured nanomaterials. Coordinated efforts and research strategies for a comprehensive exposure assessment of manufactured nanomaterials still have to be defined.

3.4. The interface between nanomaterials and biological systems

When nanomaterials come into contact with a biological fluid, the fluid usually penetrates into pores of nanomaterials regardless of whether they are single particles or agglomerate/aggregates. As a result, they may become coated with proteins (Blunk et al. 1993, Cedervall et al. 2007, Labarre et al. 2005) and other biomolecules. The coating may then influence the outcome of the biological response to the nanoparticle. Proteins have been the most widely studied in mammalian systems. The association and dissociation of proteins from the nanomaterials was found to depend on the particle hydrophobicity and size (radius of curvature) (Cedervall et al. 2007). Many proteins formed transient complexes with the nanomaterials, the binding and dissociation being dependent on protein identity. Albumin and fibrinogen displayed relatively high rates of both association and dissociation compared to apolipoprotein A-I. When there is an excess of biological fluid (serum) the lower abundance proteins with higher affinities may even eventually dominate the proteins present on the particle surface, the so-called "protein corona" (Cedervall et al. 2007).
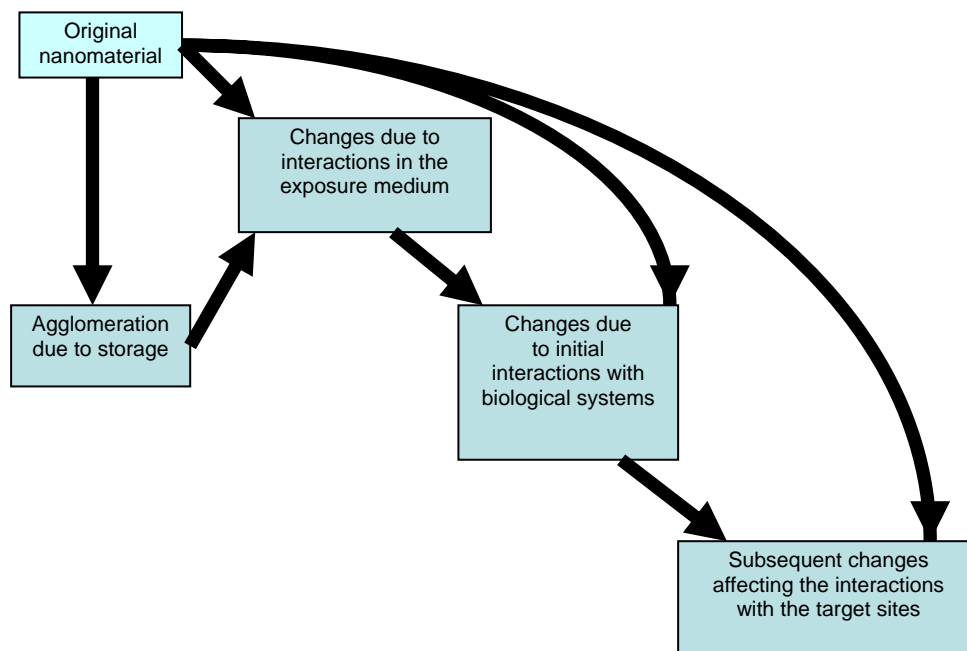
Figure 2:   The potential changes in nature of a nanomaterial due to the surrounding media

The body defences have evolved in such a way that they can deal with any conceivable kind of foreign material that enters the biological system, including bacteria, viruses and particles. The foreign surface is coated with host molecules present at portals of entry. These host molecules act in several ways. Some are opsonins and following binding to particle surfaces, they are recognised by defence cells, which possess receptors for them, resulting in phagocytosis and clearance e.g. scavenger receptors. The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles (Arredouani et al. 2004). Proteins of the complement system bind to foreign

surfaces. This results in a cascade of effects including production of opsonins and induction of inflammation. The complement system can also be involved in the response to some dusts (Warheit et al. 1991). Immunoglobulins are present in serous fluids at portals of entry. IgG is an opsosin that was found to modify the reponse to some respirable fibres but not others (Donaldson et al. 1995). In addition, a number of host proteins that are specific for certain portals of entry (e.g. Surfactant protein A) and non-specific proteins bind to the particle surface (Kendall 2007). Fine airborne urban particles (PM2.5) sequester lung surfactant and amino acids from human lung lavage. The actual role that these biomolecules play in the subsequent response is not known. However, it is prudent to consider that the outcome of the interaction of a particle with a biological system might depend on the coating that it receives and this has implications for *in vitro* work and any situation where a particle is delivered into a biolgical system under non-physiological conditions.

Inflammatory reaction is a key event that may occur following exposure to any solid material, including nanomaterials. For several nanomaterials *in vitro* induction of inflammatory cytokines was demonstrated (Carlson et al. 2008, Kim et al. 2003, Kocbach et al. 2008, Zhang et al. 2008). Such inflammatory cytokines can also bind to nanomaterials (Kim et al. 2003). This may have implications when *in vitro* assays are used for the evaluation of the inflammatory properties of nanomaterials.

Important factors for the potential translocation/absorption of nanomaterial may be the protein-nanomaterial interactions both in the lungs and in the gut. Upon contact with body fluids, some nanomaterials have been found to interact with proteins and biomolecules immediately (Linse et al. 2007, Lynch and Dawson 2008). This contact with biological matrices (including food) may determine the behaviour of the nanomaterials, in addition to the nature of the surface (charge, chemistry) itself, although this will of course influence the binding of the biomolecules (Colvin 2003, Lundqvist et al. 2004, Nel et al. 2006).

A recent systematic study of interaction of polystyrene nanoparticles with no modification (plain) or modification with positive (amine) or negative (carboxylic) charges indicates that the surface and the curvature (particle size) both influence the details of the adsorbed proteins, although in all cases, a significant fraction of the proteins bound were common across all particles (Lundqvist et al. 2008). Due to the curvature of the nanoparticle surface this may affect the tertiary structure of the binding protein resulting in malfunctioning (Lynch et al. 2006). These nanoparticle protein interactions may not be static but change during time (Cedervall et al. 2007). Such protein coatings on nanoparticle may enhance membrane crossing and cellular penetration (John et al. 2001, John et al. 2003, Panté and Kann 2002). This may even include crossing the nuclear membrane as demonstrated for gold nanoparticle up to a size of 39 nm bound to the nuclear core complex protein (Panté and Kann 2002).

A recent review has summarised much of the current state-of-the-art in protein-nanoparticle interactions (Lynch and Dawson 2008). However, there are several complicating factors, such as the fact that the biomolecules surrounding the nanomaterial, sometimes referred to as "corona", are not fixed, but are in a dynamic state. The corona equilibrates with the surroundings, with high abundance proteins binding initially, but being replaced gradually by lower abundance, higher affinity proteins. This complicates the measurement of such a protein corona. A considerable portion of the true biologically relevant biomolecules (proteins) will be associated with the nanoparticles for a sufficiently long time that they are not affected by the measurement processes – the so-called "hard-corona" (Lundqvist et al. 2008). Additionally, changes in the biomolecule environment, such as uptake or biodistribution will be reflected as changes in the corona. One may speculate whether the protein determines the fate or distribution of the absorbed nanoparticles or the environment with its specific proteins present. Indeed the coating of 500 nm polystyrene nanoparticles with a bioadhesive tomato lectin molecule did increase considerably the uptake of the particles after oral administration (Hussain et al. 1997). The uptake in the GI tract of

various polystyrene latex particles (ranging ion size from 50 nm to 3 microns) could be increased or decreased by modification of the particle surface (Florence et al. 1995).

The significance of this for nano-safety and nano-risk assessment is clear, as it implies that detailed characterisation of the nanoparticles in the relevant biological milieu is vital. Evidence is emerging in the scientific literature that coating of nanoparticles with specific proteins can direct them to specific locations – apolipoprotein E for example has been associated with transport of nanoparticles to the brain (Kreuter et al. 2002). Serum albumin has been shown to induce uptake and anti-inflammatory responses in macrophages, which were not present when the particles were pre-coated with surfactant to prevent albumin binding (Dutta et al. 2007). In addition, coating with polyethylene glycol (PEG) prevents the cellular uptake of nanomaterials and increases their half-life in blood (Niidome et al. 2006).

3.5. Human health issues

Many of the currently available OECD guidelines for the testing of chemicals are likely to be adequate to detect potential hazards of manufactured nanomaterials as well (SCENIHR 2007a). However, considering the particulate nature of the manufactured nanomaterials some adaptation of the testing methodology is likely needed (SCENIHR 2007a). This is especially the case for the expression of the dose metric as administered in the test systems. The currently available information in the use of the OECD guidelines for the evaluation of manufactured nanomaterials is, however, limited. Warheit et al. (2007) reported on a base set of toxicity tests for detection of the acute toxicity of ultrafine TiO_2 particles using assays as described in various OECD guidelines. The results of most of the studies demonstrated low hazard potential in mammals or aquatic species following acute exposures to the ultrafine TiO_2 particle-types tested. In the studies particle sizes were approximately 140 nm in diameter when TiO_2 was dispersed in water, but increased up to approximately 2000 nm when present in phosphate buffered saline (PBS), thus indicating the importance of nanoparticle characterisation as they are used in various test conditions (see section 4.2). Recently, the OECD has started a sponsorship programme in which, for 14 of the most used nanomaterials, a dossier on hazard identification will be produced (OECD 2008a). In this programme the applicability of the various OECD guidelines for nanomaterial testing will also be evaluated.

In the possible applications of validated *in vitro* assays for determination of general toxic effects of nanomaterials there has been almost no progress in relation to risk assessment. There is still a clear need for validated *in vitro* assays for nanoparticle evaluation. In the base set as reported by Warheit et al. (2007), only two *in vitro* assays were used, both of which are assays for the detection of genotoxicity. One assay (Ames test) uses bacterial cells and the other (chromosomal aberration test) uses mammalian cells. For the bacterial assays there can be reasonable doubt whether the manufactured nanomaterials in the size range used (140 nm) can enter the bacterial cells. Recent results reported by Sayes et al. (2007) did not support the use of *in vitro* assays for toxicity endpoints. For five different particle types the range of toxicity end points showed little correlation between *in vitro* and *in vivo* measurements for inhalation toxicity profiles (Sayes et al. 2007). Recent evaluations of safety assessment of manufactured nanomaterials indicated that *in vitro* assays may be useful, but mainly for screening and the evaluation of specific mechanistic pathways (ECETOC 2006, Oberdörster et al. 2005a). So, they may be used for assessing the possible reactivity, inflammatory potential and cellular uptake of nanoparticles. However, to be applicable in risk assessment, these assays need to be validated and their relevance for *in vivo* hazard identification needs to be demonstrated.

It should be noted that the *in vivo* assays as described in the various OECD guidelines are not validated for nanomaterials either. However, the experience gained in the testing

of chemicals with these assays indicates that they can be used for the detection of some potential human and ecological hazards.

–

3.5.1. Nanoparticle-protein interaction

As the protein corona may affect the nanoparticle behaviour including its biological effect, the nanoparticle may also have an effect on protein behaviour. Nanoparticles were found to have the potential to promote protein assembly into amyloid fibrils *in vitro* by assisting the nucleation process (Linse et al. 2007). This phenomenon may have implications for human disease as protein self-assembly of a variety of proteins and peptides is known to cause human amyloid disease (Chien et al. 2004, Chiti and Dobson 2006, Koo et al. 1999). Large insoluble protein fibrils are formed resulting in amyloid plaques, an example being dialysis related amyloidosis due to β_2-microglobulin (Floege and Ehlerding 1996).

Various types of nanoparticles (copolymer nanoparticles, cerium oxide particles, quantum dots, and carbon nanotubes) were found to enhance the probability of appearance of a critical nucleus for nucleation of protein fibrils from human β_2-microglobulin (Linse et al. 2007). The shorter nucleation phase depended on the amount and nature of the particle surface. There was an exchange in protein on the particle surface in which β_2-microglobulin formed multiple layers on the particle surface providing a locally increased protein concentration promoting oligomer formation. These results suggest a mechanism involving surface assisted nucleation that may increase the risk for toxic cluster and amyloid formation (Linse et al. 2007). Further research demonstrated that besides an increase in nucleation, nanoparticles may also retard the nucleation process which was attributed to an effect on the nucleation step of the amyloid beta protein while the elongation step was unaffected (Cabaleiro-Lago et al. 2008). These experiments were performed using an incubation of nanoparticles with purified β_2-microglobulin protein. Recently this effect of a shortening of the nucleation process was also demonstrated for β-amyloid after incubation with TiO_2 nanoparticles (Wu et al. 2008). Whether the observed nucleation process also occurs in an *in vivo* situation or in more complex biological fluids where competitive binding may take place remains to be determined.

There are indications that after deposition at the olfactory mucosa of the nose nanoparticles may translocate into the brain (see below). This observation raises some concern in view of the amyloid diseases of the brain in the context of the potential of nanoparticles to cause protein fibrillation *in vitro*. This is certainly an area for which additional research is urgently needed.

3.5.2. Toxicokinetics

3.5.2.1. General background

Toxicokinetics is the science dealing with absorption, distribution, metabolism and excretion (ADME) of substances in the body. This whole cascade of events occurring after an (external) exposure determines the internal exposure of organs at risk to potential toxic substances. Prominent exposure routes of nanoparticles are inhalation, ingestion and skin uptake as well as intravenous injection for medical purposes.

3.5.2.2. Translocation of nanomaterials

Translocation of manufactured nanoparticles through the epithelium is likely to depend on the physical-chemical properties of the nanoparticle, e.g. surface charge, hydrophobicity, size, presence or absence of a ligand, and physiology of the organ of intake e.g. healthy vs diseased state (where translocation may be increased or decreased depending on the illness) (Des Rieux et al. 2006). Under normal physiological conditions, paracellular transport of nanoparticles would be extremely limited, as pore size at tight junctions is

between 0.3-1.0 nm (Des Rieux et al 2006). Little is known on the behaviour and fate of nanoparticles in the gastrointestinal tract (EFSA 2008).

While some mechanisms may be of general applicability for many biological membranes, it must be noted that each membrane has specific tasks so that mechanisms related to those tasks may not be applicable to other membranes. After translocation/absorption, the distribution of nanoparticles inside the body over the various organ systems and within the organs needs to be determined. After the initial translocation/absorption of nanoparticles the systemic circulation can distribute the particles to all organs and tissues in the body.

As a model particle for nanotechnology research including toxicokinetic studies, metallic colloidal gold nanoparticles are widely used. They can be synthesised in different forms (rods, dots), are commercially available in various size ranges, and can be detected at low concentrations. Human cells can take up gold nanoparticles without cytotoxic effects (Connor et al. 2005). In particular for biomedical applications, they can be considered relevant models, since they are used as potential carriers for drug delivery, imaging molecules and even genes (Kawano et al. 2006), and for the development of novel cancer therapy products (Hainfield et al. 2004, Hirsch et al. 2003, Loo et al. 2004, O'Neal et al. 2004, Radt et al. 2004). In addition, gold nanoparticles have a history as labels for tracking protein distribution *in vivo* in which proteins are coupled to small colloidal gold beads at nanoscale dimensions (Heckel et al. 2004, Hillyer and Albrecht 1999).

3.5.2.3. Organ distribution after intravenous exposure

For systemic distribution, direct systemic exposure of organs can be obtained by the intravenous route for which the total internal dose/exposure is equal to the administered dose. Distribution of particles occurs at multiple organs including liver, spleen, heart and brain (De Jong et al. 2008, Ji et al. 2006).

When rats were intravenously injected with solutions containing various sizes of metallic colloidal gold nanoparticles (10, 50, 100 and 250 nm), the distribution of gold nanoparticles was found to be size-dependent, the smallest particles showing the most widespread organ distribution including blood, heart, lungs, liver, spleen, kidney, thymus, brain, and testis (De Jong et al. 2008). The larger nanoparticles mainly resided in spleen and liver. Intravenously injected gold nanorods (length 65 ± 5 nm; width 11 ± 1 nm) accumulated within 30 min, predominantly in the liver. The PEGylation (coating with polyethylene glycol) of these gold nanorods resulted in a prolonged circulation (Niidome et al. 2006).

When negatively charged 1.4 nm or 18 nm gold nanoparticles were injected intravenously, the 18 nm nanoparticles showed a similar pattern as described above with the highest accumulation in liver and spleen. For the 1.4 nm gold nanoparticles only half of the injected dose was present in liver and spleen, whereas the other half was found at higher fractions in the other organs mentioned above, in soft tissues and in the skeleton. Furthermore, the 1.4 nm gold nanoparticles were still circulating in the blood after 24 hours (Semmler-Behnke et al. 2008). When the same gold nanoparticles were injected intravenously in pregnant rats in their third trimester, both 1.4 nm and 18 nm particles were found in the placenta and the foetuses (Semmler-Behnke et al. 2007). For 5 and 30 nm gold colloid solutions very small fractions were found to be transferred to the rat fetus after intravenous administration (Takahashi and Matsuoka 1981). In contrast, transfer to fetal tissue could not be demonstrated by Sadauskas et al. (2007) when gold nanoparticles of 2 nm and 40 nm were injected in pregnant mice.

For intravenously administered TiO_2 nanoparticles in rats with a dose of 5 mg/kg body weight and a size range 20-30 nm, the tissue content of TiO_2 was determined 1, 14, and 28 days after administration (Fabian et al. 2008). There were no detectable levels of TiO_2 in blood cells, plasma, brain, or lymph nodes. The TiO_2 levels (µg/g organ) were highest in the liver, followed in decreasing order by the levels in the spleen, lung, and very low in the kidney, and highest on day 1 in all organs. TiO_2 levels were retained in the liver for

28 days; there was a slight decrease in TiO$_2$ levels from day 1 to days 14 and 28 in the spleen, and a return to control levels by day 14 in the lung and kidney. A limitation of this study is that most of the particles administered were in the fine fraction up to 1 µm, whereas only 10% by weight was in the nanosize range (<100nm).

The results obtained with studies using intravenous administration show that the main target organs for particles are the spleen and liver which are abundant in phagocytic cells i.e. macrophages and Kupffer cells. As was demonstrated for 10 nm particles, the smallest nanoparticles may also show distribution to other organs besides the liver and spleen.

3.5.2.4. Organ distribution after oral exposure

Oral administration of metallic colloidal gold nanoparticles of decreasing size (58, 28, 10 and 4 nm) to mice resulted in an increased distribution to other organs indicating a higher uptake with diminishing size (Hillyer and Albrecht 2001). The smallest particle (4 nm) administered orally resulted in an increased presence of gold particles in kidney, liver, spleen, lungs and even the brain. The biggest particle (58 nm) tested was detected almost solely inside the gastrointestinal tract. For 13 nm sized gold colloids, the highest amount of gold was observed in liver and spleen after intraperitoneal administration (Hillyer and Albrecht 1998). One might speculate whether such translocation of nanoparticles is accompanied by transport of food components/molecules and thus may create an (unwanted) port of entry, which may result in unexpected toxicity or other adverse affects such as induction of allergy.

TiO$_2$ particles of 500 nm were observed in all major tissues of the Gut Associated Lymphoid Tissue (GALT) including Peyer's Patches and mesenteric lymph nodes after repeated oral administration by gavage for 10 days and evaluation at day 11 (Jani et al. 1994). Systemic exposure occurred as titanium and was detected by chemical analysis in blood, liver, lungs, spleen and heart. The presence of the TiO$_2$ particles was confirmed by histology in Peyer's Patches, mesenteric lymph nodes, liver, spleen and lung. In the heart the presence of particles could not be confirmed by histology. The highest levels Ti (µg per g tissue) were present in the lymphoid tissues like Peyer's Patches, mesenteric lymph nodes and the mesentery network. The colon with lymphoid tissue as the appendix and diffuse lymphoid aggregates showed a high Ti level as well. It was concluded that the 500 nm TiO$_2$ particle uptake was primarily taking place via the Peyer's Patches (Jani et al. 1994).

In a 28 day oral toxicity study of silver nanoparticles (average 60 nm) a dose dependent accumulation of silver was observed in all organs examined, i.e. blood, brain, kidneys, liver, lungs, stomach and testes (Kim et al. 2008). The highest levels were observed in the stomach, followed by kidney and liver, lungs, testes, brain and blood. In the mid and high dose treated groups all levels measured were significantly increased compared with the non treated control group. The silver content was determined by atomic absorption spectrophotometry (AAS) while histology for the confirmation of the presence of silver particles was not performed. Silver levels in the kidneys were, for all doses investigated, twice as high in female rats than in male rats which could not be explained by the results presented (Kim et al. 2008).

It can be concluded that for some nanoparticles the size may be a limiting factor in the potential to cross the GI tract barrier, while for other nanoparticles a similar size may result in uptake from the GI tract which may occur at sizes up to 500 nm.

3.5.2.5. Organ distribution after inhalation exposure

Several inhalation studies in rodents have shown the distribution of nanoparticles to numerous organs including the liver, the spleen, the heart and the brain (Kreyling et al. 2002, Oberdörster et al. 2002,, Semmler et al. 2004; Semmler-Behnke et al. 2007).

The translocation of radiolabeled insoluble iridium nanoparticles was monitored after inhalation (Kreyling et al. 2002). For both the 15 nm and for the 80 nm particles, most of the particles remained in the lungs from which they were predominantly cleared via the airways into the GI tract and the feces. After systemic uptake from the lungs minimal particle translocation of <1% was observed to secondary organs such as the liver, spleen, heart and brain. The translocation of the 80 nm particles was about an order of magnitude lower than that of the 15 nm particles (Kreyling et al. 2002). Two studies (Semmler et al. 2004, Semmler-Behnke et al. 2007) report on long-term nanoparticle biokinetics in secondary target organs over six months after a single short-term nanoparticle inhalation. Only about 1% of the inhaled nanoparticles had crossed the air-blood-barrier and accumulated in secondary target organs (liver, spleen, kidneys, heart and brain) as well as in the soft tissue and bone. After a transient maximum in all secondary target organs between 1-2 weeks after inhalation, nanoparticle concentrations remained surprisingly constant between week 3 and six months. Unexpected back-and-forth trafficking was observed across the alveolar epithelium of the lungs of the 20 nm sized iridium nanoparticles in the lungs of adult healthy rats after a single one-hour inhalation (Semmler-Behnke et al. 2007). Although being retained in interstitial spaces Ir-nanoparticle re-appeared on the epithelium during the next six months to be cleared by macrophage-mediated transport towards the mucociliary escalator and to GI-tract after swallowing.

When negatively charged 1.4 nm or 18 nm gold nanoparticles were intratracheally instilled into rats, the distribution determined 24 hours later, showed a much higher fraction (8% of instilled dose) of the 1.4 nm particles to be translocated into the circulation and accumulated in secondary target organs than a 30-fold lower fraction of 18 nm particles (Semmler-Behnke et al. 2008). Particles were found in the liver, the spleen, the kidneys, the heart, the brain, the reproductive organs, soft tissue and the skeleton, but by far the highest fraction remained in the lungs. Furthermore, in one other study for gold nanoparticles with sizes of 5-8 nm the majority of the inhaled nanoparticles remained in the lung, although there was a very small but significant fraction being translocated to the blood (Takenaka et al. 2006). When rats were exposed by inhalation for 5 days to gold nanoparticles (majority with a size <35 nm) gold was only detected in the lung and olfactory bulb (Yu et al. 2007). After 15 days of exposure gold could also be detected in other organs including heart, liver, pancreas, spleen, kidney and testis (Yu et al. 2007).

In mice exposed by inhalation to fluorescent Fe containing magnetic nanoparticles (size 50 nm) for four weeks (4h/day, 5 days/week) the nanoparticles were distributed to various organs including liver, spleen, lung, testis and brain (Kwon et al. 2008). The results indicated that both the blood brain barrier (BBB) and the blood testis barrier (BTB) were penetrated by the nanoparticles.

Rats were exposed to carbon nanoparticles (size 20-29 nm) labelled with stable isotope C^{13} in a whole body inhalation chamber (Oberdorster et al. 2002). Only at high exposures did the label start to accumulate in the liver 30 minutes after exposure. 18 and 24 h after exposure the liver contained about five times more label than the lung. No significant increase in label was observed in the other organs examined which included heart, olfactory bulb, brain and kidney. It is worth noting that ^{13}C is naturally present in each organism at a level of about 1%. Therefore, the deposited ^{13}C labeled nanoparticles represented less than the endogenous ^{13}C content in the mouse lungs.

Ambient air particles and nanoparticles have also been demonstrated to translocate to the brain after inhalation, and thus may potentially influence the central nervous system Using ^{13}C as model particles with a diameter of 36 nm translocation into the olfactory bulb was indicated most likely originating from entry in the olfactory mucosa in the nose (Oberdörster et al. 2004). Also for gold nanoparticles translocation to the brain was observed after inhalation exposure (Yu et al. 2007). When 15-20 nm iridium nanoparticles were administered by inhalation up to 6 months after exposure iridium could be detected in the brain (Kreyling et al. 2002, Semmler et al. 2004). Whether this

was solely due to translocation via the nasal absorption is not certain as systemic distribution was also observed in the study. For diesel exhaust containing a nanosized fraction, inhalation exposure resulted in changes in brain activity as demonstrated by changes in EEG signals (Crüts et al. 2008). These observations on possible direct translocation of inhaled nanoparticles in the brain warrant further research to either confirm or reject the hypothesis of nanoparticle association with various brain diseases.

In humans, most inhaled carbon nanoparticles remain in the lung (Brown et al. 2002, Mills et al. 2006, Möller et al. 2008, Wiebert et al. 2006a, Wiebert et al. 2006b). Translocation was found to be <1%. So, although the translocation of nanoparticles from the lungs may occur after inhalation, most nanoparticles remain in the lung and only a minute fraction may reach the circulation. Other efforts to study this translocation from the lung across the air-blood-barrier in humans failed because the experimental limits of detection were about 1% of the administered dose to the lungs and hence above the translocated fraction if at all existing (Brown et al. 2002, Mills et al. 2006, Möller et al. 2008, Wiebert et al. 2006a, Wiebert et al. 2006b). Studies using radiolabelled nanoparticles may have their limitations as it is known that a dissociation of the label from the nanoparticles may occur as shown for the radio-tracer ^{99m}Tc when used for labelling carbon nanoparticles (Nemmar et al. 2002).

The results obtained with inhalation studies show that there is the potential of the smaller nanoparticles to cross the air-blood barrier and enter the systemic circulation. In general only a very small portion of the inhaled dose (< 1%) shows translocation. It should be noted that after inhalation exposure, systemic disctribution to other organs may also be due to secondary gut uptake after removal of particulates from the lung by the mucociliary mechanism. However, with repeated exposures and the low particle fraction showing migration, it could mean that based on particle numbers a considerable internal systemic exposure may occur.

3.5.2.6. Clearance of nanomaterials

A prominent clearance pathway of ingested nanoparticles is fecal excretion since every nanoparticle which is not absorbed by the gut epithelium will leave the body via this pathway. Similarly inhaled nanoparticles which deposited on the airways of the respiratory tract will be transported by mucociliary action to the larynx from where it will be swallowed entering the GI-tract. Furthermore, even insoluble nanoparticles deposited in the lung periphery (alveoli) may eventually be cleared by macrophage mediated transport to the distal end of the mucociliary escalator from where they are cleared as described above. However, note that in the human body only 20-30% of the peripherally deposited nanoparticles leave the lungs via this route (Kreyling and Scheuch 2000).

If nanoparticles enter the systemic circulation there are two potential clearance pathways for excretion:

1   Glomerular filtration in the kidneys towards the bladder into urine. In fact, Choi et al. (2007) have observed that intravenously injected quantum dots with a size below 4.5 nm and not bound to any protein due to cystein surface modification will be quantitatively excreted in urine using a rat model. Furthermore, the same clearance pathway into urine applied to intravenously injected, hydrophilically functionalised and positively surface charged SWCNT and MWCNT of up to 2 µm in length was observed in a guinea pig model (Singh et al. 2006). Besides these studies 0.09 and 0.001% fractions of intravenously injected gold nanoparticles of 1.4 and 18 nm size, respectively, were excreted in urine (Semmler-Behnke et al. 2008)

2.   Another potential pathway might be a hepato-biliary clearance of nanoparticles from the liver via the bile into the intestine and feces. While this clearance pathway is well known in pharmacology it is only postulated for nanoparticles.

3.5.2.7. Conclusions on toxicokinetics

Existing data show that nanoparticles can enter circulation from the respiratory tract or the gastro-intestinal tract. These processes are likely to depend on the physical-chemical properties of the nanoparticles such as size and on the physiological state of the organs of entry. The translocation fractions seem to be rather low; however, this is subject of current intense research.

After the nanoparticles have reached the blood circulation, the liver and the spleen are the two major organs for distribution. Circulation time increases drastically when the nanoparticles are hydrophilic and their surface is positively charged. For certain nanoparticles all organs may be at risk as, for all organs investigated so far, either the chemical component of the nanoparticles or the nanoparticles themselves could be detected, indicating nanoparticle distribution to these organs. These organs include the brain and testis/the reproductive system. Distribution to the foetus *in utero* has also been observed. As the knowledge of the long-term behaviour of nanoparticles is very limited, a conservative estimate must assume that insoluble nanoparticles may accumulate in secondary target organs during chronic exposure with consequences not yet studied. There is a specific concern considering the possible migration of nanoparticles into the brain and unborn fetus. Research in both of these areas has to be conducted in order to either confirm or reject the hypothesis of nanoparticle association with various brain diseases, and the possible reprotoxic effects of nanoparticles.

3.5.3. Effects of carbon nanotubes

The superficial resemblance between carbon nanotubes and some other high aspect ratio (long thin) nanoparticles was commented upon early in concerns over the safety of nanotubes (Donaldson et al. 2006, The Royal Society and The Royal Academy of Engineering 2004). The term HARN, or High Aspect Ratio Nanoparticles, has been used to cover such structures. For fibrous-type or asbestos-like effects the major concern is mesothelioma, an unusual endpoint that is difficult to study but arises with unusual specificity to certain fibre exposure. Because of the low rate of mesothelioma following inhalation exposure in rats (a few % similar to the prevalence in exposed humans) direct exposure of the peritoneal mesothelium by intraperitonal injection was developed as an assay in the eigthties (Miller et al. 1999, Pott 1995). Takagi et al. (2008) used this approach to examine the tumerogenicity of carbon nanotubes in p53-deficient mice.

Although Takagi et al. (2008) showed that both asbestos and carbon nanotubes caused mesotheliomas whilst fullerenes did not the methodology was criticised (Donaldson et al. 2008, Ichihara et al. 2008) on several gounds including i) the presentation of the test sample indicating that clumps of nanotubes were used that were hundreds of microns in diameter, ii) the dose of 3 mg that was injected into each mouse to obtain the dose of 10^9 fibres which was originally developed for rats but not mice and iii) the use of the p53 deficient mouse model for mesothelioma detection without using relevant controls.

Poland et al. (2008) used the mouse peritoneal model of direct mesothelial exposure. They tested the acute inflammogenic effects of carbon nanotubes and their ability to cause granulomas on the surface of the diaphragm after one week. The acute response in the peritoneal cavity, inflammation and granuloma formation, mimics the long-term mesothelioma development (Davis et al. 1986, Kane 2006). Various controls were used including long and short amosite asbestos samples that had been used in the 1980's and which produced mesotheliomas in the peritoneal cavity in the case of the long and none in the case of the short fibers (Davis et al. 1986) Nanoparticullate carbon black, graphene in compact form, as opposed to graphene in a tubular form as in carbon nanotubes, was also used. Two carbon nanotube test samples containing a fraction of long straight nanotubes were compared with two samples comprised of short or tangled nanotubes. Inflammation and granulomas were only found in the case of the long straight nanotubes whilst the short/tangled nanotubes had no effect. This property of

length-dependent inflammation and early fibrosis in a mesothelial exposure model was therefore shared by both asbestos and the specific carbon nanotubes investigated.

This provides a first support for the contention that specific types of long carbon nanotubes may be pathogenic, like hazardous asbestos, when they have similar characteristics, such as length, rigidity and biopersistence. Whether this poses a risk for humans would depend on whether there is inhalation exposure to these specific types of carbon nanotubes. In addition, the risk would also depend on the possibility for natural migration of nanotubes to the pleural mesothelium from the airspaces. It needs sufficient long straight CNTs to get airborne in workplaces to reach a threshold dose followed by translocation to the pleural mesothelium.

Pacurari et al. (2008) used mesothelial cells in culture and compared carbon nanotubes to asbestos showing that carbon nanotubes induced activation of molecular signaling pathways associated with oxidative stress, similarly to asbestos.

The above studies focus on the fibre paradigm for predicting carbon nanotubes effects. Of course carbon nanotubes occur (probably predominantly) as tangled 'particles' of nanotubes material and not as 'fibres'. So like a ball of string that can be very long yet fit in the hand, carbon nanotubes can be long but be compactly tangled into particles. There is reason to think that such nanotubes pose a 'particle-type' hazard that is greater than would be anticipated and reviewed in Donaldson et al. (2006).

Extensive research on the effects of purified and non-purified single walled carbon nanotubes on the respiratory tract of mice has been carried out at NIOSH, Pittsburg, USA. The exposure of C57BL/6 mice to non-purified single walled carbon nanotubes (iron content of 17.7% by wt) at 5 mg/m^3, 5 hr/day for 4 days was compared with pharyngeal aspiration of varying doses (5-20 μg/mouse) of the same but purified single walled carbon nanotubes. Both exposure regimens resulted in the development of multifocal granulomatous pneumonia and interstitial fibrosis. Non-purified single walled carbon nanotubes inhalation was more effective than aspiration of purified single walled carbon nanotubes in causing inflammatory response, oxidative stress, collagen deposition and fibrosis as well as mutations of K-ras gene locus in the lung of C57BL/6 mice (Shvedova et al. 2005, Shvedova et al. 2008a). Sequential exposure to the same single walled carbon nanotubes and bacteria enhanced pulmonary inflammation and infectivity (Shvedova et al. 2008b). In *in vitro* studies on a RAW 264.7 macrophage cell line the same purified and non-purified single walled carbon nanotubes showed that the presence of iron in single walled carbon nanotubes may be important in determining redox-dependent responses of macrophages (Kagan et al. 2006)

When nanotubes, possibly of any chemical composition, have similar characteristics as some types of hazardous asbestos, it was demonstrated that similar inflammatory reactions can be induced by the nanotubes as asbestos. The main characteristics required for this to occur are long thin fibrous forms (length >20 micrometer), rigidity, and non-degradability (biopersistence). Whether such nanotubes would pose a risk for humans is unknown, as besides these specific nanomaterial characteristics, inhalation exposure to such structures would also be essential. In addition, migration of such fibrous nanomaterials from the airspaces in the lung to the pleural mesothelium has to occur. In terms of occupational safety, the local air concentration also needs to be higher than threshold doses. The main conclusion of the studies on carbon nanotubes relating to a risk for mesothelioma is that such a risk cannot be excluded. So, when manufacturing nanotubes (possibly of any chemical composition) one should be aware that certain characteristics may pose such a risk and thus should be considered in the safety evaluation of that particular manufactured nanomaterial. Carbon nanotubes seem to conform to the same paradigm as some forms of asbestos, glass fibres etc., that any long, thin biopersistent fibre poses a potential mesothelioma hazard. This means that other high aspect ratio nanoparticles such as nanowires or nanorods are likely to have the same hazard if they satisfy the criteria of length and biopersistence.

3.5.4. Genotoxicity

The genotoxic effects of conventional particles are driven by two mechanisms – direct genotoxicity and indirect (inflammatory processes-mediated) genotoxicity, as reviewed by Schins et al. (2007). Nanoparticles may act via either of these pathways since they may cause inflammation (see above) and they can also enter cells and cause oxidative stress (Donaldson et al, 2005, Nel et al. 2006, Oberdörster et al. 2005a, Oberdörster et al. 2005b, Stone et al. 2007). There is some evidence that the small size may allow nanoparticles to penetrate into sub-cellular compartments that normally exclude environmental particles, like the mitochondria, and nucleus (Chen and von Mikecz 2005, Li et al. 2003, Geiser et al. 2005). The presence of nanomaterials in both mitochondria and the nucleus opens the possibility for oxidative stress mediated indirect genotoxicity, and direct interaction of nanoparticles with DNA and histones. Besides oxidative stress, additional mechanisms of genotoxicity which may be specific for nanomaterials also need to be considered, such as possible mechanical interferences during cell division, and other sources of genotoxic effects (i.e. metal release by nanomaterials) (Gonzalez et al. 2008).

Several studies with nanoparticles have indicated that some nanoparticles may be genotoxic (reviewed by Gonzalez et al. 2008, Landsiedel et al. 2008). The most frequently used test was the Comet assay demonstrating the presence of DNA damage. For several nanomaterials a positive outcome on genotoxicity was observed including C_{60} fullerene, single walled carbon nanotubes (SWCNT), nanoparticles of cobalt chrome (CoCr) alloy, TiO_2, nanosized metal oxide V_2O_3, Carbon Black (CB), and nanosized diesel exhaust particles.

The second most frequently used assay was the micronucleus assay in which the presence of micronuclei in dividing cells is indicative for chromosomal aberrations. In the micronucleus assay positive results were obtained for nanoformulations of TiO_2, SiO_2, CoCr, ZnO and multi-walled carbon nanotubes (MWCNT). For the gene mutation assays some studies showed a positive result for several nanomaterials including nano-FePt, SiO_2, TiO_2, MWCNT, and CB.

For all three assay systems used (Comet, micronucleus and gene mutation), negative results were obtained for TiO_2, CB, SiO_2, and single walled carbon nanotubes, while for some nanomaterials contrasting results were obtained (Landsiedel et al. 2008). The interpretation of the data presented in the reviewed papers was hampered by various limitations including the differences in the methodology used within one assay type, the use of non-standardised methods with different primary cells or cell lines, and by the sometimes minimal characterisation of the nanoparticles tested and the lack of information on possible contaminants.

For TiO_2 and CB it was reported that the smaller (~20 nm) particles induced DNA damage while larger particles (~200 nm) did not (Gurr et al. 2005, Mroz et al. 2008, Rahman et al. 2002). Cobalt nanoparticles have been shown to induce more DNA damage than micron sized particles using human fibroblasts in tissue culture in the alkaline comet assay (Papageorgiou et al. 2007). In the micronucleus assay Co nanoparticles showed minor changes, whereas in the Comet assay for the same Co nanoparticles, clear statistically significant positive results were observed (Colognato et al. 2008).

Some studies showed that highly purified amorphous silica, with a low surface reactivity, was negative in the Comet assay (Barnes et al. 2008). This might suggest that nanoparticles with low surface reactivity are likely to be less genotoxic than others. In addition to the negative results of Barnes et al. (2008), very mild positive results (Yang et al. 2009) on DNA damage in Comet assay were reported after exposure of mouse embryo fibrolasts 3T3 to different concentrations of SiO_2 nanoparticles (size 20 – 400 nm). In two types of genotoxicity assays i.e. the micronucleus assay and the gene mutation assay positive results were observed for silica nanoparticles (Landsiedel et al. 2008) while in the Comet assay weak positive results were obsreved (Yang et al. 2009).

A variety of genotoxicity (Ames test, clastogenicity in mammalian cells) and photo-genotoxicity (Photo-Ames test, photo-clastogenicity in mammalian cells) tests have been performed under GLP conditions on 14 different sunscreen-grade TiO_2 (anatase and rutile; coated an uncoated; particle size range 11-60nm + one pigment grade – 200 000 nm). All results were negative. They were provided as an unpublished industry safety dossier but reviewed, summarised and published in the Opinion of the Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers Concerning Titanium Dioxide (SCCNFP, 2000). Negative photo-clastogenic results were also found in chromosome aberration tests on Chinese hamster ovary cells with a variety of TiO_2 particles (anatase, rutile; particles size: 14-60 nm) (Theogaraj et al. 2007).

However, others (Rahman et al. 2002, Wang et al. 2007) documented that ultrafine TiO_2 particles increased the number of micronuclei in Syrian hamster embryo cells and a human B-cell lymphoblastoma (WIL2-NS) cells. In the latter model mutation frequency was increased in the HPRT test and DNA damage was indicated by the Comet assay. Positive results in micronucleus test and oxidative DNA damage were found recently in fish cell lines derived from rainbow trout and goldfish skin (Reeves et al 2008, Vevers and Jha 2008). It was suggested that several types of TiO_2 (anatase; particle size of 255 – 420 nm) were not genotoxic but photo-genotoxic in mouse lymphoma and Chinese hamster lung cells (Nakagawa et al. 1997). This was further supported by the study of Dunford et al. (1997) which showed DNA oxidative damage in human fibroblasts (MRC-5) using the Comet assay. Similar to silica (SiO_2) positive results were observed for TiO_2 in all three types of genotoxicity assays (Landsiedel et al. 2008).

Inconsistent results were published for the genotoxicity of zinc oxide nanoparticles (Brayner 2008, Dufour et al. 2006, Nohynek et al. 2007, SCCNFP 2003, Yang et al. 2009).

It was shown that silver nanoparticles (ca. 60 nm size) did not increase micronuclei formation in rat bone marrow in an *in vivo* 28-days oral exposure test in doses up to 1000 mg/kg (Kim et al. 2008).

The genotoxicity of C_{60} has been relatively well studied, but conclusions are conflicting. Dhawan et al. (2006) demonstrated a weak genotoxicity of colloidal C_{60} in Comet assay performed on human lymphocytes. Several types of carbon nanomaterials such as carbon nanoparticles that generate reactive oxygen species (ROS) were found to be genotoxic (Jacobsen et al. 2008). On the other hand, Zakharenko et al. (1997) reported no genotoxicity of C_{60} in concentrations as high as 450 μg/l in SOS Chromotest and slight genotoxicity (at 2.2 mg/l) when somatic mutation and recombination test in *Drosophila melanogaster* was used. Mori et al. (2006) obtained negative results for fullerenes in both a bacterial Ames test and mammalian cell chromosomal abberation tests.

In order to consider a specific engineered nanomaterial genotoxic, confirmation by two independent laboratories and in two test systems is necessary and minimal criteria should be met as proposed by Gonzalez et al. (2008). There are various inconsistencies in the results reported so far (reviewed by Gonzalez et al. 2008, Landsiedel et al. 2008). A major limitation in concluding whether a certain nanomaterial is genotoxic or not is the scarce description and minimal characterisation of the nanomaterial samples used in the various studies.

In conclusion, for some manufactured nanomaterials, *in vitro* genotoxic activity has been reported, but negative contradictory results were also obtained, and not all results could be confirmed by *in vivo* testing. One potential cause of inconsistencies is the difficulty in delivering the nanomaterials to the test systems appropriately. Most available *in vitro*/*in vivo* genotoxicity studies have been performed at high particle concentrations. In *in vivo* situations, this may be associated with marked inflammatory and proliferative responses, and hence may obscure and/or modify genotoxicity and even carcinogenicity readouts. In addition, various assays with different primary cells and cell lines were used which did not always show consistent results. Such inconsistencies may depend on physico-chemical characteristics of the test material investigated such as size, shape,

aggregation/agglomeration state, surface properties, contaminants present and the cell type used.

3.5.5. Cardiovascular effects of nanoparticles

Air pollution is increasingly recognised as an important factor for cardiovascular disease in urban communities (reviewed by Mills et al. 2009). The occurring cardiovascular events including myocardial infarction and heart failure were attributed to the exposure to combustion derived nanoparticles that incorporate reactive organic and transition metal components. It was suggested that after inhalation the resulting respiratory inflammation induces systemic effects either directly by translocation from the lung or indirectly by yet unknown mediators (Mills et al. 2009). In view of these findings also for manufactured nanomaterials a risk for interaction with the cardiovascular system can be imagined.

Varous types of carbon nanomaterials were investigated and compared to a standard urban particulate matter by Radomski et al. (2005). Mixed carbon nanoparticles, single wall carbon nanotubes, multi wall carbon nanotubes, but not C_{60} fullerenes were found to stimulate platelet aggregation *in vitro*, and to accelerate vascular thrombosis in a ferric chloride model of thrombosis in a specific rat model. No information was presented on the characteristics of the carbon nanomaterials used, which limits the value of the observations. In a recent study, modified fullerenes ($C_{60}(OH)_{24}$) were found to facilitate adenosine diphosphate (ADP)-induced platelet aggregaton *in vitro*, whereas $C_{60}(OH)_{24}$ alone or carbon black did not (Niwa and Iwai 2007).

In contrast for several nanomaterials designed for drug delivery purposes, no or limited effects on platelet function *in vitro* was noted including alcohol/polysorbate nanoparticles (Koziara et al. 2005), gadolinium nanoparticles (Oyewumi et al. 2004), and nanostructured silica hydroxyethyl methacrylate biocomposites (Liu et al. 2008).

Based on the observations some concern exists on the possible effect of manufactured nanoparticles on the cardiovascular system. However, so far this has not been clearly demonstrated to be the case for manufactured nanoparticles as well. Overall the information on the possible hazard of nanoparticles for cardiovascular effects is rather limited and needs expansion.

3.6. Environmental issues

Inevitably, production, use and disposal will lead to releases to the environment. Wastewater treatment streams, landfill and combustion of products containing nanomaterials are means through which they may end up in the environment, although it is most likely that they do so as modified forms from their primary counterpart. In addition, some nanomaterials are used in environmental remediation applications and as such they are applied as primary nanomaterials to the environment.

3.6.1. Environmental fate and behaviour

3.6.1.1. General principles

The environmental fate and behaviour of nanomaterials has been recently reviewed by Klaine et al. 2008. Knowledge from colloid science can provide information on the likely fate and behaviour of nanomaterials (Lead and Wilkinson 2006). The behaviour of nanoparticles in the environment is expected to depend not only on the physical and chemical character of the nanomaterial, but also and perhaps predominantly on the characteristics of the receiving environment (Chen et al. 2008, Chen and Elimelech 2008,

Saleh et al. 2008). It is generally known that small particles tend to aggregate or agglomerate to eventually become associated with other dissolved, colloidal and particulate matter present in the environment. Upon entry into the environment, nanoparticles may remain intact or undergo one or more of the following:

- dissolution,

- speciation (i.e. association with other ionic or molecular dissolved chemical substances),

- biological or chemical transformation to other chemicals, and/or complete mineralization (to carbon dioxide and water),

- agglomeration/disagglomeration,

- settling.

So far, there are no peer reviewed publications providing information on concentrations or amounts of nanomaterials in environmental compartments such as surface waters and soils. Estimates of quantities of nanomaterials present in surface waters and other media derive from calculated exposure scenarios based on predicted nanomaterial use and not from actual measurements (Boxall et al. 2007, Mueller and Nowack 2008). 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). The appropriate metrics of the measurement of manufactured nanomaterials in relation to environmental risk assessment is still under discussion.

In order to assess the potential effects of nanomaterials in the environment, exposure concentrations or doses should be considered realistically. All forms, in which the nanomaterial occurs, not only the free nanoparticulate form, but all physical and chemical species, should be considered. It is important to realise that there may be some 'hot spots' where nanomaterials might concentrate due to their tendency to aggregate/agglomerate and potentially to adsorb to or associate with organic matter. In addition, it is likely that some of the nanomaterials going through the standard waste treatment stream will end up associated with the solid phase and then potentially be deposited in certain areas of the environment where they might reach higher loads.

In the environment nanomaterials are expected to occur mainly associated with sediments and soils (Baalousha et al. 2008, Klaine et al. 2008). The free dispersed form of nanomaterials is of particular importance and is addressed here specifically. It is recognised that for the estimation of the possible presence of free nanoparticles knowledge on release scenarios is very important. Unfortunately, to date, there is no suitable information available on this topic. 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 processess 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.

Air

Information concerning the presence of nanomaterials in air is summarised in section 4.3.

Water

Upon release to water, dispersed nanomaterials are expected to behave according to well-understood phenomena described and explained in colloid science (Jones 2002, Lyklema 2005). Colloidal suspensions of nanomaterials are generally expected to be unstable: i.e. upon collision, particles may approach each other close enough for

attractive Van der Waals forces to become dominant over repulsive electrostatic forces and steric hindrance. As a consequence, particles adhere to each other and then settle due to gravity (Baalousha et al. 2008, Ju-Nam and Lead 2008, Saleh et al. 2008). Moreover, natural waters contain many other dissolved, colloidal and solid materials (including natural nanomaterials) to which nanomaterials can and usually will adhere. Suspensions of dispersed nanomaterials are stable only under narrow ranges of environmental conditions (Baalousha et al. 2008).

The dominant factors in colloid stability under natural conditions are known to be pH, ionic strength and presence of natural organic matter (Lead and Wilkinson 2006). In sea water with high pH and ionic strength, electric double layers of colloid particles are much smaller than in freshwater, allowing for closer interparticle approach, which usually leads to more 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.

The humic and fulvid acids of "brown waters" will cover the nanoparticles with a coating that keeps them probably longer and more dispersed (Hyung et al. 2007). For example, the presence of natural organic matter (NOM), as well as iron oxide, have a stabilising effect on aquatic suspensions of fullerene and carbon nanotubes, at least in fresh water systems (Baalousha et al. 2008, Chen and Elimech 2008, Christian et al. 2008, Saleh et al. 2008). A similar effect has been shown recently for nanoparticles of CeO_2 (Quik et al. 2008). On the other hand, Baalousha et al. (2008) have shown that the effects of humic acids and varying pH can have combined effects on the fate of iron oxide nanoparticles with increasing pH resulting in a higher level of aggregation.

Likewise, surface modification of nanomaterials can influence the environmental fate and behaviour. As carbon nanotubes are considered to be highly hydrophobic and with a tendency to aggregrate, they would be expected to settle in the natural environment. However, Kennedy et al. (2008) have indicated that surface modifications, which are widespread (e.g. functional groupings and coatings) lead to increased dispersability, increased water column stability and lower settling rate, especially in combination with natural organic matter.

To understand the fate of nanomaterial dispersions in the environment it will be necessary to characterise the nanomaterial colloidal properties and the aqueous phase physical-chemical properties to a greater degree than is necessary for gas phase or dissolved substances (e.g. present as environmental contaminants). For example, moderate changes in the ionic strength will have little effect on the solubility or many organic substances (e.g. PAHs, most pesticides) but can have major effects on the suspension stability of nanoparticles. In saline environments nanomaterials have a tendency to aggregate (Nielsen et al. 2008, Stolpe and Hassellöv, 2007) and thus would most likely tend to settle. In sedimentary systems it would be important to determine how these nanomaterials might interact with organic matter and potentially be adsorbed and sequestered. This would have influence on their bioavailability and determine their biological uptake.

The estimation of concentrations in water is essential to environmental risk assessment. In sharp contrast with the situation for conventional chemical substances, there is neither theoretical nor empirical evidence that can be used to predict residual concentrations of nanomaterials in suspension under conditions of limited colloid stability. When substances enter the environment, they distribute themselves between the various phases of the system (partitioning). The environmental distribution of substances is often predicted by the octanol-water partition coefficient (K_{ow}). However, there is no reason to assume that the K_{ow} of the substance of which the nanoparticles are made of is predictive of the extent to which nanoparticles associate themselves with other particles. The K_{ow} is probably not applicable to non-soluble nanomaterials for risk assessment purposes. Due to the interactions of nanomaterials with various components of the environmental

system, generally near-zero concentrations of the nanomaterial in its original form would be expected. It is of great importance to gain understanding of the environmental conditions under which stable colloidal suspensions of dispersed nanoparticles can be formed.

For nanomaterials which may be solubilised, K_{ow} could be applicable. Recently, Jafvert and Kulkarni (2008) have studied the octanol-water partition coefficient (log K_{ow}) of buckminsterfullerene (C_{60}) and its aqueous solubility. The authors obtained a value for log K_{ow} of 6.7, and a value for the solubility of C_{60} in water-saturated octanol of 8 ng/L. Based upon this high K_{ow}, it is expected that C_{60} has high affinity for lipids and organic matter, indicating that in the natural environment, C_{60} will tend to sorb to solid phases.

Some recent predictive modelling work has been published for TiO_2 and silver nanoparticles and carbon nanotubes (Boxall et al. 2007, Mueller and Nowack 2008). However current knowledge of the behaviour of nanoparticles in natural waters provides insufficient basis for the full assessment of environmental exposure concentrations of dispersed nanomaterials. There is an urgent need to improve knowledge in this area.

It should be noted that in wastewater treatment plants partitioning of nanomaterials into the solid biomass is likely to be an important fate for hydrophobic materials which end up in the sewage stream.

Soil and sediments

As described above, depending on receiving environment and material, nanomaterials, if not degraded or dissolved, will tend to aggregate and eventually settle onto the substrate. Within soil and sedimentary systems it is expected that these materials will adhere to solids.

3.6.1.2. Test methods for predicting environmental distribution

It is likely that the OECD test methods for a number of physico-chemical methods for environmental distribution are applicable, although this needs to be further assessed, taking into account the administration of the sample to the test system.

Methods for assessing the environmental distribution of nanomaterials have been described (Christian et al. 2008, Hassellöv et al. 2008, Klaine et al. 2008, Tiede et al. 2008;). They are progressively being developed so that the complex issues of fate in different media may be addressed. Nevertheless, much information is still needed in this area.

For reasons explained above, it is doubtful whether standard tests of vapor pressure, water solubility, octanol-water partition coefficient and ready biodegradability are adequate and sufficient to describe and predict the distribution of nanomaterials in the aquatic environment.

Vapor pressure and solubility

Vapor pressure and water solubility of conventional chemicals are used to predict air/water partitioning. If measurable at all for nanomaterials, these properties may not be very useful in predicting the extent to which nanoparticles may partition from water into air. However, solubility of nanomaterials in water, or rather: the rate of dissolution of nanoparticles in water is important for an entirely different reason. Toxic effects of the presence of nanomaterials may well result, at least in part, from the presence of dissolved species that originate from dissolution of the nanomaterials. To date, it is unclear to what extent the effects observed can be attributed to the dissolved form or to the nanoformulation the effect being a combination of fraction and size (Franklin et al. 2007, Navarro et al. 2008b). Nevertheless, Lin and Xing (2008) have suggested that phytoxicity observed on exposures to ZnO nanoparticles may not be attributed solely to dissolved zinc. Griffit et al. (2008) drew a similar conclusion with respect to nanosilver.

The OECD assay on water solubility (OECD 1995) may not be very useful in this context (OECD, 1995). Rather, standard measurement of rate and extent of dissolution under natural water conditions would be helpful. Many nano materials are highly insoluble in water, so that specialised methods are likely to be needed to measure or estimate their water solubility anyhow. For example, the solubility of fullerene is usually estimated by measuring solubility in alcohols and extrapolating to a zero carbon alcohol, i.e. water (Jafvert and Kulkarni 2008).

3.6.1.3. Test methods for degradation and transformation

Environmental persistence of nanomaterials (i.e. resistance to transformation and degradation) depends on the chemical composition of both core and surface material. Although it is possible that most nanomaterials will be persistent in their original particulate form, this cannot be assumed in general. It seems likely that the organic coatings of nanomaterials are readily transformed or degraded, but there is lack of data in this area. As mentioned above, dissolution may occur for at least some metal nanomaterials (Franklin et al. 2007, Luoma 2008). Whether or not followed by degradation of the dissolved material, the process of dissolution makes nanoparticles disappear and become less persistent.

In standard tests for ready biodegradability of chemical substances, either disappearance of dissolved organic carbon or the generation of CO_2 is measured. Therefore, it is necessary to examine first whether the nanomaterial can be utilised as an energy or nutrient source for microorganisms. Secondly, the nanomaterial must be available to microorganisms in order to be degradable. If the material is unlikely to reside in the water column or if it is not soluble in water, biodegradation is unlikely and testing in surface water may be unnecessary.

For C-containing nanomaterials, the biodegradation screening methods (e.g. for ready biodegradability) measuring dissolved carbon are not applicable. In principle, the methods measuring carbon dioxide production or oxygen uptake are applicable, but they require large amounts of test material. It is also important to consider whether carbon based nanomaterials such as fullerenes and nanotubes can be degraded at all under any conditions. However, some data indicated that fullerenes could be taken up by wood decay fungi, suggesting that the carbon from fullerenes could be metabolised (Filley et al. 2005).

Simulation tests for biological degradation in various environmental compartments are applicable in principle, but again the detection of the nanomaterials is the challenge. The possible degradation to carbon dioxide, integration into biomass or other partitioning can be followed using labelled test material. However, it should be noted that the use of labels needs specific attention in terms of association of the label with the nanomaterial.

Likewise, for hydrolysis testing, the chemical structure of the material and whether it contains groups which could be subject to hydrolysis dictate whether this test is necessary or appropriate. In view of the sometimes very long lifetime of ecosystem processes, other non biological degradation mechanisms have to be investigated (e.g. UV induced, slow dissolution).

3.6.1.4. Test methods for bioaccumulation

Current work assessing uptake has focussed on exposures in media with different nanomaterial loads over a specific time interval, followed by total body burden assessment, especially if species are small, such as *Daphnia* species, copepods or *Lumbriculus* (Fernandes et al. 2007, Petersen et al. 2008, Roberts et al. 2007). If organisms are larger, specific studies have focussed on detection, following exposures, of loads within specific organs, such as liver, kidney, muscle, gills (Handy et al. 2008a). In terms of detection, it may not always be possible to identify the form of such material. This may be particularly important for materials that may tend to get into solution such as silver (Luoma 2008, Navarro et al. 2008b).

From studies of biological exposures of nanomaterials it is clear that adsorption and aggregation of the material onto surface of the organism is commonly observed (Fernandes et al. 2007, Handy and Eddy 2004, Nielsen et al. 2008, Rosenkranz et al. 2009). This has also been shown by the aggregation of single wall carbon nanotubes on the gill mucus of rainbow trout (Smith et al. 2007) and of carbon black and titanium dioxide nanoparticles on the carapaces of *Daphnia*, (Fernandes et al. 2007), as well as the entanglement of macroalgae gametes by clusters of carbon black (Nielsen et al. 2008).

Given the tendency of nanomaterials to aggregate, and thus their likelihood to end up associated with sediment (Klaine et al. 2008) bioaccumulation studies on sediment organisms would be especially important. OECD has recently adopted a new standard test for the assessment of bioaccumulation into sediment worms using *Lumbriculus variegatus.* This method could be relevant to be used in a test battery for risk assessment as OECD has also published recently a toxicity test (OECD TG 225) based on the same species which could provide effects data (OECD 2008b).

For organic substances, there is an established relationship between octanol/water partition coefficient (K_{ow}) and bioaccumulation or bioconcentration factor (BCF). However, this relationship may not hold true for nanomaterials.

The main challenge in testing the bioaccumulation of nanoparticles is their detection and characterisation in tissues and body fluids. Radiolabelling could make detection and quantification easy but it has also limitations; for example, the labelled material can behave differently from the non-labelled particles. Petersen et al. (2008a) used radio-labelled CNTs to assess uptake and depuration by *Lumbriculus variegatus*. Another possibility could be the radio-activation of metal and metal oxide nanoparticles (Oughton et al. 2008). It enables both localisation and quantification within tissues or organisms. This technique is still at experimental stage and a key aspect is how ionisation of manufactured nanomaterials may interfere with the exposure assay and any results.

Standard BCF testing protocols such as OECD 305 (OECD 1996) may have limitations in testing of bioaccumulation of nanoparticles. It has been observed for substances dissolved in water that a large molecular size effectively (MW > 600, or effectively a diameter size > 0.5 nm) limits direct uptake. It is likely that in most cases the relatively large size (1-100 nm) of nanoparticles compared to dissolved molecules limits their direct uptake by fish gills. Fish dietary bioaccumulation factor (BAF) testing (Fisk et al. 1998; Stapleton et al. 2004) is not a standard OECD testing protocol yet. This spiked food method is suitable for testing of poorly soluble, large molecules and might be suitable for testing several classes of nanoparticles, either by itself or in combination with the OECD 305 testing. However, more data using a harmonised OECD dietary protocol, especially for testing nanomaterials, are needed. The testing results of human health endpoints should also be taken into consideration if available when generating environmental testing plans for specific nanomaterials. Uptake studies from mammalian studies may give valuable basic information on uptake characteristics, rates and mechanisms of nanoparticles also in non-mammalian species.

3.6.2. Bioavailability and exposure

3.6.2.1. General Principles

Uptake by biota is likely to be via the respiratory or digestive tracts in animals, or via the root system in plants. Uptake across epithelial surfaces is also possible. Plants and fungi have cell walls that act as an initial barrier to the entrance of nanomaterials.

In terrestrial (and water) systems some nanomaterials may preferentially bind to NOM (see section 4.6.1.1) and thus become less bioavailable. Sediment feeders may be able to uptake these nanomaterials. In fact they may preferentially ingest them if they are

associated with NOM (Roberts et al. 2007) and strip/de-associate them within the gastro-intestinal tract. Li et al (2008) have reported the reduced bioavailability, and resulting reduced antibacterial activity, associated with the increased sorption of C_{60} to soil organic matter.

In aquatic systems stabilisation by NOM may maintain nanomaterials within the water column which may result in increased bioavailability to aquatic organisms (Kennedy et al. 2008). Although such association with NOM also may reduce and even eliminate antibacterial activity (Li et al. 2008).

If the nanomaterial readily dissolves in water current protocols and guidelines developed to measure bioavailability of conventional chemicals are applicable, although in such cases the test would address the dissolved form rather than the nanoparticulate form. However, given that depending on the nanomaterial and the receiving environment the rate of dissolution could be quite variable, there is a possibility that a combination of nanoparticles (i.e. size) and substances (dissolved nanomaterial) elicits the detected toxic effects (Luoma 2008). Limbach et al. (2007) have shown that for partially soluble nanomaterials such as cobalt oxide and manganese oxide the nanoparticles may be taken up into cells preferentially to their respective ionic forms. It is then possible that once inside the cells these nanoparticles may dissolve, resulting in enhanced toxic effects. Regardless of the key causes, it should be considered that given the increased use, and thus release, of nanomaterials with different levels of solubility this will lead into potential increased levels of soluble substances which may result in undesirable environmental effects. These effects may be enhanced (depending on material, receiving environment and species) by a combination of forms, i.e. particulate and soluble.

For nanomaterials that do not dissolve readily, it should be determined whether they will form stable dispersions in air or stable suspensions in aqueous media (in both fresh and sea waters).

3.6.2.2. Exposure to nanomaterials in experimental studies

One of the major problems in aquatic ecotoxicological fate and effects testing is the absence of consistent and broadly-applicable information on how nanomaterials are suspended in various exposure media used in ecotoxicological testing. There are essentially three approaches to achieve as uniform as possible stock solutions for testing: dispersion with strong solvents and detergents, dispersion by sonication or dispersion by prolonged stirring (Crane et al. 2008, Klaine et al. 2008). Suspension methods used to date include the use of strong solvents e.g. tetrahydrofuran (THF) (Oberdörster 2004), dispersion agents e.g. sodium dodecyl sulphate (SDS) (Smith et al. 2007), bath or ultrasonication with filtration to remove aggregates (Lyon et al. 2006), stirring (Hund-Rinke and Simon 2006, Oberdörster et al. 2006), and combinations of these methods. Natural organic matter (NOM) can be a suitable dispersant and can keep the nanoparticles suspended longer than a 1% solution of SDS (Hyung et al. 2007). In addition, the time used during mixing (sonication or stirring) is also very variable (Klaine et al. 2008). Henry et al. (2007) has referred to some of the key issues regarding the use of solvents when dispersing nanomaterials in aqueous media.

In most cases, the verification of exposure and the characterisation of the nanomaterials in the resulting suspensions is limited to working on stock solutions rather than on the actual concentrations, either after a dilution series is generated or periodically over the duration of an exposure or media-renewal period. As such, it is likely that the methods reported might not produce similar results for different forms of a nanomaterial. In addition, similar to the testing for health effects also the possible temporal evolution of nanomaterials during the perfomance of the assays should be considered.

3.6.2.3. Food chain effects and secondary poisoning

Not much work has been published on potential food chain effects of nanomaterials. A recent study (Holbrook et al. 2008) on the possible transfer of quantum dots in a simplified aquatic food chain has found that these materials can be transferred to rotifers through dietary uptake of ciliated protozoans. Although there was transfer across these levels, bioconcentration (accumulation from surrounding environment) in the ciliates was limited and no biomagnification (enrichment across levels) in the rotifers detected. This study indicates potential for transfer across food chain levels but this would depend on material type and food chain, as is mostly the case for other studies of standard materials. Fortner et al. (2005) have also observed that fullerene nanoparticles accumulate in microbial cells, in worms eating those microbes and possibly in animals higher up the food chain. Furthermore, Bouldin et al (2008) have reported the transfer of quantum dots from dosed algae (*Pseudokirchneriella subcapitata*) to *Ceriodaphnia dubia*. Petersen et al. (2008b) have also indicated that CNTs were not readily bioaccumulated by the earthworm *Eisenia foetida* with results indicating bioaccumulation factors 2 orders of magnitude smaller than those measured for pyrene used for comparison.

3.6.3. Environmental effects

Ecotoxicological testing in soil and sedimentary systems has been the focus of relatively few studies. As a result, methodology and practical approaches have not been as widely discussed in the literature. Suspensions of nanomaterials in aquatic media, followed by either mixing or spraying on sediments/soils would lead to similar issues to the ones raised above. Other methods include mixing or applying nanomaterials directly to soils and sediments. It is clear that similar issues of standardisation also apply to these systems. There is a need, therefore, to address methodological variability associated with the current studies assessing the hazard of nanomaterials in environmental models. Although it is accepted that methods need to be appropriate to the materials being tested, as well as the test organisms and end points studied, it is important that standardised methology is developed and implemented so that variability can be kept to a miminum and results widely accepted and replicated.

In general, it is necessary to check that the suspensions used for aquatic testing are suitable for the test organisms. Salt concentration, pH, solvent and amount of solvent have to be within ranges tolerated by the test organisms. For organic and inorganic nanomaterials different procedures are usually applied. Inorganic nanomaterials (e.g. metals and metal oxides) are weighed into an aqueous solution; they are homogenously dispersed by ultrasound or by stirring and followed by filtration. Organic, water-insoluble nanomaterials are dissolved in solvents such as tetrahydrofuran (THF), toluene or benzene, or dispersed by detergents. By adding water and removing the solvent, a stable aqueous suspension is obtained. However, there are indications that traces of THF used to solubilise fullerenes may remain in the suspension resulting in toxicity due to the solvent (Zhu et al. 2006).

3.6.3.1. Environmental test systems

Microbial systems

Uptake of nanomaterials by microbial organisms might be via diffusion, specific or non-specific uptake, or via membrane damage (Klaine et al. 2008). The bioavailability and antibacterial activity of C_{60} fullerene in soil and water were found to be affected by the concentration of humic acids (Li et al. 2008). In general, the higher the carbon content of the soil, the stronger the likely adherence to soil/sediment. A list of some microbial effects observed is presented in Table 1.

Table 1 Effects of nanomaterials on microbial species (from Klaine et al. 2008, Wiesner 2006, and references therein)

Nanomaterial	Observed Effects
Carbon-containing	
Fullerenes	Antibacterial to a broad range of bacteria, inhibit growth of *E. coli* by interfering with energy metabolism
	Cleave plasmid DNA
	Induce DNA damage in plasmids
Carbon nanotubes	Antibacterial to *E. coli*, cell membrane damage
	Cytotoxic to microbes
Metallic	
Quantum dots, Silver	Bactericidal; viricidal
	May penetrate cells by oxidative damage to membrane
Gold	Low toxicity to *E. coli* and *Staphylococcus aureus*
Metal oxides	
TiO_2 , MgO, CeO_2 ZnO	Generalised anti-bacterial effect
Others	
SiO_2	Mild toxicity due to ROS production

Generalised microbial effects reported have been: disruption of membrane/membrane potential, production of ROS, oxidation/damage to proteins, interference with electron transport/respiration, potential DNA damage, with in general more serious effects observed on Gram-positive species (Klaine et al. 2008 and references therein).

Terrestrial systems

Few studies on the effects of nanomaterials on soil organisms have been published to date. One of the first studied the effects of aluminium oxide nanoparticles on the emergence and growth of plants (Yang and Watts 2005). The authors observed clear effects but it was later debated whether these effects were due to the nanoparticle form of aluminium or to a soluble fraction of aluminium ions (Murashov 2006). Regardless of the key cause, it is clear that a negative effect was observed that resulted from the exposure to aluminium in a nanoparticulate form.

Recently, Cañas et al. (2008) studied the effects of single walled carbon nanotubes on the root elongation of crop species. A few minor effects were detected on some species after rather short exposure times of 24 and 48 hours. However no uptake of single walled carbon nanotubes was observed. Lee et al. (2008), using a plant agar test system, indicated a toxic effect of copper nanoparticles as demonstrated by a reduced growth of seedlings of mung bean (*Phaseolus radiatus*) and wheat (*Triticum aestivum*). Using transmission electron microscopy and energy dispersive spectroscopy, the authors observed the accumulation of copper particles in the cells.

Reduced enzyme activities for catalase (CAT) and glutathione-S-transferase (GST) were observed after ingestion of titanium dioxide nanoparticles (anatase) by terrestrial isopods (*Porcellio scaber*) (Jemec et al. 2008). However the overall endpoints like survival and growth were not affected. Scott-Fordsmand et al. (2008) detected effects on the

reproduction of earthworms (*Eisenia veneta*) when the worms were exposed to double-walled carbon nanotubes in food.

Aquatic systems

Studies focussing on the hazard of nanomaterials to a range of aquatic species were reviewed by SCENIHR (2006). Further developments took place through 2008 with new data and knowledge generated. Most of the recent studies have indicated that key aspects of aggregation may result in exposures not reflecting the highest doses. Issues considering solvents and the role of NOM, as a stabiliser of nanomaterials, have also been highlighted (see above). Nevertheless, it is important to note that increased dispersion has not led to increased bioavailability and hazard in all studies. Results depend on nanomaterial composition (Franklin et al. 2007, Navarro et al. 2008a). Exposure to raw carbon nanotubes resulted in the viability of *Daphnia magna* being reduced, but this was not found in stable dispersions when compared with functionalised and stabilised forms of carbon nanotubes (Kennedy et al. 2008). In contrast, Kang et al. (2008) indicated that some methods used to stabilise the dispersion of CNTs (e.g. functionalisation) resulted in increased toxicity for the bacterial systems they were studying.

Recent results have indicated the physical interference (e.g. movement hindrance, clogging) of nanomaterials with biota (e.g. Nielsen et al. 2008). Sublethal effects observed included lipid peroxidation, altered haematology, changes in behaviour with some effects being observed at different stages in the life cycle (Zhu et al. 2007).

Interactions of nanomaterials with other pollutants

It has been suggested that nanomaterials may interact with contaminants which may result in toxic effects on biota (Cheng et al. 2007, Xia et al. 2004). Baun et al. (2008) indicated the potential of nanomaterials to enhance the toxic effects of organic contaminants.

3.6.3.2. Methods of assessment in vitro

Methods of assessment *in vitro* have mirrored work in the area of mammalian toxicology. For example, several approaches used to study oxidative assessment have been used, and more slowly work is taking place in the area of genomics and proteomics. Other methods, borrowed from mammalian work, such as assessment of effects of nanomaterials on fish hepatocyte function evaluated by enzyme lactate dehydrogenase (LDH) as a marker for cell toxicity (Bopp and Lettieri 2008) or assessment of lysosome stability in mollusc hemocyte cells, may also be used (Castro et al. 2004).

Linking effects at different levels of organisation is important in the assessment of potential long term effects, as well as to improve knowledge on how some toxic effects may occur. *In vivo* exposures, followed by assessment of specific endpoints at organ and organelle levels, as well as biochemical endpoints, provide a comprehensive approach to assessment of effects. *In vitro* studies, such as the ones described above, allow a focused assessment of mechanistic effects at a specific level of organisation.

3.6.3.3. Methods of assessment in vivo

There is still much debate in the literature (Fernandes et al. 2007, Handy et al. 2008b, Klaine et al. 2008) regarding what may be considered optimal approaches for exposures. Exposure media, mixing or suspension of materials within the media and consideration of realistic exposures, have all been a particular focus of attention. In this context, an important point of consideration has been the characterisation of the nanomaterials in the exposure studies. This has been particularly debated in studies of sedimentary systems. Mixing of nanomaterials with sediments/soils, as well as characterisation over time, are still areas at a very early stage of development.

In this context, consideration of detection, in a background of natural abundance of specific types of materials such as carbon-based products, zinc and silicon, is an area that is still currently advancing in technical terms.

A wide range of methods have been used to assess the hazard of nanomaterials on environmental species. The approaches were chosen according to the species studied. Laboratory studies have focussed on the effects of a range of nanomaterials on standard species used in ecotoxicology. Most have focussed on aquatic species including: primary producers (mainly *Pseudokirchneriella subcapitata* (Franklin et al. 2007, Van Hoecke et al. 2008) and *Desmodesmus subspicatus* (Hund-Rinke and Simon 2006); invertebrates, mainly *Daphnia* species but also other crustacean (Fernandes et al. 2007, Hund-Rinke and Simon 2006, Lovern and Klaper 2006, Rosenkranz et al. 2009), and fish (such as rainbow trout *Oncorhynchus mykiss*, zebra fish *Danio rerio*, largemouth bass *Micropterus salmoides*, fathead minnow *Pimephales promelas* and Japanese medaka *Oryzias latipes*;(Federici et al. 2007, Griffit et al. 2007, Lee et al. 2007, Oberdörster 2004, Smith et al, 2007, Warheit et al. 2007, Zhang et al. 2007). The toxicity to various microbial organisms has also been studied (e.g. Lyon et al. 2006, Lyon and Alvarez 2008, Sondi and Salopek-Sondi 2004). In some of these studies, toxic effects were observed.

The integration of endpoints such as mortality, growth, feeding and reproduction, are widely used in ecotoxicology. In addition, specific biomarkers, such as means of assessing oxidative stress (in a specific organ, or whole body; e.g. lipid peroxidation), genetic damage, CYT P450 levels, gene expression, damage to specific cell organelles (e.g. mitochondria or nucleus) are all widely used in the assessment of effects of nanomaterials. Cytological responses such as cellular apoptosis and necrosis have also been used. Although the methods employed in these studies tend to be standard methods used routinely in ecotoxicology studies, modifications have been implemented to address the specific particle issue, or to address the effects of interference of the materials with reading of results.

Less research has taken place using soil or sedimentary species but work is now progressing at a steady pace in these systems. The procedures that were adopted have in general followed OECD guidelines, particularly when using standard species, but also when other species were used. Depending on the aims of the study, different durations of exposure have been used.

The number of scientific studies assessing the environmental effects of nanomaterials has increased dramatically in the period 2007-2008. The main focus is still on micro-organisms and invertebrates, followed closely by studies on fish species. Still very much lacking are studies on soil systems and terrestrial species in general, including primary producers. There is also a general paucity of studies on marine species. This is not surprising given the complexity associated with dispersing and suspending nanomaterials in exposure media. Nevertheless, published results to date indicate clearly the potential for hazardous effects, at lethal and sublethal levels, including behaviour, reproduction, growth and development, ROS production, induction of inflammatory responses and cytotoxic effects. In addition, a small number of studies have indicated the potential for transfer to embryos, accumulation and potential food chain transfer.

Nevertheless, the exposure levels organisms may endure in their natural environments and how the results in the laboratory can be extrapolated to assess hazard in the field is less clear. Information on environmental loads is at present lacking. One important aspect in this context is the understanding of any interactions of nanomaterials with micro-organisms in sewage treatment plants, and the consequent effects on the treatment process.

A few key issues need to be brought out when assessing critically the results obtained to date on the environmental hazard of nanomaterials in order to focus on what is important and optimise the approach and design of future studies.

A first issue, which has already been widely discussed, concerns the protocols used in laboratory exposures and a related link to the current lack of standardised protocols. The

use of mechanical or chemical means to suspend nanomaterials may lead to changes in the physical-chemical properties of the test material. It is unclear what the extent of these may be and how they may impact any effects observed.

Arguably, dispersants/surfactants/solvents may need to be used in certain situations; however, it is important that they must not add to the toxicity of studied materials. It is suggested that results of studies where THF was used should be treated with caution, as at least in one study, the observed toxicity was due to traces of THF (Zhu et al. 2006). The same caution may apply to other dispersants for which there is lack of knowledge regarding their interaction with the test material (e.g. SDS). Further work with humic and fulvic acids, as well as widely used detergents (which are likely to be encountered in the environment) should be undertaken.

Related to this topic is the use in hazard assessment of ready-made (off-the-shelf) suspensions of nanomaterials. It is unclear what the interactions might be of the used preparation dispersants on the properties (and thus behaviour) of the test material (as described above). Thus any reported effects might not be comparable with effects observed on exposures of the same species to the same component material but which is in a different form (i.e. solid and suspended nanomaterials in the laboratory vs nanomaterials obtained as a suspension).

Therefore, standardisation of protocols, as possible, is desirable for the comparability of studies as well as reliability of results, and the derivation of information to risk assessment and risk management.

Regarding experimental design and approach, characterisation of exposures, via appropriate method(s) should be carried out and chemical analyses undertaken, as possible. The assessment of the solubility of the nanomaterials being studied is very important in this context so that any observed effects can be attributed to the different fractions. This is particularly important in the case of certain metal nanomaterials, as well as in the case of CNTs and quantum dots.

Some studies have highlighted the importance of assessing contamination of the nanomaterials being studied. This should be undertaken for similar reasons. Another important point is the comparison of effects between nano and equivalent, larger, material. This has not been consistently incorporated in the published studies and would also allow the correct attribution of effects.

There is a lack of information regarding the fate and form of the test nanomaterials within biological systems following in-vivo exposures. It is unclear what particular form (e.g. soluble or particulate) is preferentially taken up into tissues and cells. It is likely that this would depend on the material composition; nevertheless these studies are not routinely carried out.

Studies should be conducted on a range of guilds and endpoints, with fate within the body and tissues assessed and depuration quantified, as possible. Micro/mesocosms studies should be undertaken. Furthermore, dietary studies, the role of nanomaterials' coatings in uptake and translocation within the body, should be conducted, as well as the assessment of the role, if any, of their interaction with other environmental contaminants.

In this context it is crucial to ascertain the fate of nanomaterials in the environment so that their availability for environmental exposure can be assessed. Environmental fate and load assessment of nanomaterials must, therefore, be undertaken. The use of the current approach to the derivation of K_{ow} in the assessment of environmental fate is unlikely to be beneficial to risk assessment. Nevertheless, the derivation of alternative approaches may be useful and may allow the development of appropriate predictive modelling. Finally, further information on the degradability (bio and abiotic) of nanomaterials should be derived.

3.7. NANOTECHNOLOGIES- RISK ASSESSMENT

A suitable framework for the assessment of all engineered nanomaterials requires exposure and hazard data on a wide range of products. At present there have been insufficient published studies to establish a detailed framework. Nonetheless in the previous SCENIHR opinion an outline for such a framework, in the form of an algorithm, was presented (SCENIHR 2007a). This framework remains appropriate although a few further details can be added in the light of recent publications.

3.7.1. Relevant physicochemical properties

The most important properties of a nanomaterial to characterise, from a risk assessment viewpoint, are:

- Size and size distribution of free particles and fibres/rods/tubes. These may be produced during the manufacture, use (including wear) and/or disposal/recycling of the nanoproduct.
- Specific surface area
- Stability in relevant media (including the ability to aggregate and disaggregate)
- Surface adsorption properties
- Water solubility

In addition, suitable measurements of chemical reactivity are needed although at present the most relevant ones for a particular nanoparticle/nanofibre are best judged on a case by case basis bearing in mind the likely applications of the product (see read across).

Depending on the nature of the nanoparticle/nanofibre it may also be appropriate to consider:

- *Photoactivation*. Recent data have indicated that some nanoparticles may, by virtue of their relatively large surface area and reactive potential, become activated by light. This is relevant both in considering their stability and their potential to be photo activated when in contact with the skin/external surfaces of other species.
- *Potential to generate active oxygen*. Production of active oxygen is one accepted general mechanism for the adverse effects of nanoparticle/nanofibre. Thus the *in vitro* measurement of the ability of a particular nanoparticle/nanofibre to generate active oxygen species may be considered.

3.7.2. Read-across

There is insufficient information to identify opportunities for read-across based on the general chemical composition of nanomaterials. Nonetheless there are some properties for which read-across is appropriate in determining the experimental studies that need to be considered.

i)   *Fibres, rods and tubes*. In the light of experience with asbestos, and the recent studies on carbon nanotubes (reported above) if there is a potential exposure to free fibres, rods and tubes that are chemically/ biologically persistent, are rigid and have a high aspect ratio (i.e. micrometres in length and nanometres in diameter) the possibility should be considered that they may have similar properties to asbestos.

ii)   *Particles.* There is a large amount of data on airborne fine particles generated as a result of combustion to indicate that comparable particles may cause respiratory and cardiovascular effects. Situations in which there is production of fine nanoparticle/nanofibre with reactive surfaces could cause comparable effects.

iii) *Nanomaterials of comparable dimensions and surface properties.*The database for extrapolation is very limited, however, for the assessment of a specific nanomaterial it may be possible to highlight relevant properties that require particular assessment.

iv) *Bulk material.* For any nanomaterial for which significant exposure of man or other species could occur, it is appropriate to consider the toxicological/ ecotoxicological properties of the material in other physical forms, unless there is good evidence that no bulk material will be released in biological systems.

3.7.3. Development of the risk assessment framework

3.7.3.1. Development of the SCENIHR algorithm

In the previous opinion of the SCENIHR (2007a) a four tier algorithm was presented in which the initial consideration was the potential for exposure of man and/or other environmental species to the nanomaterial. There are no new data that would suggest a significant change in the SCENIHR exposure driven framework to be appropriate, other than the aspects discussed above.

This four stage algorithm offered a framework for the case by case evaluation of the potential risks due to exposure of humans and other species to nanomaterials. The algorithm is exposure–driven. To be of practical value a thorough assessment of the potential exposure of humans and other environmental species during the entire life cycle is vital. This must include not only the current use but also possible further applications. In addition, it must take into account the potential for nanomaterials to be released during use (e.g. as a consequence of wear and tear), and the range of end-use fates, that may occur (e.g. waste disposal or waste recycling options).

It is anticipated that as the scientific knowledge improves, it may be possible to classify nanomaterials into specific risk categories that might become the subject of category specific risk assessments. However, at present this categorisation is not possible. New significant developments have come to light (as identified above), which allows further work on Stage III (Hazard identification and characterisation). These include:

i) Cell/tissue uptake tests;
ii) Bioaccumulation tests to assess prolonged exposure;
iii) Selection of a test system(s) for nanofibers/tubes that are biopersistent, are rigid and have a high aspect ratio (HAR – L>20µm);
iv) Ability of nanomaterials to trigger one or more of the putative mechanisms of toxicity (e.g. generation of reactive oxygen species).

3.7.3.2. Addressing deficiencies in the data base

As discussed above, one of the major challenges for nanotechnologies (and indeed for other emerging issues) is how to characterise the risks where the database is very limited.

The traditional approach to address such a situation is to adopt a traditional risk assessment framework and either:

– introduce a default value for each major data gap

– compare the new/potential nano product with the conventional (non-nano) product

However, other approaches to the risk assessment of nanomatertials exist. There have been several recent reviews of the emerging health issues from products of nanotechnologies (Hannah and Thompson 2008, Hoyt and Mason 2008, Linkov et al. 2007, Sweet and Strohm 2006, Wardak et al. 2008,). Two approaches for coping with the large gaps in the data have emerged from these publications:

— Application of lifecycle methodology currently used to evaluate sustainability that has a lesser data requirement.

Sweet and Strohm (2006) have proposed a structured approach that combines risk assessment and risk management approaches viz:

— Is it likely that the product system contributes to actual harm in the life cycle?

— How much does each product or stage contribute?

— Do relevant toxicity data exist for risk assessment?

— What is the potential upstream and downstream technology units impacted?

— What opportunities are available for upstream or downstream improvements (e.g. environmental quality, emission reductions)?

— What opportunities to control the risks by selecting less risky options or by restricting access to the hazard or life cycle stage of concern?

Application of this approach, using worked examples would enable the general utility of this approach to be assessed

Von Gleich et al. (2008) have applied life cycle inventory analysis to assess the potential risks and benefits of several eco-efficient nano surface coatings for metals compared to the non nano surface coating equivalents. This approach appears to be promising to identify the general environmental impacts of a nanomaterial in terms of use of resources including energy, but the value of its application to issues concerning human health is as yet uncertain.

— Use of expert judgment to fill the critical gaps.

One interesting aspect of this approach is its use to identify measures that should be taken for containment or environmental control in the workplace. The approach uses a matrix to characterise the level of concern and the consequent action that should be taken (See Table 2). It involves a two dimensional matrix of the likelihood of exposure and the likelihood of effects and severity. With modification it could be applied to identify the potential impacts of individual nanomaterials on human health and on other species along the following lines:

Table 2: Expert Judgement matrix

EXPOSURE TO NP/NF

E		probable	likely	Less likely	Extremely unlikely
F	v. high	4	4	3	3
F	high	4	4	3	2
E	medium	4	4	2	2
C	low	3	3	1	1
T	v. low	2	1	1	1

Where 4 =highest priority for a detailed assessment/ preventative action, 1= the lowest priority for detailed assessment/regulatory action. This type of methodology has been applied to air fresheners incorporating nanoproducts and to a range of other consumer products (Wardak et al. 2008) although these authors use graphic plots rather than a matrix format to present the findings.

Such a methodology could prove useful as a first step, for example, in achieving consensus among experts and risk managers on priorities. It may also be of value in a matrix or a graphic form to compare products/exposure situations in a transparent manner.

Von Gleich et al. (2008) have proposed that the assessment of the potential impacts of nanomaterials should run closely in parallel with the research and technical work to develop them. They refer to this process as 'leitbuilder'. This is a logical concept that is worthy of further elucidation.

A more structured way of selecting which nanoproducts to be developed has been proposed by Linkov et al. (2007) using multi-criteria decision analysis. Their approach is that, prior to comparison of individual nanomaterials; expert judgment is used to set weighted values for relevant parameters of health and ecological effects (e.g. public health effects, effects related to occupational exposure, environmental effects), importance to society and stakeholder preference. The challenge with such an approach is to set the most appropriate weighting, in a manner that is both transparent and acceptable to all the key stakeholders.

The Swiss Federal Office of Public Health (2008) has proposed a structured scoring system for the categorisation of risks posed by nanomaterials into two classes A and B. The approach is compatible with the above approach. The key parameters incorporated into this system are:

- Exposure of human beings / release into the environment

- Potential effects (e.g. stability in biological systems (biopersistence), redox activity)

- Nano-relevance (e.g. physico-chemical properties) and

- Information about the life-cycle (e.g. is the future life-cycle known?).

The approach is sound but needs to be evaluated using actual case histories. One possible concern is the use of a single total numerical score to assign nanomaterials as either low risk or possible risk.

3.7.4.   Conclusion for the risk assessment

The development of a widely accepted and robust methodology that would be used at the R&D stages to identify and mitigate potential human health (including occupational health) and environmental risks, associated with individual nanomaterials should be given high priority. For this purpose it is vital to develop a data bank of case histories to assess its validity.

3.8. Research needs

The research needs as indentified by SCENIHR in its Opinion on the evaluation of the Technical Guidance Documents (TGDs) are still valid (SCENIHR 2007a). They include amongst others the availability of validated *in vitro* assays, development of an approach for using quantitative structure activity relationship (QSAR), studies on potential cardiovascular effects, evaluation of bacterial genotoxicity assays, methodology on prediction of environmental concentrations (PEC), and environmental species used for ecotoxicity testing. Some progress has been made in the areas of properties needed to determine for nanomaterial characterisation, toxicokinetics and genotoxicity testing with mammalian cells, and environmental behaviour of nanomaterials. Recent research has also identified new concerns in the areas of protein fibrillation, potential hazards of

certain nanotubes, and potential for transfer across the food chain in environmental species.

3.8.1. Characterisation of nanomaterials

There is a need for comparable, reproducible and repeatable harmonised methods for measuring and characterising nanomaterials (SCENIHR 2006), especially for measuring concentrations and characteristics of nanomaterials in biological and environmental media. Being able to address these gaps is important for providing meaningful data which can produce a system of reliable risk assessment. This requires also defining the metrics most appropriate for hazard characterisation and exposure, including the methodology to perform the measurements.

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.

3.8.2. Determination of human exposure

Exposure determinations/estimations at workplace and the ambient environment need improvement. Therefore more specific measurement methodologies need to become available to discriminate beween background and manufactured nanomaterials. This can be achieved by working on the feasibility for routine assessments, development of reliable measurement techniques, standardisation of measurement techniques, measurement strategies, and implementation of screening/monitoring of nanoscale particles in sensitive work areas. Challenges are currently especially seen in the detection and assessment of nanoparticles from products in the environment.

Exposure estimates from food and consumer products remain difficult. Information on the presence of manufactured nanomaterials solely relies on information (claims) provided by manufacturers. In addition, exposure estimation is also hampered by lack of information on product use and use of multiple products containing manufactured nanomaterials. Coordinated efforts and research strategies for a comprehensive exposure assessment of manufactured nanomaterials need to be defined.

3.8.3. Identification of human hazards

The effect of nanoparticles on protein behaviour as demonstrated *in vitro* needs further investigation. It is necessary to elucidate whether the *in vitro* observed effects on protein fibrillation processes (both enhancement and retardation) also occur in an *in vivo* situation or in more complex biological fluids where competitive binding may take place.

There are indications that after deposition at the olfactory mucosa of the nose, ambient air and nanoparticles may translocate into the brain. This may offer a potential route of entry for medicinal products into the brain. This observation may also raise some concern in view of the amyloid diseases of the brain in the context of the potential of nanoparticles to cause protein fibrillation in vitro. This is certainly an area for which additional research is urgently needed.

Additional studies on the potential hazards of nanofibers/nanotubes need to be performed.

3.8.4. Environmental exposure

The estimation of relevant environmental exposure concentrations is seriously hampered by lack of the two essential pieces of information/knowledge. Firstly, there is no quantitative knowledge on the rates of release of nanomaterials to the environment. Secondly, there is neither knowledge nor theory that can be used to predict concentrations of nanomaterials in the ambient environment from release rates. Well-established knowledge of distribution and fate of chemical substances, as it is applied in the current EU guidelines for environmental risk assessment of conventional chemicals, cannot be used for nanomaterials without modification. It is recommended that research be initiated to develop quantitative theory and models that predict residual concentrations of free nanoparticles from release rates, and implement such models in the current EU guidelines for environmental exposure assessment of nanomaterials.

One of the unknown processess relevant to the environmental exposure assessment of nanomaterials is the extent/rate of dissolution of nanomaterials in water. It is unlikely that the standard OECD methods for measuring solubility of nanomaterials in water can provide the required information and it is recommended to revise these methods to accommodate the measurement of the rate of dissolution of nanomaterials in the natural environment.

Most urgently needed are analytical methods to detect and measure ambient concentrations of free nanomaterials. Currently, no standard methods exist for this purpose, although efforts are being developed in this area and environmental exposure levels are still unknown.

3.8.5. Environmental hazards

Studies on soil systems and terrestrial species in general, including primary producers are still lacking. There is also a general paucity of studies on marine species.

One important aspect in this context is the understanding of any interactions of nanomaterials with micro-organisms in sewage treatment plants, and the consequent effects on the treatment process.

Further work on the establishment of standard protocols is required. The use of mechanical or chemical means to suspend nanomaterials may lead to changes in the physical-chemical properties of the test material. It is unclear what the extent of these may be and how they may impact any effects observed.

Arguably, dispersants/surfactants/solvents may need to be used in certain situations; however, it is important that they must not add to the toxicity of studied materials. It is suggested that results of studies where THF was used should be treated with caution. The same caution may apply to other dispersants for which there is lack of knowledge regarding their interaction with the test material (e.g. SDS). Further work with humic and fulvic acids, as well as widely used detergents (which are likely to be encountered in the environment) should be undertaken.

Related to this topic is the use in hazard assessment of ready-made (off-the-shelf) suspensions of nanomaterials. It is not clear how the dispersants used in the preparation of the nanomaterials might interact with the test material, and what effects they may have on the properties (and thus behaviour) of the test material (as described above). Thus any reported effects might not be comparable with effects observed on exposures of the same species to the same component material but which is in a different form (i.e. solid and suspended nanomaterials in the laboratory vs nanomaterials obtained as a suspension).

Regarding experimental design and approach, characterisation of exposures, via appropriate method(s) should be carried out and chemical analyses undertaken, as possible. The assessment of the solubility of the nanomaterials being studied is very

important in this context so that any observed effects can be attributed to the different fractions. This is particularly important in the case of certain metal nanomaterials, as well as in the case of CNTs and quantum dots.

The importance of assessing contamination of the nanomaterials has been highlighted. The comparison of effects between nano and equivalent, larger, material needs to be undertaken. This has not been consistently incorporated in the published studies and would allow the correct attribution of effects.

There is lack of information regarding the fate and form of the test nanomaterials within biological systems following *in vivo* exposures. It is unclear what particular form (e.g. soluble or particulate) is preferentially taken up into tissues and cells. It is likely that this would depend on the material composition; nevertheless these studies are not routinely carried out.

Studies should be conducted on a range of guilds and endpoints, with fate within the body and tissues assessed and depuration quantified, as possible. Micro/mesocosms studies should be undertaken. Furthermore, dietary studies, the role of nanomaterials' coatings in uptake and translocation within the body, should be conducted, as well as the assessment of the role, if any, of their interaction with other environmental contaminants.

In this context it is crucial to ascertain the fate of nanomaterials in the environment so that their availability for environmental exposure can be assessed. Environmental fate and load assessment of nanomaterials must, therefore, be undertaken. The use of the current approach to the derivation of K_{ow} in the assessment of environmental fate is unlikely to be beneficial to risk assessment. Nevertheless, the derivation of alternately approaches may be useful and may allow the development of appropriate predictive modelling. Finally, further information on the degradability (bio and abiotic) of nanomaterials should be derived.

4. OPINION

While risk assessment methodologies for the evaluation of potential risks of substances and conventional materials to man and the environment are widely used and are generally applicable to nanomaterials, specific aspects related to nanomaterials still require further development. This will remain so until there is sufficient scientific information available to characterise the harmful effects of nanomaterials on humans and the environment. The methodology for both exposure estimations and hazard identification needs to be further developed, validated and standardised. The highest risk, and thus concern, is considered to be associated with the presence or occurrence of free (non bound) insoluble nanoparticles either in a liquid dispersion or airborne dusts.

Characterization of manufactured nanomaterials

For the characterisation of manufactured nanomaterials, several issues are important. A consensus is now emerging about what properties need to be determined for risk assessment purposes. In biological test systems nanomaterials may change their properties. In particular, they may partly dissolve or agglomerate/aggregate so that the particle size distribution changes. For (partially) soluble nanomaterials the toxicity may be governed at least in part by the soluble species/fraction released from the nanomaterial. For low solubility or a slow release, the particulate nature of the substance may be relevant with regard to potential tissue distribution and local release of toxic species which should then be considered in the risk assessment of such nanomaterials. When using nanomaterials, an extensive characterisation is necessary, including the nanomaterial as produced and the nanomaterials as used in test systems and the nanomaterial as present in final products. The characterisation 'as manufactured' provides information for the material safety data sheet (MSDS) of the product itself. The characterisation 'as used' in biological systems is needed as properties of nanomaterials may considerably change, notably the size distribution due to agglomeration/aggregation of the particles. An issue of specific importance are the properties of the nanomaterial as it is actually used in products and to which consumers may be exposed. For the risk assessment the latter characterization is of highest relevance.

Legally, in the EU, nanomaterials are covered by the definition of substance within the REACH regulation (Regulation (EC) No. 1907/2006) (European Commission 2006). However, the definition of nanoscale is still under debate. Various organisations have proposed definitions of nanoscale using an upper limit of about 100nm. It should be noted that most currently proposed definitions use the size of the primary particle/structure as a starting point. However, when a nanomaterial is in particulate form, the particles may be present either as single particles or as agglomerates or aggregates. Depending on the nanomaterial the majority of the particles may be agglomerates or aggregates. This may lead to the misinterpretation that agglomerates or aggregates of nanoparticles that have external dimensions well beyond 100nm are not considered nanomaterials. Yet they retain specific physicochemical properties which are characteristic of nanomaterials, most likely due to their large specific surface area (SSA). Therefore, when describing a nanomaterial it is important to describe not only the mean particle size but also the size of the primary particles. In addition, information on the presence of agglomerates or aggregates should be presented. Besides size, the specific surface area as determined by the BET method is a good metric to describe particulates as it is independent of the primary versus the agglomerated state. Hence, it should be considered to complement the current definition based on physical size by adding a limit of the specific surface area. Solid spheres of 100 nm with unit density have a specific surface area of 60 m²/g.

There is currently a need for reference nanomaterials. Some are available but they are spherical model materials which are certified primarily for size and are used mainly to calibrate instruments which measure particle size. The absence of well-defined parameters to measure and of standardised test protocols is identified as a major obstacle for reference material production. It should be noted that for use in biological

systems certain compounds need to be added which may have an effect on nanomaterial composition and properties resulting in changes in (toxic) behaviour.

Human exposure

One of the main limitations in the risk assessment of nanomaterials is the general lack of high quality exposure and dosimetry data both for humans and the environment. One of the issues is the difficulty to determine the presence on nanomaterials and properly measure them. In contrast to the situation for other exposure routes, for air-borne nanomaterials, analytical instruments are generally available to determine exposure (size distribution of mass and number). This is particularly true in the context of test atmospheres. However, differentiation between background and incidental exposure is generally not possible in real life situations as the methods employed mainly measure the presence of (ultrafine) particles and do not discriminate between the different types of particles that may be present. To date most information on particle measurements comes from airborne measurements at the workplace. No quantitative or qualitative measurements of manufactured nanomaterials in ambient air outside of workplaces have been identified. Even when outside measurements are available these may be confounded, an example being carbon nanotubes that also may originate from general combustion processes and thus can be found in ambient locations. This illustrates the difficulty of identifying exposure levels of airborne manufactured nanomaterials. There is a need to establish reliable and standardised measurement techniques, to develop measurement strategies, and to implement screening/monitoring of nanoscale particles in sensitive work areas. Challenges are currently especially seen in the detection and assessment of manufactured nanoparticles in the environment. This is even more urgent for exposure of humans and ecosystems via natural water, sediment and soil.

Exposure estimates for consumers from food and consumer products remains difficult. Information on the presence of manufactured nanomaterials solely relies on information (claims) provided by manufacturers. In addition, exposure estimation is also hampered by lack of information on product use and use of multiple products containing manufactured nanomaterials. In a similar fashion to air measurements, determination of manufactured nanomaterials in consumer products suffers from the difficulty of discrimination between background and intentionally added manufactured nanomaterials. Coordinated efforts and research strategies for a comprehensive exposure assessment of manufactured nanomaterials still have to be defined. The main issues may be summarised as problems in replicating actual exposure conditions in laboratory tests and the lack of general availability of robust and specific measurement methods. Exposure assessment needs to consider each stage in the life-cycle.

Human hazard

When nanomaterials come into contact with a biological fluid they may become coated with proteins and other biomolecules. The coating may then influence the outcome of the biological response to the nanoparticle. Coating proteins have been most widely studied in mammalian systems. The significance of nanomaterial coating for nanomaterial safety and nanomaterial risk assessment is clear, as it implies that detailed characterisation of the nanoparticles in the relevant biological environment is necessary. The coating of nanoparticles may be used for therapeutic purposes to prolong circulation time (by PEGylation) or to target specific locations (e.g. apolipoprotein E for brain, immunoglobulins for tumors).

As the protein coating may affect the nanomaterial behaviour including its biological effect, it may be anticipated that nanomaterials may have an effect on protein behaviour. Some nanoparticles have been found to have the potential to promote and to retard protein assembly into amyloid fibrils *in vitro.* These experiments were performed using an incubation of various nanoparticles with purified β_2-microglobulin or β-amyloid protein. Whether the observed nucleation process also occurs in an *in vivo* situation or in more complex biological fluids where competitive binding may take place remains to be

determined.

Existing data show that nanoparticles can enter the circulation from the respiratory tract or the gastro-intestinal tract but typically in minimal amounts (less than 1% percentage of the dose as expressed in mass units). However, although minimal in percentage this may result in a systemic availability of a considerable number of nanoparticles. Nanoparticle migration is likely to depend on the physico-chemical properties of the nanoparticles such as size and on the physiological state of the organs of entry. When the nanoparticles reach the blood circulation, the liver and the spleen are the two major organs for distribution. Circulation time increases drastically when the nanoparticles are hydrophilic and their surface is positively charged. A coating like polyethylene glycol (PEGylation) also increases the residence time in the circulation. For certain nanoparticles all organs may be at some risk. For all organs investigated so far, either the chemical component of the nanoparticles or the nanoparticles themselves could be detected, as demonstrated for the brain and the testes. In the case of distribution to the foetus *in utero,* contradicting results were observed. The knowledge on toxicokinetics has been increased showing that, for a given substance, the smaller nanoparticles do have a much wider organ distribution than the larger nanoparticles.

There are indications that after deposition at the olfactory mucosa of the nose nanoparticles may translocate into the brain. This may offer a potential route of entry for medicinal products into the brain. Because of the potential of nanoparticles to cause protein fibrillation *in vitro*, this observation may raise some concern in view of the amyloid diseases of the brain. This is certainly an area for which additional research is urgently needed.

Based on the observations on the effects of particulate matter present in air pollution some concern exists on the possible effect of manufactured nanoparticles on the cardiovascular system. However, this has not been clearly demonstrated to be the case for manufactured nanoparticles so far. Overall the information on the possible hazard of nanoparticles for cardiovascular effects is rather limited and needs expansion.

When carbon nanotubes have physico-chemical and biopersistence characteristics similar to those of hazardous asbestos fibres, it was demonstrated that they can induce similar inflammatory reactions. The main characteristics for this to occur are a long thin fibrous form (length >20 μm), rigidity, and no degradability (biopersistence). Whether inhalation exposure to such carbon nanotubes would pose a risk to humans is unknown. Thus, manufacturers of nanotubes (possibly of any chemical composition) should be aware that certain characteristics (e.g. length, rigidity, biopersistence) may pose a risk and the possibility for chronic inflammation and mesothelioma induction and consequently should be considered in the safety evaluation.

The genotoxic effects of conventional particles are driven by two mechanisms: direct and indirect (mediated by inflammatory processes). Nanoparticles may act via either of these pathways since they can cause inflammation and can also enter cells and cause oxidative stress. There is some evidence that the small size may allow nanoparticles to penetrate into sub-cellular compartments like the mitochondria and nucleus. The presence of nanomaterials in mitochondria and the nucleus opens the possibility for oxidative stress mediated genotoxicity, and/or direct interaction with DNA. For some manufactured nanomaterials genotoxic activity has been reported, mainly associated to reactive oxygen species (ROS) generation, while for others contradicting results were obtained. Besides oxidative stress, additional mechanisms of genotoxicity which may be specific for nanomaterials also need to be considered, such as possible mechanical interferences during cell division, and other sources of genotoxic effects (i.e. metal release by nanomaterials).

The main issues for human hazard identification may be summarised as a need to ensure that each test system is appropriate for nanomaterials and to ensure that endpoints of potential particular concern (e.g. cardio-vascular effects) are properly addressed.

Environmental exposure

The increasing production, use and disposal of nanomaterials will lead to an increase in environmental exposure. Similar to human health risks, fate and behaviour of the manufactured nanomaterials in the environment itself is crucial for the potential ecotoxic effects in various environmental species. Estimation of relevant exposure concentrations is seriously hampered by lack of the two most important pieces of information/knowledge. Firstly, there is no quantitative knowledge on the rates of release of nanomaterials to the environment. Secondly, there is hardly any knowledge on the concentrations of nanomaterials in the ambient environment. There is also no theory that can be used to estimate such concentrations from release rates. The main problem is that the well-established knowledge of distribution and fate of chemical substances, as it is applied in the current EU guidelines for environmental risk assessment of conventional chemicals, cannot be used for nanomaterials without modification. Most certainly, the K_{ow} is of limited use as a predictor of the extent to which nanomaterials adhere to solid surfaces.

A hypothesis to describe fate and distribution of nanomaterials is slowly being developed, mostly from classical knowledge of colloid science. It is recognised that the main factors that influence the colloidal behaviour of nanoparticles (aggregation/agglomeration, sedimentation) are, besides the physical and chemical properties of the nanomaterial, the properties of the receiving environment: pH, ionic strength, prensence of natural organic matter. Depending of the nanomaterial characteristics either an increased sedimentation or improved dispersion of nanomaterials in water may occur. Exposure estimates are hampered by difficulties in distinguishing manufactured nanomaterials from background levels of naturally occurring nanomaterials. For the environmental risk assessment the estimation of water concentrations is essential. The assessment of exposure concentrations of dispersed nanomaterials requires detailed insight into the processes that act on the particles in the environment. However, currently available knowledge of these processes is insufficient to allow quantitative predictions of the environmental fate of nanomaterials.

The solubility of the nanomaterials is an important property that needs to be addressed. Knowledge of the extent to which nanomaterials dissolve and the rate at which this takes place is essential in two respects: (i) it is a direct control of the concentrations of nanomaterials in the environment and of the time that the nanomaterials reside in the environment and in organisms, and (ii) it determines the concentrations of dissolved species that originate from the nanomaterials. Knowledge of the extent to which nanomaterials dissolve in water, and of the rate at which this occurs, is essential to predicting the environmental fate and the effects of nanomaterials. It is doubtful whether currently available standard methods for measuring the (rate of) dissolution can adequately deliver this knowledge.

Unlike in the assessment of exposure concentrations of conventional (dissolved) chemical substances, the octanol-water partition coefficient K_{ow} is likely to have a limited role in predicting water-solids partitioning. An alternative theory to predict the exposure levels of nanomaterials in water is yet to be developed. Based on well-established knowledge of colloid science, it is expected that pH, ionic strength and presence of natural organic matter in the water compartment (freshwater versus marine environments) are important factors influencing the residual levels of nanomaterials in suspension. Depending on these factors and the chemistry of the manufactured nanomaterial increased aggregation and thus sedimentation or in contrast enhanced dispersion may occur.

For some nanomaterials (i.e. quantum dots) the transfer across environmental species was demonstrated indicating a potential for bioaccumulation in the species at the end of that part of the food chain. The main issues may be summarised as the development of suitable methods to assess the distribution of nanomaterials in the environment and the lack of portable monitoring equipment to measure levels of nanomaterials in different environmental media.

In addition, for many manufactured nanomaterials the methods currently used (carbon dioxide production, integration into biomass) for determining biological degradation will not be applicable.

Environmental hazard

Ecotoxic effects on environmental species have been demonstrated; aquatic species have been most studied. One of the major problems in ecotoxicological fate and effects testing is the absence of consistent and broadly-applicable information on how nanomaterials are to be suspended in various exposure media used in testing. Exposure media, mixing of materials with the media and consideration of realistic exposures, need a particular focus of attention. In this context, the characterisation of the nanomaterials in the eco(toxico)logical studies is important. Mixing of nanomaterials with sediments/soils, as well as characterisation over time, are areas which are still at a very early stage of development. In addition, there is the problem of the presence of background levels of nanomaterials and how to distinguish them from the nanomaterials being tested.

The common endpoints used in ecotoxicology such as mortality, growth, feeding, and reproduction can also be used for the evaluation of ecotoxicity by nanomaterials. In addition, some biomarkers similar to those used in the assessment of mammalian toxicity, such as oxidative stress, genetic damage and gene expression, may provide some insight in toxic mechanisms of nanomaterials.

The main issues for environmental hazard assessment may be summarised as the need for validation of laboratory test systems for characterising the effects of nanomaterials and the need for studies of the impacts of specific nanomaterials on ecosystems.

Risk assessment

Health and environmental hazards have been demonstrated for a variety of manufactured nanomaterials. The identified hazards indicate potential toxic effects of nanomaterials for man and the environment. However, it should be noted that not all nanomaterials induce toxic effects. Some manufactured nanomaterials have already been in use for a long time (e.g. carbon black, TiO_2) showing low toxicity. Therefore, the hypothesis that smaller means more reactive, and thus more toxic, cannot be substantiated by the published data. In this respect nanomaterials are similar to normal chemicals/substances in that some may be toxic and some may not. As there is not yet a generally applicable paradigm for nanomaterial hazard identification, a case-by-case approach for the risk assessment of nanomaterials is still recommended.

5. MINORITY OPINION

6. LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectrophotometry
ADME	Absorption, distribution, metabolism and excretion
ADP	Adenosine diphosphate
BAF	Bioaccumulation factor
BBB	Blood Brain Barrier
BCF	Bioconcentration factor
BTB	Blood Testis Barrier
CAT	Catalase
CB	Carbon black
CEN	European Committee for Standardisation
CNT	Carbon Nanotube
CYT	Cytochrome
DNA	Deoxyribonucleic acid
ECHA	European Chemicals Agency
EEG	Electroencephalography
EFSA	European Food Safety Authority
EMEA	European Medicines Agency
ETP	European Technology Platform
g	gram
GALT	Gut Associated Lymphoid Tissue
GI	Gastrointestinal
GLP	Good Laboratory Practice
GST	Glutathione-S-transferase
h	hour
HAR	High Aspect Ratio
HARN	High Aspect Ratio Nanoparticles
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
ICRP	International Commission on Radiological Protection
IgG	Immunoglobulin G
IRMM	Institute for Reference Materials and Measurements
ISO	International Organization for Standardisation
JRC	Joint Research Centre
kg	kilogram
K_{ow}	Octanol-water partition coefficient
l	litre
LDH	Lactate dehydrogenase
m^2	square metre
m^3	cubic metre
MARCO	Macrophage receptor with collagenous structure
mg	milligram

min	minute
MSDS	Material Safety Data Sheet
MWCNT	Multi-walled Carbon Nanotube
NIOSH	National Institute for Occupational Safety and Health
NIST	National Institute of Standards and Technology
nm	nanometre
NOM	Natural Organic Matter
OECD	Organisation for Economic Cooperation and Development
PAH	Polycyclic Aromatic Hydrocarbons
PBS	Phosphate buffered saline
PEC	Predicted Environmental Concentration
PEG	Polyethylene glycol
PM2.5	Particulate matter below 2.5 µm in diameter (respirable fraction)
QSAR	Quantitative Structure Activity Relationship
REACH	Registration, Evaluation and Authorisation of Chemicals
RM	Reference Material
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SCCNFP	Scientific Committee on Cosmetic Products and Non-Food Products
SCCP	Scientific Committee on Consumer Products
SCENHIR	Scientific Committee on Emerging and Newly Identified Health Risks
SSA	Specific Surface Area
SWCNT	Single Walled Carbon Nanotube
TG	Test Guideline
TGD	Technical Guidance Document
THF	Tetrahydrofuran
µg	microgram
µm	micrometre
WWICS	Woodrow Wilson International Centre for Scholars

7. REFERENCES

Arredouani M, Yang Z, Ning Y, Qin G, Soininen R, Tryggvason K, et al. The scavenger receptor MARCO is required for lungs defense against Pneumococcal Pneumonia and inhaled particles. J Exp Med 2004; 200:267-72.

Baalousha M, Manciulea A, Cumberland S, Kendall K, Lead JR. Aggregation and surface properties of iron nanoparticles: influence of pH and natural organic matter. Environ Toxicol Chem 2008; 27:1875-82.

Barnes CA, Elsaesser A, Arkusz J, Smok A, Palus J, Leśniak A, et al. Reproducible comet assay of amorphous silica nanoparticles detects no genotoxicity. Nano Lett 2008; 8:3069-74.

Baun A, Sörensen SN, Rasmussen RF, Hartmann NB, Koch CB. Toxicity and bioaccumulation of xenobiotic organic compounds in the presence of aqueous suspensions of aggregates of nano-C_{60}. Aquatic Toxicol 2008; 86:379-87.

Bihari P, Vippola M, Schultes S, Praetner M, Khandoga AG, Reichel CA, et al. Optimized dispersion of nanoparticles for biological in vitro and in vivo studies. Part Fibre Toxicol 2008; 5:14.

Blunk T, Hochstrasser DF, Sanchez JF, Müller BW, Müller RH. Colloidal carriers for intravenous drug targeting: plasma protein adsorption patterns on surface-modified latex particles evaluated by two dimensional polyacrylamide gel electrophoresis. Electrophoresis 1993; 14:1382-7.

Bopp SK, Lettieri T. Comparison of four different colorimetric and fluorometric cytotoxicity assays in a zebrafish liver cell line. BMC Pharmacol 2008; 8:8.

Borm PJ, Robbins D, Haubold S, Kuhlbusch T, Fissan H, Donaldson K, et al. The potential risk of nanomaterials: A review carried out for ECETOC. Part Fibre Toxicol 2006; 3:11.

Bouldin JL, Ingle TM, Sengupta A, Alexander R, Hanningan RE, Buchanan RA. Aqueous toxicity and food chain transfer of quantum dots in freshwater algae and Ceriodaphnia dubia. Environ Toxicol Chem 2008; 27:1958-63.

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

Brayner R. The toxicological impact of nanoparticles. Nano Today 2008; 3:48-55.

Brouwer DH, Gijsbers JH, Lurvink MW. Personal exposure to ultrafine particles in the workplace: exploring sampling techniques and strategies. Ann Occup Hyg 2004; 48:439-53.

Brown JS, Zeman KL, Bennet WD. Ultrafine particle deposition and clearance in the healthy and obstructed lung. Am J Respir Critic Care Med 2002; 166:1240-7.

Brunauer S, Emmett PH, Teller E. Adsorption of gases in multimolecular layers. J Amer Chem Soc 1938; 60:309-19.

Buford MC, Hamilton RF Jr, Holian A. A comparison of dispersing media for various engineered carbon nanoparticles. Part Fibre Toxicol 2007; 4:6.

Cabaleiro-Lago C, Quinlan-Pluck F, Lynch I, Lindman S, Minogue AM, Thulin E, et al. Inhibition of amyloid protein fibrillation by polymeric nanoparticles. J Am Chem Soc 2008; 130:15437-43.

Cañas JE, Long M, Nations S, Vadan R, Dai L, Luo M, et al. Effects of functionalized and nonfunctionalized single walled carbon nanotubes on root elongation of selected crop species. Environ Toxicol Chem 2008; 27:1922-31.

Carlson C, Hussain SM, Schrand AM, Braydich-Stolle LK, Hess KL, Jones RL, et al. Inuque cellular interactions of silver nanoparticles: size dependent generation of reactive oxygen species. J Phys Chem B 2008; 112,13608-19.

Castro M, Santos MM, Monteiro NM, Vieira N. Measuring lysosomal stability as an effective tool for marine coastal environmental monitoring. Mar Environ Res 2004; 58:741-5.

Cedervall T, Lynch I, Lindman S, Berggård T, Thulin E, Nilson H, et al. Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. Proc Natl Acad Sci USA 2007; 104:2050-5.

Chen M, von Mikecz A. Formation of nucleoplasmic protein aggregates impairs nuclear function in response to SiO_2 nanoparticles. Exp Cell Res 2005; 305:51-62.

Chen KL, Elimelech M. Interaction of fullerene (C_{60}) nanoparticles with humic acid and alginate coated silica surfaces: measurements, mechanisms, and environmental implications. Environ Sci Technol 2008; 42:7607-14.

Chen Z, Westerhoff P, Herckes P. Quanitification of C_{60} fullerene concentrations in water. Environ Toxicol Chem 2008; 27:1852-9.

Cheng J, Flahaut E, Cheng SH. Effects of carbon nanotubes on developing zebrafish (Danio rerio) embryos. Environ Toxicol Chem 2007; 26:708-16.

Chien P, Weissman JS, DePace AH. Emerging principles of conformation based prion inheritance. Ann Rev Biochem 2004; 73:617-56.

Chiti F, Dobson CM. Protein misfolding, functional amyloid, and human disease. Ann Rev Biochem 2006; 75:333-66.

Choi HS, Liu W, Misra P, Tanaka E, Zimmer JP, Ipe BI, et al. Renal clearance of quantum dots. Nat Biotechnol 2007; 25:1165-70.

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

Colvin VL. The potential environmental impact of engineered nanomaterials. Nat Biotechnol 2003; 21:1166-70.

Colognato R, Bonelli A, Ponti J, Farina M, Bergamaschi E, Sabbioni E, et al. Comparative genotoxicity of cobalt nanoparticles and ions on human leukocytes in vitro. Mutagenesis 2008; 23:377-82.

Connor EE, Mwamuka J, Gole A, Murphy CJ, Wyatt MD. Gold nanoparticles are taken up by human cells but do not cause acute cytotoxicity. Small 2005; 1:325-7.

Crane M, Handy DR, Garrod J, Owen R. Ecotoxicity test methods and environmental hazard assessment for engineered nanoparticles. Ecotoxicology 2008; 17:421-37.

Crüts B, Van Etten L, Törnqvist H, Blomberg A, Sandström T, Mills NL, et al. Exposure to diesel exhaust induces changes in EEG in human volunteers. Part Fibre Toxicol 2008; 5:4.

Davis JM, Addison J, Bolton RE, Donaldson K, Jones AD, Smith T. The pathogenicity of long versus short fibre samples of amosite asbestos administered to rats by inhalation and intraperitponeal injection. Br J Exp Pathol 1986; 67:415-30.

De Jong WH, Hagens W, Krystek P, Burger M, Sips A, Geertsma R. Particle size-dependent organ distribution of gold nanoparticles after intravenous administration. Biomaterials 2008; 29:1912-9.

Des Rieux A, Fievez V, Garinot M, Schneider YJ, Préat V. Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach. J Control Release 2006; 116:1-27.

Dhawan A, Taurozzi JS, Pandey AK, Shan W, Miller SM, Hashsham SA, et al. Stable colloidal dispersions of C_{60} fullerenes in water: evidence for genotoxicity. Environ Sci Technol 2006; 40:7394-401.

Donaldson K, Hill IM, Beswick PH. Superoxide anion release by alveolar macrophages exposed to respirable industrial fibres: modifying effect of fibre opsonisation. Exp Toxicol Pathol 1995; 47:229-31.

Donaldson K, Tran L, Jimenez LA, Duffin R, Newby DE, Mills N, et al. Combustion-derived nanoparticles: A review of their toxicology following inhalation exposure. Part Fibre Toxicol 2005; 2:10.

Donaldson K, Aitken R, Tran L, Stone V, Duffin R, Forrest G, et al. Carbon nanotubes: a review of their properties in relation to pulmonary toxicology and workplace safety. Toxicol Sci 2006; 92:5-22.

Donaldson K, Stone V, Seaton A, Tran L, Aitken R, Poland C. Re: Induction of mesothelioma in p53+/- mouse by intraperitoneal application of multi-wall carbon nanotube (Comment on: J Toxicol Sci 2008; 33:105-16). J Toxicol Sci 2008; 33:385.

Dufour EK, Kumaravel T, Nohynek GJ, Kirkland D, Toutain H. 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. Mutat Res 2006; 607:215-24.

Dunford R, Salinaro A, Cai L, Serpone N, Horikoshi S, Hidaka H, et al. Chemical oxidation and DNA damage catalysed by inorganic sunscreen ingredients. FEBS Lett 1997; 418:87-90.

Dutta D, Sundaram SK, Teeguarden JG, Riley BJ, Fifield LS, Jacobs JM. Adsorbed proteins influence on biological activity and molecular targeting of nanomaterials. Toxicol Sci 2007; 100:303-15.

ECETOC. Testing Strategies to Establish the Safety of Nanomaterials—7–8 November 2005, Barcelona. Workshop Report No. 7. European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels, Belgium; 2006.

EFSA (European Food Safety Authority). Draft Opinion of the Scientific Committee on the risks arising from nanoscience and nanotechnologies on food and feed safety. Endorsed for public consultation on 14 October 2008. EFSA, Parma, Italy; 2008.

European Commission. Regulation (EC) No 1907/2006 of the European Parliament, and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. Official Journal of the European Union L396/1 dd 30.12.2006, European Commission, Brussels, Belgium; 2006.

European Commission. Communication from the Commission to the European Parliament, the Council and the European Economic and Social Committee - Regulatory aspects of nanomaterials, 2008, [SEC(2008) 2036], COM/2008/0366 final. European Commission, Brussels, Belgium; 2008.

Fabian E, Landsiedel R, Ma-Hock L, Wiench K, Wohlleben W, van Ravenzwaay B. Tissue distribution and toxicity of intravenously administered titanium dioxide nanoparticles in rats. Arch Toxicol 2008; 82:151-7.

Federici G, Shaw BJ, Handy RD. Toxicity of titanium dioxide to rainbow trout (Oncorhynchus mykiss): gill injury, oxidative stress, and other physiological effects. Aquat Toxicol 2007; 84:415-30.

Fernandes TF, Christofi N, Stone V. The environmental implications of nanomaterials. In: Monteiro-Riviere NA, Tran CL, editors. Nanotoxicology: characterization, dosing and health effects. Taylor and Francis, CRC Press, USA; 2007.

Filley TR, Ahn MM, Held BW, Blanchette RA. Investigations of fungal mediated (C_{60}-C_{70}) fullerene decomposition. Preprints of extended abstracts presented at the 229th ACS National Meeting, American Chemical Society; 2005 Mar 13-17; San Diego, CA, USA; 45:446-450.

Fisk AT, Norstrom RJ, Cymbalisty CD, Muir DC. Dietary accumulation and depuration of hydrophobic organochlorines: Bioaccumulation parameters and their relationship with the octanol/water partition coefficient. Environ Toxicol Chem 1998; 17:951-61.

Floege J, Ehlerding G. Beta-2-microglobulin associated amyloidosis. Nephron 1996; 72: 9-26.

Florence AT, Hillery AM, Hussain N, Jani PU. Factors affecting the oral uptake and translocation of polystyrene nanoparticles: histological and analytical evidence. J Drug Target 1995; 3:65-70.

Fortner JD, Lyon DY, Sayes CM, Boyd AM, Falkner JC, Hotze EM, et al. C_{60} in water: nanocrystal formation and microbial response. Environ Sci Technol 2005; 39:4307-16.

Franklin N, Rogers N, Apte S, Batley G, Gadd G, Casey P. Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and $ZnCl_2$ to a freshwater microalga (Pseudokirchneriella subcapitata): the importance of particle solubility. Environ Sci Technol 2008; 41:8484-90.

Fujitani Y, Kobayashi T, Arashidani K, Kunugita N, Suemura K. Measurement of the physical properties of aerosols in a fullerene factory for inhalation exposure assessment. J Occup Environ Hyg 2008; 5:380-9.

Geiser M, Rothen-Rutishauser B, Kapp N, Schurch S, Kreyling W, Schulz H, et al. Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells. Environ Health Perspect 2005; 113:1555-60.

Gonzalez L, Lison D, Kirsch-Volders M. Genotoxicity of engineered nanomaterials: a critical review. Nanotoxicology 2008; 2:252-73.

Griffitt RJ, Weil R, Hyndman KA, Denslow ND, Powers K, Taylor D, et al. Exposure to copper nanoparticles causes gill injury and acute lethality in Zebrafish (Danio rerio). Environ Sci Technol 2007; 41:8178-86.

Griffitt RJ, Luo J, Gao J, Bonzongo JC, Barber DS. Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. Environ Toxicol Chem 2008; 27:1972-8.

Gurr JR, Wang AS, Chen CH, Jan KY. Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells. Toxicology 2005; 213:66-73.

Hainfeld JF, Slatkin DN, Smilowitz HM. The use of gold nanoparticles to enhance radiotherapy in mice. Phys Med Biol 2004; 49:N309-15.

Handy RD, Eddy FB. Transport of solutes across biological membranes in eukaryotes: an environmental perspective. In: van Leeuwen HP, Köster W, editors. Physiological kinetics and transport at chemical-biological interphases. IUPAC series; John Wiley, Chichester; 2004. p.337-56.

Handy RD, Henry TB, Scown TM, Johnston BD, Tyler CR. Manufactured nanoparticles: their uptake and effects on fish – a mechanistic analysis. Ecotoxicology 2008a; 17:396-409.

Handy RD, von der Kammer F, Lead JR, Hassellöv M, Owen R, Crane M. The ecotoxicology and chemistry of manufactured nanoparticles. Ecotoxicology 2008b; 17:287-314.

Hannah W, Thompson PB. Nanotechnology, risk and the environment: a review. J Environ Monit 2008; 10:291-300.

Hassellöv M, Readman JW, Ranville JF, Tiedje K. Nanoparticle analysis and characterization methologies in environmental risk assessment of engineered nanoparticles. Ecotoxicology 2008; 17:344-61.

Heckel K, Kiefmann R, Dörger M, Stoeckelhuber M, Goetz AE. Colloidal gold particles as a new marker of early acute lung injury. Am J Physiol Lung Cell Mol Physiol 2004; 287:L867-78.

Henry TB, Menn FM, Fleming JT, Wilgus J, Compton RN, Sayler GS. Attributing effects of aqueous C60 nano-aggregates to tetrahydrofuran decomposition products in larval zebrafish by assessment of gene expression. Environ Health Perspect 2007; 115:1059-65.

Hillyer JF, Albrecht RM. Correlative instrumental neutron activation analysis, light microscopy, transmission electron microscopy, and X-ray microanalysis for qualitative and quantitative detection of colloidal gold spheres in biological specimens. Microsc Microanal 1998; 4:481-90.

Hillyer JF, Albrecht RM. Gastrointestinal persorption and tissue distribution of differently sized colloidal gold nanoparticles. J Pharm Sci 2001; 90:1927-36.

Hirsch LR, Stafford RJ, Bankson JA, Sershen SR, Rivera B, Price RE, et al. Nanoshell-mediated near-infrared thermal therapy of tumors under magnetic resonance guidance. Proc Natl Acad Sci USA 2003; 100:13549-54.

Holbrook RD, Murphy KE, Morrow JB, Cole KD. Trophic transfer of nanoparticles in a simplified invertebrate food web. Nat Nanotechnol 2008; 3:352-5.

Hoyt VW, Mason E. Nanotechnology emerging health issues. J Chem Health Safety 2008; 15:10-5.

Hund-Rinke K, Simon M. Ecotoxic effects of photocatalytic active nanoparticles (TiO_2) on algae and daphnids. Environ Sci Pollut Res Int 2006; 13:225-32.

Hussain N, Jani PU, Florence AT. Enhanced oral uptake of tomato lectin-conjugated nanoparticles in the rat. Pharm Res 1997; 14:613-8.

Hyung H, Fortner JD, Hughes JB, Kim JH. Natural organic matter stabilizes carbon nanotubes in the aqueaous phase. Environ Sci Technol 2007; 41:179-84.

Ichihara I, Castranova V, Tanioka A, Miyazawa K. Re: Induction of mesothelioma in p53+/- mouse by intraperitoneal application of multi-wall carbon nanotube (Comment on: J Toxicol Sci 2008; 33:105-16). J Toxicol Sci 2008; 33:381-2.

ICRP. Human respiratory tract model for radiological protection. Publication 66. Ann. ICRP 24 (1-3), Pergamon Press, Oxford, 1994.

Jacobsen NR, Pojana G, White P, Møller P, Cohn CA, Korsholm KS, et al. Genotxicity, cytotoxicity, and reactive oxygen species induced by single-walled carbon nanotubes and C_{60} fullerenes in the FE1-Muta^{TM} Mouse lung epithelial cells. Environ Mol Mutagen 2008; 49:476-87.

Jafvert C, Kulkarni P. Buckminsterfullerene's (C_{60}) octanol-water partition coefficient (Kow) and aqueous solubility. Environ Sci Technol 2008; 42:5945-50.

Jani PU, MacCarthy DE, Florence AT. Titanium dioxide (rutile) particle uptake from the rat GI tract and translocation to systemic organs after oral administration. Int J Pharm 1994; 105:157-68.

Jemec A, Drobne D, Remskar M, Sepcic K, Tisler T. Effects of ingested nano-sized titanium dioxide on terrestrial Isopods (Porcellio scaber). Environ Toxicol Chem 2008; 27:1904-14.

Ji ZQ, Sun H, Wang H, Xie Q, Liu Y, Wang Z. Biodistribution and tumor uptake of $C_{60}(OH)_x$ in mice. J Nanopart Res 2006; 8:53-63.

John TA, Vogel SM, Minshall RD, Ridge K, Tiruppathi C, Malik AB. Evidence for the role of alveolar epithelial gp60 in active transalveolar albumin transport in the rat lung. J Physiol 2001; 533:547-59.

John TA, Vogel SM, Tiruppathi C, Malik AB, Minshall RD. Quantitative analysis of albumin uptake and transport in the rat microvessel endothelial monolayer. Am J Physiol Lung Cell Mol Physiol 2003; 284:L187-96.

Jones RA. Soft condensed matter. Oxford University Press: New York; 2002.

Ju-Nam Y, Lead JR. Manufactured nanoparticles: An overview of their chemistry, interactions and potential environmental implications. Sci Total Environ 2008; 400:396-414.

Kagan VE, Tyurina YY, Tyurin VA, Konduru NV, Potapovich AI, Osipov AN, et al. Direct and indirect effects of single walled carbon nanotubes on RAW 264.7 macrophages: role of iron. Toxicol Lett 2006; 165:88-100.

Kane AB. Animal models for malignant mesothelioma. Inhal Toxicol 2006; 18:1001-4.

Kane DB, Oktem B, Johnston MV. Nanoparticle detection by aerosol mass spectrometry. Aerosol Sci Technol 2001; 34:520-7.

Kang S, Mauter MS, Elimelech M. Physicochemical determinants of multiwalled carbon nanotube bacterial cytotoxicity. Environ Sci Technol 2008; 42:7528-34.

Kawano T, Yamagata M, Takahishi H, Niidome Y, Yamada S, Katayama Y, et al. Stabilizing of plasmid DNA in vivo by PEG-modified cationic gold nanoparticles and the gene expression assisted with electrical pulses. J Control Release 2006; 111:382-9.

Kendall M. Fine airborne urban particles (PM 2.5) sequester lung surfactant and amino acids from human lung lavage. Am J Physiol Lung Cell Mol Physiol 2007; 293:L1053-8.

Kennedy AJ, Hull MS, Steevens JA, Dontsova KM, Chappell MA, Gunter JC, et al. Factors influencing the partioning and toxicity of nanotubes in the aquatic environment. Environ Toxicol Chem 2008; 27:1932-41.

Kim H, Liu X, Kobayashi T, Kohyama T, Wen FQ, Romberger DJ, et al. Ultrafine carbon black particles inhibit human lung fibroblast-mediated collagen gel contraction. Am J Resp Cell Mol Biol 2003; 28:111-21.

Kim YS, Kim JS, Cho HS, Rha DS, Kim JM, Park JD, et al. Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats. Inhal Toxicol 2008; 20:575-83.

Klaine SJ, Avarez PJ, Batley GE, Fernandes TF, Handy RD, Lyon DY, et al. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. Environ Toxicol Chem 2008; 27:1825-51.

Kocbach A, Herseth JI, Låg M, Refsnes M, Schwarze PE. Particles from wood smoke and traffic induce differential pro-inflammatory response patterns in co-cultures. Toxicol Appl Pharmacol 2008; 232:317-26.

Koo EH, Lansbury PT, Kelly JW. Amyloid diseases: abnormal protein aggregation in neurodegeneration. Proc Natl Acad Sci USA 1999; 96:9989-90.

Koziara JM, Oh JJ, Akers WS, Ferraris SP, Mumper RJ. Blood compatibility of cetyl alcohol/polysorbate-based nanoparticles. Pharm Res 2005; 22:1821-8.

Kreuter J, Shamenkov D, Petrov V, Ramge P, Cychutek K, Koch-Brandt C, et al. Apolipoprotein-mediated transport of nanoparticle-bound drugs across the blood-brain barrier. J Drug Target 2002; 10:317-25.

Kreyling W, Scheuch G. Clearance of particles deposited in the lungs. In: Heyder J, Gehr P, editors. Particle lung interactions. New York, USA: Marcel Dekker; 2000. p.323-76.

Kreyling WG, Semmler M, Erbe F, Mayer P, Takenaka S, Schulz H. Translocation of ultrafine insoluble particles from lung epithelium to extrapulmonary organs in size dependent but very low. J Toxicol Environ Health A 2002; 65:1513-30.

Kuhlbusch TA, Neumann S, Fissan H. Number size distribution, mass concentration, and particle composition of PM1, PM2.5, and PM10 in bag filling areas of carbon black production. J Occup Environ Hyg 2004; 10:660-71.

Kuhlbusch TA, Fissan H. Particle characteristics in the reactor and pelletizing areas of carbon black production. J Occup Environ Hyg 2006; 3:558-67.

Kuhlbusch TA, Fissan H, Asbach C. Measurement and detection of nanoparticles in the environment. In: Krug H, editor. Nanotechnology, Volume 2: Environmental Aspects. Wiley-VCH; 2008a. p.229-265.

Kuhlbusch TA, Fissan H, Asbach C. Nanotechnologies and Environmental Risks: Measurement Technologies and Strategies. In: Linkov I, Steevens J, editors. Nanotechnology: Risk and Benefit. Springer, Dordrecht, The Netherlands; 2008b. p.235-47, in press.

Kwon JT, Hwang SK, Jin H, Kim DS, Minai-Tehrani A, Yoon HJ, et al. Body distribution of inhaled fluorescent magnetic nanoparticles in the mice. J Occup Health 2008; 50:1-6.

Labarre D, Vauthier C, Chauvierre C, Petri B, Muller RH. Interactions of blood proteins with poly(isobutylcyanoacrylate) nanoparticles decorated with a polysaccharidic brush. Biomaterials 2005; 26:5075-84.

Landsiedel R, Kapp MD, Schulz M, Wiench K, Oesch F. Genotoxicity investigations on nanomaterials: Methods, preparation and characterization of test material, potential artifacts and limitations – many questions, some answers. Mutat Res 2008; Nov 11. [Epub ahead of print].

Lead JR, Wilkinson KJ. Natural aquatic colloids: current knowledge and future trends. Environ Chem 2006; 3:159-71.

Lee KL, Nallathamby PD, Browning LM, Osgood CJ, Xu XH. In vivo imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. ACS Nano 2007; 1:133-43.

Lee WM, An YJ, Yoon H, Kweon HS. 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 2008; 27:1915-21.

Li N, Sioutas C, Cho A, Schmitz D, Misra C, Sempf J, et al. Ultrafine particulate pollutants induce oxidatiove stress and mitochondrial damage. Environ Health Perspect 2003; 111:455-60.

Li D, Lyon DY, Li Q, Alvarez P.J. Effect of soil sorption and aquatic natural organic matter on the antibacterial activity of a fullerene water suspension. Environ Toxicol Chem 2008; 27:1888-94.

Limbach LK, Wick P, Manser P, Grass RN, Bruinink A, Stark WJ. Exposure of engineered nanoparticles to human lung epithelial cells: influence of chemical composition and catalytic activity on oxidative stress. Environ Sci Technol 2007; 41:4158-63.

Lin D, Xing B. Root uptake and phytotoxicity of ZnO nanoparticles. Environ Sci Technol 2008; 42:5580-5.

Lindfors K, Kalkbrenner T, Stoller P, Sandoghdar V. Detection and spectroscopy of gold nanoparticles using supercontinuum white light confocal microscopy. Phys Rev Lett 2004; 93:037401.

Linkov I, Satterstrom FK, Steevens J, Ferguson E, Pleus RC. Multi-criteria decision analysis and environmental risk assessment for nanomaterials. J Nanoparticle Res 2007; 9:543-54.

Linse S, Cabaleiro-Lago C, Xue WF, Lynch I, Lindman S, Thulin E, et al. Nucleation of protein fibrillation by nanoparticles. Proc Natl Acad Sci USA 2007; 104:8691-6.

Liu YY, Liu TY, Chen SY, Liu DM. Synthesis and characterization of nanoporous SiO(2)/pHEMA biocomposites. J Mater Sci Mater Med 2008; 19:2903-11.

Loo C, Lin A, Hirsch L, Lee MH, Barton J, Halas N, et al. Nanoshell-enabled photonics-based imaging and therapy of cancer. Technol Cancer Res Treat 2004; 3:33-40.

Lovern SB, Klaper R. Daphnia magna mortality when exposed to titanium dioxide and fullerene (C_{60}) nanoparticles. Environ Toxicol Chem 2006; 25:1132-7.

Lundqvist M; Sethson I; Jonsson BH. Protein adsorption onto silica nanoparticles: conformational changes depend on particles curvature and protein stability. Langmuir 2004; 20:10639-47.

Lundqvist M, Stigler J, Elia G, Lynch I, Cedervall T, Dawson KA. Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. Proc Natl Acad Sci USA 2008; 105:14265-70.

Luoma SN. Silver Nanotechnologies and the environment: old problems or new challenges? Project on emerging technologies (PEN) 15. Pew Charitable Trusts, Woodrow Wilson International Center for Scholars; 2008.

Lyklema J. Pair Interactions. In: Lyklema J, editor. Fundamentals of interface and colloid science, Volume IV, Particulate colloids. Elsevier Academic Press, Amsterdam; 2005.

Lynch I, Dawson KA. Protein-nanoparticle interactions. Nano Today 2008; 3:40-7.

Lynch I, Dawson KA, Linse S. Detecting cryptic epitopes created by nanoparticles. Sci STKE 2006; 327:pe14.

Lyon DY, Adams LK, Falkner JC, Alvarez PJ. Antibacterial acitivity of fullerene water suspensions: effects of preparation method and particle size. Environ Sci Technol 2006; 40:4360-6.

Lyon DY, Alvarez P.J. Fullerene water suspension (nC_{60}) exerts antibacterial effects via ROS-independent protein oxidation. Environ Sci Technol 2008; 42:8127-32.

Maynard AD, Baron PA, Foley M, Shvedova AA, Kisin ER, Castranova V. Exposure to carbon nanotube material: aerosol release during the handling of unrefined single-walled carbon nanotube material. J Toxicol Environ Health A 2004; 67:87-107.

Miller BG, Searl A, Davis JM, Donaldson K, Cullen RT, Bolton RE, et al. Influence of fibre length, dissolution and biopersistence on the production of mesothelioma in the rat peritoneal cavity. Ann Occup Hyg 1999; 43:155-66.

Mills NL, Amin N, Robinson SD, Anand A, Davies J, Patel P, et al. Do inhaled carbon nanoparticles translocate directly into the circulation in humans? Am J Resp Crit Care Med 2006; 173:426-31.

Mills NL, Donaldson K, Hadoke PW, Boon NA, MacNee W, Cassee FR, et al. Adverse cardiovascular effects of air pollution. Nat Clin Pract Cardiovasc Med 2009; 6:36-44.

Möller W, Felten K, Sommerer K, Scheuch G, Meyer G, Meyer P, et al. Deposition, retention, and translocation of ultrafine particles from the central airways and lung periphery. Am J Respir Crit Care Med 2008; 177:366-7.

Mordas G, Sipilä M, Kulmala M. Nanoparticle Detection Using Nucleation Regime of the CPC. Book Nucleation and Atmospheric Aerosols: Springer; Netherlands, 2008. DOI 10.1007/978-1-4020-6475-3 Copyright 2008 ISBN 978-1-4020-6474-6 (Print) 978-1-4020-6475-3 (Online) Part I DOI 10.1007/978-1-4020-6475-3_43. p.209-13.

Mori T, Takada H, Ito S, Matsubayashi K, Miwa N, Sawaguchi T. Preclinical studies on safety of fullerenen upon acute oral administration and evaluation for no mutagenesis. Toxicology 2006; 225:48-54.

Mroz RM, Schins RP, Li H, Jimenez LA, Drost EM, Holownia A, et al. Nanoparticle-driven DNA damage mimics irradiation-related carcinogenesis pathways. Eur Respir J 2008; 31:241-51.

Mueller N, Nowack B. Exposure modeling of engineered nanoparticles in the environment. Environ Sci Technol 2008; 42:4447-53.

Murasov V. Comments on "Particle surface characteristics may play important role in phytotoxicity of alumina nanoparticles". Toxicol Lett 2006; 164:185-7.

Murr LE, Bang JJ, Esquivel EV, Guerrero PA, Lopez D.A. Carbon nanotubes, nanocrystal forms, and complex nanoparticle aggregates in common fuel-gas combustion sources and the ambient air. J Nanoparticle Res 2004; 6:241-51.

Murr LE, Guerrero PA. Carbon nanotubes in wood soot. Atmos Sci Lett 2006; 7:93-5.

Nakagawa Y, Wakuri S, Sakamoto K, Tanaka N. The photogenotoxicity of titanium dioxide particles. Mutat Res 1997; 394:125-32.

Navarro E, Baun A, Behra R, Hartmann NB, Filser J, Miao AJ, et al. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. Ecotoxicology 2008a; 17:372-86.

Navarro E, Piccapietra F, Wagner B, Marconi F, Kaegi R, Odzak N, et al. Toxicity of Silver Nanoparticles to Chlamydomonas reinhardtii. Environ Sci Technol 2008b; 42:8959-64.

Nel A, Xia T, Madler L, Li N. Toxic potential of materials at the nanolevel. Science 2006; 311:622-7.

Nemmar A, Hoet PH, Vanquickenborne B, Dinsdale D, Thomeer M, Hoylaerts MF, et al. Passage of inhaled particles into the blood circulation in humans. Circulation 2002; 105:411-4.

Nielsen HD, Berry LS, Stone V, Fernandes TF. Interactions between carbon black nanoparticles and the brown algae Fucus serratus: inhibition of fertilization and zygotic development. Nanotoxicology 2008; 2:88-97.

Niidome T, Yamagata M, Okamoto Y, Akiyama Y, Takahishi H, Kawano T, et al. PEG-modified gold nanorods with a stealth character for in vivo application. J Control Release 2006; 114:343-7.

Niwa Y, Iwai N. Nanomaterials induce oxidized low-density lipoprotein cellular uptake in macrophages and platelet aggregation. Circ J 2007; 71:437-44.

Noble CA, Prather KA. Real-time single particle mass spectrometry: a historical review of a quarter century of the chemistry of aerosols. Mass Spectrom Rev 2000; 4:248-74

O'Neal DP, Hirsch LR, Halas NJ, Payne JD, West JL. Photo-thermal tumor ablation in mice using near infrared-absorbing nanoparticles. Cancer Lett 2004; 209:171-6.

Oberdörster G, Sharp Z, Atudorei V, Elder A, Gelein R, Lunts A, et al. Exrapulmonary translocation of ultrafine carbon particles following whole body inhalation exposure of rats. J Toxicol Environ Health Part A 2002; 65:1531-43.

Oberdörster E. Manufactured nanomaterials (fullerenes, C_{60}) induce oxidative stress in brain of juvenile largemouth bass. Environ Health Perspect 2004; 112:1058-62.

Oberdörster G, Sharp Z, Atudorei V, Elder A, Gelein R, Kreyling W, et al. Translocation of Inhaled Ultrafine Particles to the Brain. Inhal Toxicol 2004; 16:437-45.

Oberdörster G, Maynard A, Donaldson K, Castranova V, Fitzpatrick J, Ausman K, et al. Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Part Fibre Toxicol 2005a; 2:8.

Oberdörster G, Oberdörster E, Oberdörster J. Nanotoxicology: an emerging discipline evolving form studies of ultrafine particles. Environ Health Perspect 2005b; 113:823-39.

Oberdörster E, Zhu S, Blickley TM, McClellan-Green P, Haasch ML. Ecotoxicology of carbon-based engineered nanoparticles: effects of fullerene (C_{60}) on aquatic organisms. Carbon 2006; 44:1112-20.

OECD. Water Solubility: OECD Test Guideline 105. Organization for Economic Coordination and Development. Paris, France; 1995.

OECD. Bioconcentration: Flow-through Fish Test: OECD Test Guideline 305. Organization for Economic Coordination and Development. Paris, France; 1996.

OECD. Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment. OECD Environment, Health and Safety Publications Series on Testing and Assessment No. 34. Organization for Economic Coordination and Development. Paris, France; 2005

OECD. List of manufactured nanomaterials and list of endpoints for phase one of the OECD testing programme. OECD Environment, Health and Safety Publications Series on the safety of manufactured nanomaterials No 6. Document ENV/JM/MONO(2008)13. Organization for Economic Coordination and Development. Paris, France; 2008a

OECD. Sediment-water Lumbriculus toxicity test using speked sediment. OECD Test Guideline 225. Organization for Economic Coordination and Development. Paris, France; 2008b.

Oughton DH, Hertel-Aas T, Pellicier E, Mendoza E, Joner EJ. Neutron activation of engineered nanoparticles as a tool for tracing their environmental fate and uptake in organisms. Environ Toxicol Chem 2008; 27:1883-7.

Oyewumi MO, Yokel RA, Jay M, Coakley T, Mumper RJ. Comparison of cell uptake, biodistribution and tumor retention of folate-coated and PEG-coated gadolinium nanoparticles in tumor-bearing mice. J Control Release 2004; 95:613-26.

Pacurari M, Yin XJ, Zhao J, Ding M, Leonard SS, Schwegler-Berry D, et al. 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 2008; 116:1211-7.

Panté N, Kann M. Nuclear pore complex is able to transport macromolecules with diameters of about 39 nm. Mol Biol Cell 2002; 13:425-34.

Papageorgiou I, Brown C, Schins R, Singh S, Newson R, Davis S, et al. The effects of nano- and micro-sized particles of cobalt-chromium alloy on human fibroblasts in vitro. Biomaterials 2007; 28:2946-58.

Petersen EJ, Huang Q Weber WJ. Ecological uptake and depuration of carbon nanotubes by Lumbriculus variegates. Environ Health Perspect 2008a; 116:496-500.

Petersen EJ, Huang Q, Weber WJ Jr. Bioaccumulation of radio-labeled carbon nanotubes by Eisenia foetida. Environ Sci Technol 2008b; 42:3090-5.

Poland C, Duffin R, Kinloch I, Maynard A, Wallace W, Seaton A, et al. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. Nat Nanotechnol 2008; 3:423-8.

Pott F. Detection of mineral fibre carcinogenicity with the intraperitoneal test. Recent results and their validity. Ann Occup Hyg 1995; 39:771-9.

Quik JT, Lynch I, Salvati A, Miermans K, Van de Meent D. Behavior of ceriumdioxide nanoparticles under environmentally relevant conditions. Submitted to Environ Sci Technol 2008.

Radomski A, Jurasz P, Alonso-Escolano D, Drews M, Morandi M, Malinski T, et al. Nanoparticle-induced platelet aggregation and vascular thrombosis. Br J Pharmacol 2005; 146:882-93.

Radt B, Smith TA, Caruso F. Optically addressable nanostructured capsules. Adv Mater 2004; 16:2184-9.

Rahman Q, Lohani M, Dopp H, Pemsel L, Jonas D, Weiss G. Evidence that ultrafine titanium dioxide induces micronuclei and apoptosis in syrian hamster embryo fibroblasts. Environ Health Perspect 2002; 110:797-800.

Reeves JF, Davies SJ, Dodd NJ, Jha AN. Hydroxyl radicals ($\bullet$ OH) are associated with titanium dioxide (TiO_2) nanoparticle-induced cytotoxicity and oxidative DNA damage in fish cells. Mutat Res 2008; 640:113-22.

Roberts A, Mount AS, Seda B, Souther J, Qiao R, Lin S, et al. In vivo biomodification of lipid-coated carbon nanotubes by Daphnia magna. Environ Sci Technol 2007; 41:3025-9.

Rosenkranz P, Stone V, Chaudry Q, Fernandes TF. A comparison of nanoparticle and fine particle uptake by Daphnia magna. Environ Toxicol Chem 2009. In Press.

Royal Society and Royal Academy of Engineering. Nanoscience and nanotechnologies: opportunities and uncertainties. UK; July 2004.

Sadauskas E, Wallin H, Stoltenberg M, Vogel U, Doering P, Larsen A, et al. Kupffer cells are central in the removal of nanoparticles from the organism. Part Fibre Toxicol 2007; 4:10.

Sager TM, Porter DW, Robinson VA, Lindsley WG, Schwegler-Berry DE, Castranova V. Improved method to disperse nanoparticles for in vitro and in vivo investigation of toxicity. Nanotoxicology 2007; 1:118-29.

Saleh NB, Pfefferle LD, Elimelech M. Aggregation kinetics of multiwalled carbon nanotubes in aquatic systems: measurements and environmental implications. Environ Sci Technol 2008; 42:7963-9.

Sayes CM, Reed KL, Warheit DB. Assessing toxicity of fine and nanoparticles: Comparing in vitro measurements to in vivo pulmonary toxicity profiles. Toxicol Sci 2007; 97:163-80.

SCCNFP. Opinion of the Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers Concerning Titanium Dioxide. 2000. Available from: URL: http://ec.europa.eu/health/ph_risk/committees/04_sccp/04_sccp_en.htm

SCCNFP. Opinion of the Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers Concerning Zinc Oxide. 2003. Available from: URL: http://ec.europa.eu/health/ph_risk/committees/04_sccp/04_sccp_en.htm

SCENIHR. The appropriateness of existing methodologies to assess the potential risks associated with engineered and adventitious products of nanotechnologies. 2006. Available from: URL: (http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_003b.pdf)

SCENIHR. Opinion on the appropriateness of the risk assessment methodology in accordance with the technical guidance documents for new and existing substances for assessing the risks of nanomaterials. 2007a. Available from: URL: http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_010.pdf

SCENIHR (2007b), Opinion on the scientific aspects of the existing and proposed definitions relating to products of nanoscience and nanotechnologies, at http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_012.pdf

Schins RP, Knaapen AM. Genotoxicity of poorly soluble particles. Inhal Toxicol 2007; 19 (Suppl 1):189-98.

Scott-Fordsmand JJ, Krogh PH, Scaefer M, Johansen A. The toxicity testing of double-walled nanotubes-contaminated food to Eisenia veneta earthworms. Ecotox Environ Safety 2008; 71:616-9.

Semmler M, Seitz J, Erbe F, Mayer P, Heyder J, Oberörster G, et al. Long term clearance kinetics of inhald ultrafine insoluble iridium particles from the rat lung, including transient translocation into secondary organs. Inhal Toxicol 2004; 16:453-9.

Semmler-Behnke M, Fertsch S, Schmid O, Wenk A, Kreyling WG. Uptake of 1.4 mm versus 18mm gld particles by secondary target organs is size dependent in control and pregnants rats after intratracheal or intravenous application. Proceedings of Euro Nanoforum - Nanotechnology in Industrial Applications 2007, p.102-4. Available from: URL: http://www.euronanoforum2007.de/download/Proceedings ENF2007.pdf.

Semmler-Behnke M, Kreyling WG, Lipka J, Fertsch S, Wenk A, Takenaka S, et al. Biodistribution of 1.4 nm and 18 nm gold particles in rats. Small 2008; 71:616-9.

Shvedova AA, Kisin ER, Mercer R, Murray AR, Johnson VJ, Potapovich AI, et al. Unusual inflammatory and fibrogenic pulmonary responses to single walled carbon nanotubes in mice. Am J Physiol Lung Cell Mol Physiol 2005; 289:L698-708.

Shvedova AA, Fabisiak JP, Kisin ER, Murray AR, Roberts JR, Tyurina YY, et al. Sequential exposure to carbon nanotubes and bacteria enhances pulmonary inflammation and infectivity. Am J Respir Cell Mol Biol 2008b; 38:579-90.

Shvedova AA, Kisin ER, Murray AR, Johnson VJ, Gorelik O, Arepalli S, et al. Inhalation versus aspiration of single walled carbon nanotubes in C57BL/6 mice: inflammation, fibrosis, oxidative stress and mutagenesis. Am J Physiol Lung Cell Mol Physiol 2008a; 295:L552-65.

Singh R, Pantarotto D, Lacerda L, Pastorin G, Klumpp C, Prato M, et al. Tissue biodistribution and blood clearance rates of intravenously administered carbon nanotube radiotracers. Proc Natl Acad Sci USA 2006; 103:3357-62.

Smith CJ, Shaw BJ, Handy RD. Toxicity of single walled carbon nanotubes on rainbow trout, (Oncorhynchus mykiss): respiratory toxicity, organ pathologies, and other physiological effects. Aquat Toxicol 2007; 82:94-109.

Smith JN. Atmospheric nanoparticles: formation and physicochemical properties. In: Schwarz JA, Contescu CI, Putyera K. Dekker encyclopedia of nanoscience and nanotechnology, 10.1081/E-ENN-120023640. 2004.

Sondi I, Salopek-Sondi B. Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria. J Colloid Interface Sci 2004; 275:177-82.

Stapleton HM, Letcher RJ, Li J, Baker JE. Dietary accumulation and metabolism of polybrominated diphenyl ethers by juvenile carp (Cyprinus carpio). Environ Toxicol Chem 2004; 23:1939-46.

Stolpe B, Hassellöv M. Changes in size distribution of fresh water nanoscale colloidal material and associated elements on mixing with seawater. Geochim Cosmochim Acta 2007; 71:3292-301.

Stone V, Kinloch I, Clift M, Fernandes TF, Ford A, Christofi N, et al. Nanoparticle toxicology and ecotoxicology: the role of oxidative stress. In: Zhao Y, Nalwa HS, editors. Nanotoxicology. American Scientific Publishers, USA; 2007.

Sweet L, Strohm B. Nanotechnology-life cycle risk management. Human Ecotox Risk Assess 2006; 12:528-51.

Swiss Federal Office of Public Health. Guidelines on the precautionary matrix for synthetic nanomaterials. Bern, Switzerland; 2008. Available from: URL: http://www.bag.admin.ch/themen/chemikalien/00228/00510/05626/index.html?lang=en

Takagi A, Hirose A, Nishimura T, Fukomori N, Ogata A, Ohashi N, et al. Induction of mesothelioma in p53+/- mouse by intraperitoneal application of multiwall carbon nanotube. J Toxicol Sci 2008; 33:105-16.

Takahashi S, Matsuoka O. Cross placental transfer of [198]Au-colloid in near term rats. J Radiat Res (Tokyo) 1981; 22:242-9.

Takenaka S, Karg E, Kreyling WG, Lentner B, Möller W, Behnke-Semmler M, et al. Distribution pattern of inhaled ultrafine gold particles in the rat lung. Inhal Toxicol 2006; 18:733-40.

Theogaraj E, Riley S, Hughes L, Maier M, Kirkland D. An investigation of the photo-clastogenic potential of ultrafine titanium dioxide particles. Mutat Res 2007; 634:205-19.

Tiede K, Boxall A, Tear S, Lewis J, David H, Hassellöv M. Detection and characterization of engineered nanoparticles in food and the environment. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2008; 25:795-821.

Tsai SJ, Ashter A, Ada E, Mead JL, Barry CF, Ellenbecker MJ. Airborne nanoparticle release associated with the compounding of nanocomposites using nanoalumina as fillers. Aerosol and Air Quality Res 2008; 8:160-77.

Van Dijk MA, Tchebotareva AL, Orrit M, Lippitz M, Berciaud S, Lasne D, et al. Absorption and scattering microscopy of single metal nanopartcles. Phys Chem Chem Phys 2006; 8:3486-95.

Van Hoecke K, de Schamphelaere KA, van der Meeren P, Lucas SP, Janssen CR. Ecotoxicity of silica nanoparticles to the green alga Pseudokirchneriella subcapitata: importance of surface area. Environ Toxicol Chem 2008; 27:1948-57.

Vevers WF, Jha AN. Genotoxic and cytotoxic potential of titanium dioxide (TiO_2) nanoparticles on fish cells in vitro. Ecotoxicology 2008; 17:410-20.

Von Gleich A, Steinfeldt M, Petschow U. A suggested three-tiered approach to assessing the implications of nanotechnology and influencing its development. J Cleaner Prod 2008; 16:899-909.

Wake DD, Mark C. Northage: ultrafine aerosols in the workplace. Ann Occup Hyg 2002; 46 (Suppl 1):235-8.

Wallace WE, Keane MJ, Gautam, Shi XC, Murray D, Ong T. Dispersion of nanoparticles in pulmonary surfactants for in vitro toxicity studies: Lessons from ultrafine diesel exhaust particles and fine dusts. In: Monteiro-Roviere NA, Tran CL, editors. Nanotoxicology: characterization, dosing and health effects. 2007. p.153-72.

Wang JJ, Sanderson BJ, Wang H. Cyto- and genotoxicity of ultrafine TiO_2 particles in cultured human lymphoblastoid cells. Mutat Res 2007; 628:99-106.

Wardak A, Gorman ME, Swami N, Deshpande S. Identification of risks in the life cycle of nanotechnology-based products. J Ind Ecol 2008; 12:435-48.

Warheit DB, Carakostas MC, Bamberger JR, Hartsky MA. Complement facilitates macrophage phagocytosis of inhaled iron particles but has little effect in mediating dilica induced lung inflammatory and clearance responses. Environ Res 1991; 56:186-203.

Warheit DB, Reed KL, Webb TR. Pulmonary toxicity studies in rats with triethoxyoctylsilane (OTES)-coated, pigment grade titanium dioxide particles: bridging studies to predict inhalation hazard. Exp Lung Res 2003; 29:593-606.

Warheit DB, Hoke RA, Finlay C, Donner EM, Reed KL, Sayes CM. Development of a base set of toxicity tests using ultrafine TiO_2 particles as a component of nanoparticle risk assessment. Toxicol Lett 2007; 171:99-110.

Wick P, Manser P, Limbach LK, Dettlaff-Weglikowska U, Krumeich F, Roth S, et al. The degree and kind of agglomeration affect carbon nanotube cytotoxicity. Toxicol Lett 2007; 168:121-31.

Wiebert P, Sanchez-Crespo A, Falk R, Philipson K, Lundin A, Larsson S, et al. No significant translocation of inhaled 35-nm carbon particles to the circulation in humans. Inhalation Toxicol 2006a; 18:741-7.

Wiebert P, Sanchez-Crespo A, Seitz J, Falk R, Philipson K, Kreyling WG, et al. Negligible clearance of ultrafine particles retained in healthy and affected human lungs. Eur Respir J 2006b; 28:286-90.

Wiesner MR. Responsible development of nanotechnologies for water and wastewater treatment. Water Sci Technol 2006; 53:45-51.

Wijnhoven S, Herberts C, Hagens W, Oomen A, Heugens E, Roszek B, et al. Nano-silver. A review of available data and knowledge gaps. Report 360003001/2008 National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands; 2009.

Wu WH, Sun X, Yu YP, Hu J, Zhao L, Liu Q, et al. TiO_2 nanoparticles promote beta-amyloid fibrillation in vitro. Biochem Biophys Res Comm 2008; 373:315-8.

WWICS. Woodrow Wilson International Centre for Scholars: Project on emerging nanotechnologies, Consumer product Inventory. Washington, USA; 2008. [Accessed 1st October 2008] Available from: URL: http://www.nanotechproject.org/inventories/consumer/

Xia T, Korge P, Weiss JN, Li N, Venkatesen MI, Sioutas C, et al. Quinones and aromatic chemical compounds in particulate matter induce mitochondrial dysfunction: implications for ultra-fine particle toxicity, Environ Health Perspect 2004; 112:1347-58.

Yang L, Watts DJ. Particle surface characteristics may play important role in phytotoxicity of alumina nanoparticles. Toxicol Lett 2005; 158:122-32.

Yang H, Liu Ch, Yang D, Zhang H, Xi Z. Comparative study of cytotoxicity, oxidative stress and genotoxicity induced by four typical nanomaterials: the role of particle size, shape and composition. J Appl Toxicol 2009; 29:69-78.

Yeganeh B, Kull CM, Hull MS, Marr LC. Characterization of airborne particles during production of carbonaceous nanomaterials. Environ Sci Technol 2008; 42:4600-6.

Yu LE, Yung LY, Ong CN, Tan YL, Balasubramaniam S, Hartono D, et al. Translocation and effects of gold nanoparticles after inhalation exposure in rats. Nanotoxicology 2007; 1:235-42.

Zakharenko LP, Zakharov IK, Vasyunina EA, Karamysheva TV, Danilenko AM, Nikiforov AA. Determination of fullerene C_{60} and fullerol by the somatic mutation and recombination test in Drosophila melanogaster and SOS Chromotest. Genetica 1997; 33:405-9.

Zhang X, Sun H, Zhang Z, Niu Q, Chen Y, Crittenden JC. Enhanced bioaccumulation of cadmium in carp in the presence of titanium dioxide nanoparticles. Chemosphere 2007; 67:160-6.

Zhang LW, Yu WW, Colvin VL, Monteiro-Riviere NA. Biological interactions of quantum dot nanoparticles in skin and in human epidermal keratinocytes. Toxicol Appl Pharmacol 2008; 228:200-11.

Zhu S, Obördorster E, Haasch ML. Toxicity of an engineered nanoparticle (fullerene, C_{60}) in two aquatic species, Daphnia and fathead minnow. Mar Environ Res 2006; 62 (Suppl):S5-9.

Zhu X, Zhu L, Li Y, Duan Z, Chen W, Alvarez PJ. Developmental in Zebra fish (Danio rerio) embryos after exposure to manufactured nanomaterials: buckminsterfullerene aggregates (n C_{60}) and fullerol. Environ Toxicol Chem 2007; 26:976-9.