



Scientific Committee on Emerging and Newly-Identified Health Risks

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



The SCENIHR adopted this opinion at the 19th plenary on 21-22 June 2007

after the public consultation

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ABSTRACT

The Scientific Committee on Emerging and Newly Identified Health Risks of DG Sanco of the European Commission was asked, in the light of current scientific knowledge and in relation to the general information and practices of chemicals risk assessment, to assess the appropriateness of risk assessment methodologies described in the current Technical Guidance Documents of the chemicals legislation for the risk assessment of nanomaterials, and to provide suggestions for improvements to the methodologies.

The Committee has examined the existing base of scientific knowledge and recognised that this subject is new and rapidly developing. The Technical Guidance Documents currently make very little reference to substances in particulate form. With respect to human health, the current methodologies described in the Technical Guidance Documents are generally likely to be able to identify the hazards associated with the use of nanoparticles. For the determination of dose – response relationships, special attention should be given to the expression of the metrics of the nanoparticle dose since mass concentration is not necessarily the best description of dose for these materials and number concentration and surface area are likely to be more appropriate. Not all nanoparticle formulations have been found to induce a more pronounced toxicity than the bulk formulations of the same substance. This suggests that the evaluation of nanoparticle formulations should be carried out on a case by case basis and it is important that it is determined whether test procedures will be predictive for human health hazards for all types of nanoparticles.

With respect to environmental exposure, the validity and appropriateness of existing technologies are not always clear. In the absence of sufficient data on the fate and effect of nanoparticles on the environment, it is neither feasible nor appropriate to propose firm rules on how substances in nanoparticle form should be evaluated. Instead the applicability of existing methods for risk assessment of nanoparticles should be evaluated.

A series of recommendations for improved methodologies and areas urgently requiring additional data and scientific knowledge are presented, including observations on the applicability of *in vitro* test procedures, QSAR approaches to nanoparticles, the prediction of environmental concentrations, the need for new ecotoxicity tests and the assessment of bioavailability.

With respect to the performance of the risk assessment of nanomaterials, it is recommended that the staged, or tiered, approach is adopted in order to identify different adverse effects and different exposure data with nanoparticles. It is suggested that due consideration be given to the possibilities now emerging that translocation of nanoparticles away from the portal of entry may occur in humans and other species, and that the passage of nanoparticles across membranes could give rise to adverse effects, for example within the cardiovascular system or following passage across the blood – brain barrier.

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EXECUTIVE SUMMARY

The Commission Strategy and Action Plan on Nanotechnologies emphasises the importance of a safe and responsible approach to risk assessment with every step of the life cycle of nanotechnology-based products. In 2005, the Commission requested the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) for an opinion on the appropriateness of existing risk assessment methodologies. SCENIHR concluded that nanomaterials may have different (eco-) toxicological properties than the substances in bulk form and therefore their risks need to be assessed on a case by case basis. There is now a need to assess the suitability of current risk assessment methods in more detail in order to guide how to deal in practice with nanomaterials in an appropriate manner. Therefore DG Environment requested SCENIHR to assess the current risk assessment methodology as laid down in the Technical Guidance Documents, to provide an opinion on their appropriateness and make suggestions for improvements where appropriate. The Committee has examined the currently available scientific data and knowledge, and provides in this Opinion a series of observations of the appropriateness of current methodologies, with clear recommendations on how the Technical Guidance Documents should be modified to reflect current knowledge, and with suggestions for specific improvements in methodologies and with respect to the need for new knowledge.

The Technical Guidance Documents currently make very little reference to substances in particulate form. With respect to human health, the current methodologies described in the Technical Guidance Documents are generally likely to be able to identify the hazards associated with the use of nanoparticles. For the determination of dose – response relationships, special attention should be given to the expression of the metrics of the nanoparticle dose since mass concentration is not necessarily the best description of dose for these materials and number concentration and surface area are likely to be more appropriate. Not all nanoparticle formulations have been found to induce a more pronounced toxicity than the bulk formulations of the same substance. This suggests that the evaluation of nanoparticle formulations should be carried out on a case by case basis. In considering the applicability of existing methodologies to nanoparticles, special attention should be given to the changes in the nanoparticle physico-chemical characteristics that may occur under local environmental conditions. Such changes may include, but are not limited to agglomeration, dissociation and adsorption of environmental substances, all of which may have an impact on the ultimate toxicity of the nanoparticles. Depending on the experimental conditions, such alterations to nanoparticles may be difficult or even impossible to measure under the experimental conditions used.

With respect to environmental exposure, the validity and appropriateness of existing technologies are not always clear. In the absence of sufficient data on the fate and effect of nanoparticles on the environment it is neither feasible nor appropriate to propose firm rules on how substances in nanoparticle form should be evaluated. Instead the applicability of existing methods for risk assessment of nanoparticles should be evaluated.

One of the main problems encountered in the testing of the ecotoxicity of nanoparticles has been the lack of appropriate standardised protocols. The environmental effects of nanoparticles need to be evaluated through the establishment of typical scenarios reflecting their production and use. The exposure and dose-effect models may need to be adapted, taking into account their changing physico-chemical properties over time, including their slow degradation.

In relating exposure dose concentration of nanoparticles to their effects, the traditional use of mass or mass per unit volume alone is unlikely to be appropriate. Surface area and/or particle number per volume in addition to mass should be considered. Additionally, the uptake, distribution, clearance and effects of nanoparticles may differ from those of the

substances for which the Technical Guidance Documents were initially developed. From this and the lack of information regarding species sensitivities towards nanoparticles, it is concluded that at present no clear guidance can be given on the appropriateness of the key standard test taxa and recommended procedures to assess adequately the effects of nanoparticles on the various environmental compartments. The risk characterisation methodology recommended in the Technical Guidance Documents can be followed for nanoparticles, if and only if PECs and PNECs can be calculated with confidence. These are not generally available at present, negating the possibility of a full quantitative risk characterisation as presently required and defined in the Technical Guidance Document.

Improvements to the methodologies should take into account factors such as the following. First, physical parameters such as number concentration and surface area are likely to be more significant than mass concentration in the determination of exposure. Secondly, nanoparticles may agglomerate and disagglomerate in different environments, such processes affecting their properties. Thirdly, impurities within, and adsorbed species on the surface of, nanoparticles may have significant effects on risks and these possibilities should be taken into account. Fourthly, biological processes involving nanoparticles, including translocation, cellular uptake and toxicological mechanisms are still largely unknown and testing methodologies have to address these possibilities. It should also be noted that reference materials for the evaluation of nanoparticles have not yet been identified.

With respect to specific concrete suggestions, there is a clear need for validated *in vitro* assays for nanoparticle evaluation. *In vitro* tests should address key properties of the nanoparticles such as genotoxicity, biopersistence, free radical generation, cellular toxicity, cell activation and other generic endpoints. *In vitro* tests should also provide target cell-specific endpoints such as effects on the action potential of nerve cells or the phagocytic capacity of macrophages.

Inhalation studies require improvement with respect to nanoparticles. They should take into account the fact that nanoparticles with large surface area may rapidly cause saturation of lung clearance. It is generally crucial for risk assessment of nanoparticles to determine the precise tissue distribution profile as there is so little information on translocation. Also specific comments on nanoparticle metabolism and excretion are required, taking into consideration the limits of detection. Similarly, since there is some evidence that nanoparticles can translocate from the lungs to the blood and the brain, assays for the monitoring of blood and brain transfer of nanoparticles, and their consequences, have to be developed. For blood, markers of thrombosis and atherogenesis need to be considered and potential degenerative effects and oxidative stress on the brain should be assessed within these new methods. With respect to mutagenicity, genotoxicity and carcinogenicity, it is necessary to be very cautious about the interpretation and extrapolation of experimental data obtained with nanoparticles, especially with *in vitro* investigations. Since it is not clear whether existing tests are sufficient to detect the mutagenicity of nanoparticles, further developments are required. Similarly, the extrapolation of genotoxicity and relevant toxicity data from macromaterials to nanoparticles needs to be approached with caution.

Concerning the environment, it is not clear at this stage how predicted environmental concentrations for nanoparticles can be calculated. It is recommended that the validity of the current emission factors and models should be evaluated and, if necessary, a modified or new approach should be then be developed. The commonly used mathematical models of dispersal of vapour and large particulate matter will need adaptation for the assessment of the environmental distribution and dispersal of nanoparticles. This implies incorporation into the models of the key physico-chemical characteristics relevant to nanoparticles such as surface area and morphology; charge, number of particles, size, solubility and potential chemical and physical conversion into other forms, as described earlier. With respect to bioavailability, no clear guidance can be given on the appropriateness of the key standard test taxa and recommended procedures to assess adequately the effects of nanoparticles on

the various environmental compartments. There may therefore be a need for new standardized ecotoxicity tests for nanoparticles.

Finally, it is recommended that the staged, or tiered, approach is adopted in order to identify different adverse effects and different exposure data with nanoparticles recognising that the evaluation should be carried out on a case by case basis. It is suggested that due consideration be given to the possibilities now emerging that translocation of nanoparticles away from the portal of entry may occur in humans and other species, and that the passage of nanoparticles across membranes could give rise to adverse effects, for example within the cardiovascular system or following passage across the blood – brain barrier.

1. BACKGROUND

The Commission Strategy and Action Plan on Nanotechnologies underline the importance of a safe and responsible approach and integration of risk assessment into every step of the life cycle of nanotechnology-based products. Due to the novel properties of the nanotechnology products, the Commission requested the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) for an opinion on the appropriateness of existing risk assessment methodologies.

The SCENIHR opinion (SCENIHR 2006) concluded that nanomaterials may have different (eco-) toxicological properties than the substances in bulk form and therefore their risks need to be assessed on a case by case basis. The SCENIHR also foresaw that current risk assessment methodologies require some modification in order to deal with the hazards associated with nanotechnology. In particular, the existing toxicological and ecotoxicological methods may not be sufficient to address all of the issues arising with nanoparticles. For exposure evaluation, additional information is needed, including the number of nanoparticles and/or their surface area. Equipment for routine measurements of substances in various media may be inadequate to detect representative exposure to free nanoparticles. In addition, existing exposure assessment methods may not be fully appropriate to determine the environmental fate of nanoparticles.

The practical implementation of different areas of Community legislation dealing with chemical substances, including the legislation on new and existing substances, may be eventually affected by nanotechnologies. So far, the chemicals legislation does not have specific provisions or testing requirements for substances on a nanoscale. However, on the basis of the SCENIHR opinion, there is a need to assess the suitability of current risk assessment methods, when applied for nanomaterials, in more detail in order to guide how to deal in practice with nanomaterials in an appropriate manner.

Therefore DG Environment requests the SCENIHR to assess the current risk assessment methodology as laid down in the Technical Guidance Documents, to provide an opinion on their appropriateness and make suggestions for improvements where appropriate.

2. TERMS OF REFERENCE

The SCENIHR is asked, in the light of current scientific knowledge and in relation to the general information and practices of chemicals risk assessment, to:

1. assess the appropriateness of risk assessment methodologies (effects and exposure assessment) described in the current Technical Guidance Documents of the chemicals legislation, for the risk assessment of nanomaterials;

2. where current risk assessment methodology may be improved for assessment of nanomaterials, and taking into account the practical limitations of the information available for risk assessments, provide concrete suggestions for improvement of the methodology. Distinctions should be made between improvements that can be made based on current knowledge, improvements that would require specific information on the nanomaterials, and improvements that will require scientific research before they can be implemented;

3. where possible, provide practical examples of how risk assessment of nanomaterials can be performed and of nanomaterials, forms of nanoparticles etc that may cause significantly different adverse effects or different exposure behaviour.

3. SCIENTIFIC RATIONALE

3.1. General introduction

In accordance with the Terms of Reference, the Committee Opinion given in Chapter 4 will provide comments on those factors related to the risk assessment for human health and the environment that are specifically and directly concerned with nanomaterials, and provide recommendations on the nature of additions and alterations that might be made to the Technical Guidance Documents. In this Chapter some preliminary comments are given on the nature of nanomaterials themselves and on the scientific basis for treating nanomaterials differently to bulk substances. It is emphasised at the outset that nanomaterials, and especially nanoparticles, are not necessarily the same as bulk substances and, as set out in the 2006 SCENIHR Opinion, do not necessarily pose the same risk to human health or to the environment as their chemically equivalent bulk substances. It is, however, necessary to place these general statements within the context of the scientific understanding of nanomaterials and the purpose of Technical Guidance Documents.

It is noted that the Technical Guidance Documents are concerned with 'substances', which are defined for their purposes as;

Chemical elements and their compounds in the natural state or obtained by any manufacturing process including any additive necessary to preserve the stability of the products & 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'(European Commission 2003.)

Under the current legislation the decisive criterion whether a nanomaterial is a new or existing substance is the same as for all other substances, that is whether or not the substance is included in the European INventory of Existing Commercial chemical Substances, EINECS (EINECS 2007)). The difference between new and existing substances will disappear after implementation of REACH, the Regulation, Evaluation, Authorisation and Restriction of Chemicals, (European Commission 2006), and therefore this has not been given consideration in this Opinion.

In the 2006 SCENIHR Opinion (SCENIHR 2006), the nanoscale was considered to relate to at least one dimension of the order of 100 nm or less. In line with other published contemporary definitions in nanotechnology, that Opinion also concurred with the view that nanomaterials were materials with more than one external dimension or internal structure at the nanoscale that could exhibit novel characteristics compared to the same material without nanoscale features. Obviously most substances will have internal structures that individually could be considered as being at the nanoscale, for example molecules, crystals or domains, but these do not, *a priori*, qualify for classification as nanomaterials. For example, simply because a polymer may have individual molecules of nanometre dimensions does not necessarily confer nanomaterial status on that substance.

The majority of concerns about the health and environmental risks of nanomaterials, and indeed the majority of data and information on this subject, relate to nanoparticles. It is recognised, of course, that these are not necessarily the only forms of nanomaterials and that solid materials with surface nanoscale features associated with coatings, or with other nanotopographical features, including engineered nanotopographical features, may also have specific and unique physicochemical properties. However, in order to avoid confusion in an area where there is so little data, and to maintain relevance to the questions concerning the Technical Guidance Documents, this Opinion refers only to nanoparticles. With respect to manufactured nanoparticles, it should be noted that there are several possible forms of nanoparticle, including spheres, rods and tubes, and that prominent examples include carbon nanotubes, metallic nanoparticles, particles of oxides such as

titanium dioxide, and quantum dots. All of these are considered to be 'substances' in the context of the Council Directive on Dangerous Substances (European Commission 1967).

The nanomaterial status is characterised by at least one dimension below 100 nm, which may be accompanied by new physico-chemical properties. However, it is recognised that at this stage in the rapid evolution of nanoscience and nanotechnology it is not possible to be scientifically precise over inclusion and exclusion criteria for defining a substance as a nanomaterial. For example, most samples of nanoparticles will be polydisperse and may well include a minority of particles greater than 100 nm in diameter as well as the majority that are below this limit.

The current approaches to the control of substances are comprehensive and should apply to nanomaterials. If any new information concerning the nanoscale characteristics of substances linked to risk assessment becomes available it has to be provided for that assessment. It is possible that nanomaterials may require a different classification and labelling compared to the bulk material. A continuous review of the applicability of testing methods and risk assessment methods at international level, with active input from all relevant parties, is therefore required. In this respect, the SCENIHR opinion on risk assessment methodologies (SCENIHR 2006) has already provided a substantial and essential input to this review. Consequently and as a follow-up to that Opinion, the European Commission (DG Environment) has requested SCENIHR to assess the current risk assessment methodologies laid down in the Technical Guidance Documents, in order to determine their appropriateness for nanomaterials and to make detailed proposals for improvements where possible and appropriate.

Technical Guidance Documents are concerned with procedures for risk assessment for all substances. It is emphasised here that nanoparticles, as defined above in relation to the term 'substances', may be encountered in several different situations and forms, and information on manufactured nanoparticles has to be placed in the context of the exposure to all forms of nanoparticles. Specifically there are many naturally occurring types of nanoparticle and also those which arise from various combustion processes. The risk assessment with respect to new manufactured nanoparticles has to take this natural background into account.

It is also noted that nanoparticles may undergo dynamic interactions within any environment in which they are in contact, such that their characteristics may change over time. Phenomena such as dissolution, agglomeration, disagglomeration, coalescence have to be taken into account, as does the possibility of the adsorption of other substances onto their surfaces. As emphasised in the previous SCENIHR opinion (SCENIHR 2006), the behaviour of nanoparticles is critically dependent on several characteristics, including size, surface area and surface reactivity, and the risk assessments related to both human health and the environment have to be based on these characteristics.

3.2. Physicochemical properties relevant for hazard characterisation of nanoparticles

3.2.1. Special characteristics of nanoparticles

One of the principal reasons why nanoparticles are of interest is the propensity for some of their properties to change as particle size decreases. Properties such as the dynamics of dispersion, the rate of dissolution, the characteristics of nanoparticle aggregates, the surface area and the potential to adsorb substances onto nanoparticle surfaces are all relevant to the behaviour of, and responses to, nanoparticles in biological and ecological systems.

Surface area is the total area of the material that is exposed to the environment. Surface area can be external (geometric surface area) as well as internal if the material is porous or is an agglomerate of primary particles. Porous powders of any pore size exhibit higher surface areas than nonporous powders. The total surface area, and the effective porosity, of nanoparticles in the biological environment can change as the result of the adsorption of species such as biomolecules or the agglomeration of particles themselves.

3.2.2. General principles for approaching nanoparticle characterisation

A wide range of physical and chemical properties should be provided for the hazard characterisation of manufactured nanoparticles, including elemental composition, density, crystal structure, solubility, charge, conductivity, melting point, hardness, magnetic and optical properties, morphology, size and size distribution, surface area and surface layer composition. An indication of the chemical reactivity of nanoparticle surfaces is also desirable. Where relevant, the description of these characteristics needs to take into account known or anticipated variations over time and under varying conditions.

There are several general principles and procedures for approaching basic particle characterisation that also apply to nanoparticles and which are endorsed by national and international standardisation bodies such as ISO and ASTM.

First, it is imperative that the sample of particles measured is representative of the substance. The broader the size distribution, the more significant will be the errors if the sample is not representative. Sufficient sample size must be measured to ensure that the desired limits of accuracy and precision will be achieved. Standard statistical techniques can be used in the determination of representative sample sizes.

Secondly, particle size and shape characteristics should be measured in the most relevant dispersed state. Consideration should be given to the possibility that the material characteristics may change during the product cycle.

Thirdly, the most appropriate metrics and the methods of their evaluation should be used for the particle and hazard characterisation. The commonly used mass metric for substances is not necessarily sufficient for nanoparticles. Number concentration and surface area may be more appropriate parameters for the calculation of dose in terms of the dose - response relationship to be used as a metric for the inflammatory or other toxic endpoints for nanoparticle effects. The BET method of Brunauer, Emmett and Teller (1938) may be used to estimate the surface area.

Ideally, the nanoparticle characteristics should be measured under conditions that mimic those of the potential human and environmental exposure.

3.3. Exposure assessment of nanomaterials

3.3.1. Exposure assessment algorithm

In order to measure exposure to manufactured nanoparticles, it is necessary to take into account the background exposure to ambient nanoparticles such as combustion derived nanoparticles. At present there are limited data from occupational and environmental monitoring of manufactured nanoparticles, in general, for an assessment of their contribution to the overall exposure to be made. Consequently, it is difficult to develop models for the specific prediction of human and environmental exposure to manufactured nanoparticles. Furthermore, data currently available are usually expressed on a mass basis and are therefore not necessarily sufficient for the assessment of the impact of manufactured nanoparticles on human health and the environment.

The previous SCENIHR Opinion (SCENIHR 2006) provided an exposure assessment algorithm, as presented below in Figure 1. The algorithm sets out a sequence of eight questions, the answers to which enable the need for further experiments that will characterise exposure to a particular nanoparticle form, termed the product of interest, to be identified.

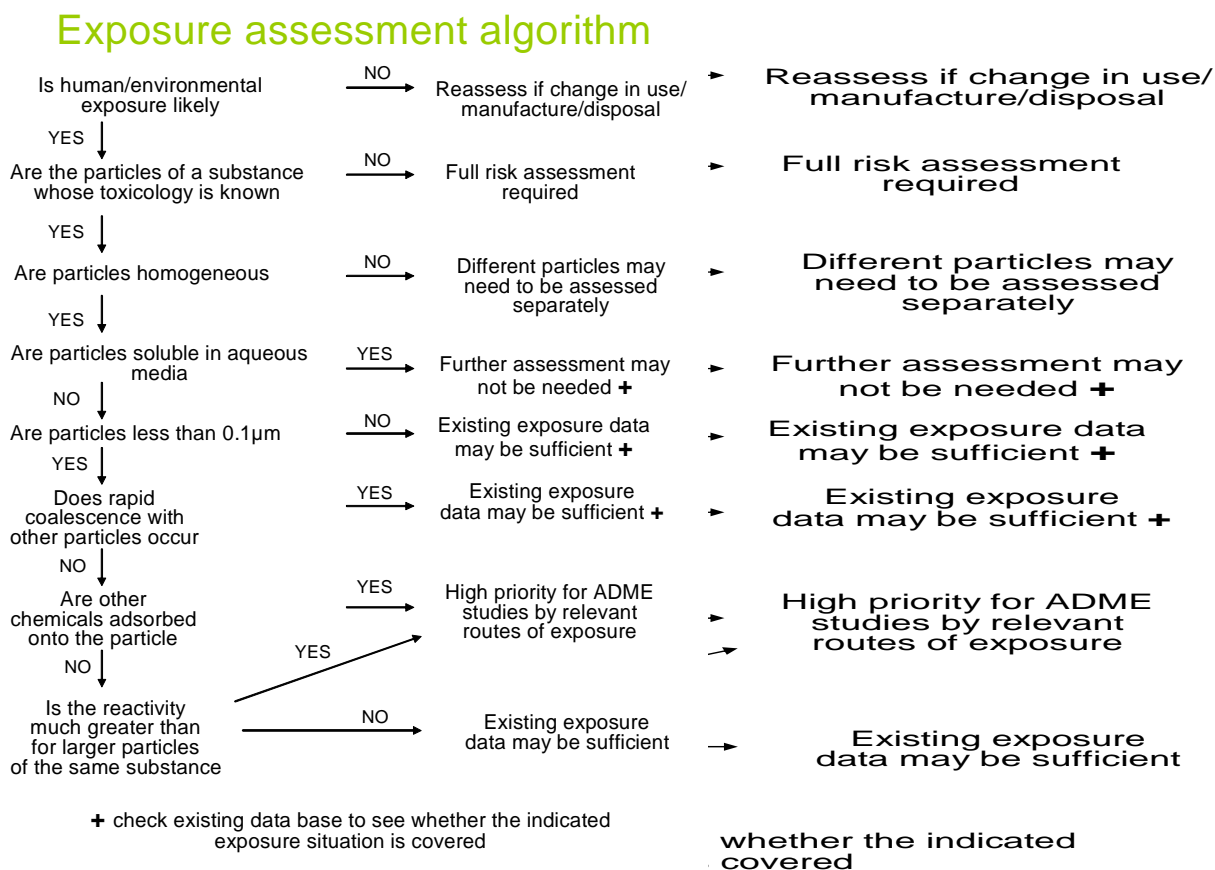


Figure 1. Exposure Assessment Algorithm, Reproduced from SCENIHR 2006

3.3.1.1. Explanation of Terms in Figure 1

A number of the terms used in Figure 1 need to be explained in the context of the current Opinion:

Homogeneity: This refers to the possibility of a large variation in the particle properties in any one product.

Separate assessment: If this is necessary, for each separate entity, the absorption, distribution, metabolism, and excretion characteristics (ADME) should be evaluated with reference to the relevant routes of exposure.

Soluble particles: If nanoparticles are water soluble, their characteristics over time should resemble those of the respective bulk chemical, in which case traditional risk assessment procedures may be applied. For particles of low solubility, translocation may result in local release and exposure beyond the portal of entry. Further assessment may be needed in such cases.

Particles <0.1 µm: This refers to individual particles. Agglomerates of nanoparticles may be larger.

Coalescence: In the algorithm coalescence should be interpreted as agglomeration. This refers to the collision and merger of particles into larger entities. Different physical or chemical effects may be associated with this process, the resulting structure being referred to as an agglomerate.

Other chemicals: This refers to both intentional coating and unintentional contamination with chemical species.

3.3.1.2. Steps in the Exposure Assessment Algorithm

The sequential steps in the algorithm, in which specific questions are asked, require more detailed analysis

Step 1. Is human and environmental exposure likely?

An evaluation should be made of the likelihood of human and/or environmental exposure resulting from emissions throughout the life-cycle, including the manufacturing process, the various anticipated use situations and the final disposal or recycling processes. If this evaluation indicates that exposure to free nanoparticles from the product of interest, at each of the different stages of the life cycle, is highly unlikely, then no further evaluation of exposure is necessary. The evaluation will have to be reassessed if, at a future date, the life cycle features change substantially or the product of interest is changed significantly.

Step 2. Are the particles of a substance whose toxicology is known?

This question addresses whether the published literature, in combination with any additional reliable data, is sufficient to characterise the toxicological properties of the product of interest. If this information is deemed to be sufficient, then only specific properties need to be addressed further, although these may require additional toxicological information. If this is not the case, the deficiencies in the general toxicological information should be remedied. However, for insoluble or poorly soluble nanoparticles, the extrapolation of toxicological results obtained with larger particles is not necessarily reliable or predictive, and the evaluation of nanoparticles should be undertaken on a case by case basis, as emphasised in the previous SCENIHR Opinion. (SCENIHR 2006) For example, surface coatings have a profound impact on the biological properties of the product.

Step 3. Are the particles homogenous?

In order to identify what further information is required, it must be established whether the free nanoparticles of interest are homogeneous or whether it is anticipated that either human and /or environmental exposure will occur to a number of distinctly different types of nanoparticle. If heterogeneity is likely, then it should be considered whether there are any forms that need to be assessed separately

Step 4. Are particles soluble in aqueous media?

The rate and completeness of solubilisation in an aqueous medium should be determined for each type of nanoparticle associated with the product of interest. If rapid and complete water solubility can be demonstrated (at room temperature) for any one type of nanoparticle, then the risk assessment can be considered substantially different to that for other forms of the chemical(s) that make up the nanoparticles. Therefore no further specific assessment of exposure to the nanoparticle is likely to be required.

Step 5. Are particles less than 0.1 µm?

For water insoluble particles, the particle size distribution and any temporal variation in this distribution needs to be identified in order to assess the potential for entry into the human body or uptake by other environmental species, and for subsequent translocation. In view of the evidence that some types of nanoparticles can pass across cell membranes, particular attention needs to be given to the situations in which nanoparticles from the product of interest come into contact with target cells. Since it is not yet possible to identify with confidence a cut off point based on particle size (or indeed any other physicochemical characteristics) that determines such cellular uptake, all nanoparticles must be considered to have the potential to enter cells.

Step 6. Does rapid agglomeration occur?

It is recognised that free nanoparticles may combine with one another or with other material present to form larger particles. If this can be demonstrated to be both rapid and complete for the free nanoparticles from the product of interest (at each life cycle stage), then further specific assessment of the nanoparticle form is not likely to be necessary. The reverse process, disagglomeration should also be considered.

Step 7. Are other chemicals adsorbed onto the particle?

Nanoparticles have a large surface area such that there is the potential for other chemicals to become rapidly adsorbed onto the surface (termed here modified nanoparticles). This process may have an influence on the toxicological properties since the adsorbed species may themselves be toxic and could alter the particle behaviour. If such adsorption is likely, then it is necessary to obtain further information on how such modified particles could behave in the body or organism (via the ADME) or to test directly the toxicity of the modified nanoparticles.

Step 8. Is the reactivity much greater than for larger particles of the same substance?

For free nanoparticles from products of interest that are not eliminated from further consideration by any of the above criteria, the final considerations from an exposure viewpoint are the nature and the extent of the toxic response to the free nanoparticles compared with those of larger particles of the same chemical(s). If the nanoparticle form of the product of interest constitutes a substantially higher risk, or is substantially different in nature, compared to that of larger particles, a full assessment of exposure of humans and/or environmental species is likely to be necessary.

3.3.1.3. Routes of Exposure

Depending on the use of the nanoparticles, the routes of exposure may be inhalation (for example work place air), dermal (sunscreens), oral (food) or parenteral (medical use). The inhalation route has generally been considered the most significant as far as the health impact of manufactured nanoparticles is concerned. However, the exposure by the other routes is becoming increasingly important. Exposure to nanoparticles may occur from occupational and environmental sources as well as through food and consumer products and medical technologies (Maynard and Michelson 2006). Protection is usually achieved by a series of actions, which all depend on monitoring methods to assess necessity and efficacy (Maynard et al. 2006).

The risk associated with nanoparticles is dependent on both the exposure and the hazards, and is mainly driven by the uptake of nanoparticles by these different routes. Nanoparticles can distribute from the site of entry to other sites in the body. Recent studies have shown that nanoparticles can accumulate in areas with increased permeability and cross barriers

such as the olfactory mucosa and the blood brain barrier (Semmler et al. 2004, Kreyling et al. 2006, Oberdörster 2004, Elder 2006). Little is known about conditions in which co-exposures may cause increased uptake and altered distribution of nanoparticles. Also there is a need for simple methods to assess airborne exposure to nanoparticles and to assess their contribution to the total body burden of nanoparticles.

Simple techniques for the online measurement of nanoparticles should help to identify those industrial operations and procedures that may give rise to emissions of nanoparticles. If the health impact of nanomaterials is related to a combination of physical and chemical properties, measurements need to be interpreted in the context of the nature of the air being sampled. In the case of measurements such as aerosol surface-area, these need to be related to the surface activity of a given material. Similarly, the size range of particles to which a particular measurement method is sensitive should be considered. The size distribution of nanoparticles in air will change depending on the time elapsed after their release and on the local environmental conditions. Thus, while it is likely that metrics such as number and surface-area concentration will provide biologically relevant exposure measurements, for many airborne nanoparticles the value of these measurements will depend on the limitations of the measurement methods, and on the underlying biological activity they represent.

3.3.2. *Exposure control measures*

3.3.2.1. Containment

The main method used for the control of nanoparticles during production involves containment within closed systems or within a fluid matrix. This has a beneficial effect of minimizing the release of nanoparticles into the workplace air during production. However, if there are any leaks in the system, nanoparticles may pass through with the efficiency of a gas and become widely dispersed into the workplace atmosphere. By the time the particles reach the end of the process, they may have formed agglomerates that are not easily dispersed into the air but inhalation exposure to these agglomerates may occur during bagging, maintenance and cleaning processes. Materials such as carbon black, fumed silica, ultra fine TiO₂, carbon nanotubes and metal and metal oxide nanoparticles are generally manufactured under total containment conditions, although individual circumstances for closed systems and total containment conditions should be taken into account.

3.3.2.2. Local Exhaust Ventilation (LEV)

LEV systems, enclosures and fume hoods are used to control emissions from materials handling processes such as mixing, weighing and bagging. For nanoparticles, the specification and quality of these systems should be similar to that used for gases. The collection efficiency is expected to be high, provided that the emissions are not entrained in a high velocity jet. However for LEV systems, it is essential that the entry hood is always positioned correctly and adequate capture velocity is maintained. Again, maintenance and cleaning of the systems may pose an additional risk of exposure.

3.3.2.3. Filtration

Impaction and interception forces dominate filtration processes with large particles. As particle size decreases, the efficiency generally reduces but then increases again at about 200 nm, when diffusion begins to become important. There is a minimum efficiency of interception at a particle size known as the most penetrating particle size (MPPS), and most filter efficiency tests are carried out using aerosols with mass median diameters equal to the MPPS. Thus it is expected that correctly specified fibrous filters will be good collectors of nanoparticles. Problems may occur, however, if the filter material has pinhole leaks or if

the filter housing has poor seals, because nanoparticles with behaviour close to gases will penetrate these. The efficiency is particularly relevant for cleaners used in production systems and materials handling areas, and for filters installed into collection systems that recirculate the air back into the workplace.

3.3.2.4. Personal protective equipment

Respirators for control of exposure to airborne dust use low pressure drop filters, with a range of efficiencies dependent upon the particle size distribution of the dust and the specific hazard that this poses. The same problems that were discussed for filters apply to respirators, where the efficiency for nanoparticles is expected to be high. For respirators, however, the problem of seal leakage is more severe around the face. It is possible that leakage will occur for nanoparticles and the protection factors of respirators for nanoparticles needs elucidation. Skin lesions may facilitate skin penetration. The use of protective clothing such as chemical suits and gloves should be considered on a case by case basis, especially at the bagging stages of the process and during maintenance and cleaning. There is currently no information on the penetration of nanoparticles through protective clothing materials.

3.3.3. *Exposure assessment for the environment*

Release of nanoparticles into the environment from a variety of sources is likely to lead to their deposition on environmental substrata. Within the sedimentary system they may be buried and adhere to organic or inorganic materials, depending on their physical and chemical properties. They may be transported, depending on run-off, and the impact on the local biota will depend on their bioavailability. Within aquatic media, nanoparticles may adhere to organic material depending on environmental conditions. It is important that information on partitioning and the fate of nanoparticles between and within different environmental compartments is obtained so that the appropriate risk assessment methodology can be followed. An approach to PBT (persistence, bioaccumulation and toxicity) assessment in relation to nanoparticles must be developed and evaluated and such an approach must focus not only on the particle concentration and distribution, but also on the decomposition products.

Environmental exposure may also be an issue at the end of the life-cycle of some products. The release or redispersion of free nanoparticles that are embedded in solid matrices of various nanotechnology products seems unlikely during the break-down, although there may be release of nanoparticles from fluid matrices to the environment during consumer use.

Some of the current uses of nanoparticles are deliberately directed at interactions with various parts of ecosystems, including remediation that involves the removal of pollutants from contaminated water or soil where large quantities are used, in water treatment filters and during the control of algal growth in water systems (Bergeron and Archambault 2005, Biswas and Wu 2005). However, in addition to the desirable and beneficial effects of such applications, there may be unintentional adverse consequences. The rapid growth of nanotechnological applications may also lead to increased accidental and purposeful release of nanoparticles into the environment. Once organisms are exposed, short or long-term toxic effects may be observed. The latter may be reflected at population level and potentially at food chain level, and include bioaccumulation and biomagnification effects that potentially lead to disturbances in the balance within ecosystems. In particular, the persistence or accumulation of non-degradable nanoparticles may result in prolonged exposure, supporting the need for chronic testing.

It is therefore important that the overall impact and risks of engineered nanoparticles released into the environment are addressed (Colvin 2003, Nature 2003, Oberdörster et al. 2005, Tran et al. 2005).

3.4. Effects assessment of nanomaterials

3.4.1. General approach

There is some information regarding the human health and environmental effects of engineered nanoparticles. Toxicological studies have been conducted on the respiratory system and results demonstrate that the toxicity of some nanoparticles is related to their ability to induce oxidative stress and inflammation in the lung leading to respiratory effects. There is epidemiological and indirect experimental evidence to associate nanoparticles in ambient air to cardiovascular effects (Oberdörster et al. 1994, Stone et al. 1998, Brown et al. 2004). For nanoparticles made from low toxicity materials, it has been suggested that potency, toxicity and the ability to generate oxidative stress and inflammation may be dependent upon their surface area and reactivity (Oberdörster et al. 1994, Duffin et al. 2002). Particles, including nanoparticles, may induce a toxic response through their chemical and physical properties as well as indirectly through degradation products.

3.4.2. Toxicokinetics

For an effects assessment, the exposure (dose) and the toxicokinetics of substances in general, as expressed as absorption, distribution, metabolism and excretion (ADME) are very important. ADME is the paradigm used to chart the fate of substances from their entry into the body, the changes they undergo and their final excretion or storage in the body. It therefore focuses on the way the body handles the substance, not the effects of the substance on the body.

Conventional particles, however, are not generally absorbed from their portal of entry, nor are they excreted through the bile, urine or milk. There is evidence that this situation may be different for nanoparticles (Oberdörster et al. 2005).

For airborne particles, the major portal of entry is the respiratory tract. Insoluble particles deposit throughout the respiratory tract at sites that depend on their aerodynamic behaviour and their size, and they then may be cleared by macrophages and the mucociliary system to the gut for excretion. However, certain particles can enter the interstitium of the lungs and subsequently transfer to the draining lymph nodes. For example, there are cases where very high exposures, such as those seen in coalminers, have led to the presence of particles in the liver and spleen. Similarly, in silicosis, quartz is found in the liver, kidney and spleen, although this is a result of exceptionally high lung burden, leading to pulmonary inflammation and release from the lymph nodes via the lymph efflux into the blood.

Soluble components of particles, such as ionisable metals, may well gain access to the blood where they can form complexes with metal-binding protein for excretion. Organic components released from particles may undergo metabolism in the liver or the lungs where Clara cells and bronchial epithelial cells contain cytochrome P450s and Phase 2 enzymes such as GST (Gluthathione S-Transferase). It is unknown whether this occurs with nanoparticles.

For nanoparticles, both fundamental and practical studies of toxicokinetics are hampered by the difficulty of tracing particles at realistic exposures within complex organisms using current detection techniques

3.4.2.1. Absorption

As noted above, nanoparticles normally have three possible portals of entry, the lungs, skin and gut. The uptake of nanoparticles is also possible after absorption at the nasal epithelium and transported by the olfactory nerve (see below). In addition, direct parenteral application may be used within various medical procedures.

In the lungs, the site and extent of deposition will depend on the thermodynamic and aerodynamic diameters of the particles. The aerodynamic diameter is important for determining which compartments of the respiratory system would be exposed, the upper respiratory tract, the airways, or the alveoli (ICRP 1994). Deposition is a complex phenomena; in general, smaller particles are more efficiently deposited in the lung than are larger airborne particles. The probability of particle deposition depends on the aerodynamic size and the geometry of the airways. For inhaled nanoparticles smaller than about 30 nm, an increasing mass fraction of particles is predicted to be deposited in the upper respiratory airways of humans (ICRP 1994). Migration of nanoparticles from the surface of the lungs across the epithelium to the interstitium inside the body, is of fundamental importance. This propensity to translocate to the interstitium may differentiate nanoparticles from larger particles.

Transport of nanoparticles through the healthy skin is widely questioned in the literature, although there is evidence that particle formulations are prone to transdermal transport if the skin is flexed. There is some evidence that smaller particles, for example quantum dots of around 7 nm in diameter, can enter the dermis (Ryman-Rasmussen et al. 2006). Nanoparticle charge has reported to be the determining factor in skin penetration (Kohli and Alper 2004) where nanoparticles could encounter dendritic cells and reach the blood via the lymph nodes. For the transdermal pathway, fine and ultrafine particle formulations may reach clefts of the healthy and the damaged skin, and potentially deposit there for a time, which can be sufficiently significant for slow degradation processes to take place.

In general, both nanoparticles and microparticles (0.1-3 µm) are ingested at high levels every day and it is estimated that 10^{12} - 10^{14} particles, mainly silicates and titanium dioxide from products such as foods and toothpaste, are ingested per person per day in the Western world (Lomer et al. 2004). There are several potential routes for nanoparticles to be taken up from the gut, including the M-cells overlying the Peyer's patches and lymphoid follicles, and also there may be uptake by intestinal epithelium.

Translocation may be influenced by various physico-chemical properties such as size, surface charge and shape. Translocation of ingested particles from the gastrointestinal tract to the blood is suggested by studies in rats and humans, which have shown that ingested TiO₂ particles in the range 150–500 nm can translocate to the blood and accumulate in the liver and spleen (Jani et al. 1994). Earlier studies described a mechanism of 'persorption' by gastrointestinal tract epithelial cells whereby even larger particles are taken up into lymphatic and blood circulation and translocated to the liver and other organs (Volkheimer 1974).

Ultrafine metal particles did not show a significant translocation to the blood circulation from the gastrointestinal tract and thereby to other organs (Semmler et al. 2004); in this study, after oesophageal administration of a suspension of 18 nm ¹⁹²Ir particles, virtually the whole of the dose was found in faecal excretion within 2–3 days. During the 6-day observation period no detectable ¹⁹²Ir was observed in urine, nor was it detected in any organ or tissue of the body, it being concluded that for these iridium particles there was no uptake and/or absorption from the gastrointestinal tract.

3.4.2.2. Distribution

Translocation of nanoparticles from the upper respiratory tract into the brain has been demonstrated (Oberdörster 2004). Additional studies in rats with radiolabelled nanoparticles suggest a high efficiency of their deposition in the nose, followed by some migration to the olfactory neurons, entering the olfactory bulb of the brain (Elder et al. 2006). The nanoparticles seem to be able to redistribute to the olfactory lobes and to the cerebellum via either olfactory nerves or the blood circulation. MnO nanoparticles (30 nm) have been shown in the bulb and the frontal cortex, which was associated with cytokine and antioxidant up regulation (Elder et al. 2006). The uptake of inhaled 18 nm iridium

nanoparticles in the brain from the circulation was shown by Kreyling et al (2002) and Semmler et al (2004). At this stage it is not possible to conclude whether exposure of cerebellum takes place via olfactory nerves rather than the circulation.

Nanoparticles that reach the circulation from the bronchiolar walls or the alveoli will redistribute with the blood flow around the body. Macrophages of the reticuloendothelial system within organs such as the liver, spleen and bone marrow have evolved to remove antigens and other matter from the blood. These organs are likely to receive a considerable dose from blood-borne nanoparticles. The iridium studies mentioned above have shown that inhaled nanoparticles may translocate, at low mass but high particle number, from rat lungs to liver, kidney, spleen, heart and brain and are retained there for six months after a single exposure. Studies with nanoparticles for drug delivery purposes emphasise that certain surface configurations, for example coating with polyethylene glycol, diminish the uptake into the tissue for any particle present in the blood, thus potentially prolonging the circulation of nanoparticles (Bazile D et al. 1995, Niidome et al. 2006, Peracchia et al. 1999).

The distribution of nanoparticles to the placenta and foetus is not known at this time.

3.4.2.3. Metabolism

In general, substances in the form of persistent solid particles are not metabolized in the same way as soluble substances, which are processed by cellular and biochemical pathways, resulting in biotransformation which allows for metabolites to be removed via bile and /or urine. However it is not clear what is the metabolic fate of any persistent nanoparticles. Some nanoparticles, such as carbon nanotubes, may either be excreted directly or undergo transformation. In addition it can be assumed that phagocytosis and the resulting production of reactive oxygen species may cause formation of derivatised nanoparticles. Lipophilic (hydrophobic) nanoparticles have the potential to accumulate in adipose tissues. Some carbon-based nanoparticles, for example carbon nanotubes and carbon black, are likely to have carboxyl and hydroxyl groups on their surfaces, originating from defects in the graphene sheet, which may allow metabolism. To date there is no published research on the size dependency of the metabolism or biological degradation pathways of nanoparticles.

3.4.2.4. Excretion

In view of their size or surface functionalisation, it is possible that nanoparticles could undergo glomerular filtration and be excreted in the urine (Singh et al. 2006). Persistent nanoparticles may be secreted into the bile or filtered in the kidneys and enter the urine, although. There is little data to indicate whether this actually occurs. Singh has showed that derivatised carbon nanotubes became water soluble and were rapidly and efficiently excreted in the urine (Singh et al. 2006).

3.4.3. Toxicity

At this stage, very little consistent data on the toxicological characteristics of nanoparticles is available, although information is rapidly accumulating. Some very important practical data, for example, concerning the effects of repeated doses of nanoparticles, are not generally available at this time. Most information has been derived from inhalation toxicity studies and the following discussion is based on data derived from this route.

3.4.3.1. Reproductive toxicity and Teratogenity

There is nothing published yet about the potential reproductive and teratogenic effects of manufactured nanoparticles.

3.4.3.2. Immunotoxicity

Particulate matter is known to possess adjuvant activity under some circumstances, which may exacerbate responses to allergens (De Haar et al. 2006, Steerenberg et al. 2006, Granum and Lovik 2002, Nygaard et al. 2004, Alessandrini et al. 2006), this being demonstrated both with model particles and ambient air particles. Furthermore, smaller particles were found to cause the stronger adjuvant effects. In addition to eliciting pulmonary inflammation in healthy subjects, the airway exposure to engineered nanomaterials may also influence the development and severity of other allergic pulmonary diseases. Atopic individuals may be more prone to respiratory symptoms compared to non-atopic people when exposed to particulate matter. The effects of airway exposure to nanoparticles may therefore depend on the atopic status of the exposed individuals. Similar questions have to be raised when assessing the effects of nanoparticle exposure on the skin.

3.4.3.3. Neurotoxicity

It is possible that nanoparticles could gain access to the brain by two different mechanisms, the trans-synaptic transport after deposition on the olfactory or bronchial epithelium, and uptake from the blood through the blood-brain barrier (Kreuter et al. 2002). The physiological obstacle that the blood-brain barrier provides may limit the distribution of some proteins and viral particles after transvascular delivery to the brain, suggesting that the healthy blood brain barrier contains defense mechanisms that protect it from being breached by blood borne nanoparticles.

The potential impact of nanoparticles on human neuronal tissue has not yet been investigated in detail. Ultrafine paramagnetic nanoparticles are being used for MRI imaging of different cell types within neural tissue. Nanoparticles may induce the production of reactive oxygen species and oxidative stress *in vitro*. There are indications that nanoparticles may migrate to the brain and have some effects on the brain although whether these effects result in disease remains unknown.

A number of pathologies, including hypertension and allergic encephalomyelitis, have been associated with increases in the permeability of the blood brain barrier to nanoparticles in experimental animals, increasing their susceptibility to diseases. In addition, the nanoparticle surface charge has been shown to alter blood-brain integrity (Lockman 2004), indicating that such factors should be considered with respect to brain toxicity and brain distribution profiles for nanoparticles.

3.4.3.4. Mutagenicity and Genotoxicity

In relation to the genotoxicity of particles, the chemical composition and surface reactivity are known to play major modifying roles. Many particles present as an insoluble or poorly soluble core onto which various adsorbed mutagens (or carcinogens) can be carried from the environment into and throughout the human body. DNA adduct formation has been linked to specific combustion-generated nanoparticles, including diesel exhaust particles or commercial carbon black (Borm et al. 2004). The possible genotoxic and mutagenic effects of particle-associated organics or metals will depend on their bioavailability. This concept of solubility also holds for the core particles themselves since, if the entire particle is readily soluble, any possible genotoxic effect is expected to be related to that of the non-particulate chemical nature.

Of major importance for genotoxicity is the formation of reactive oxygen species (Nel et al. 2006, Knappen et al. 2004). In addition, nanoparticles have been shown to penetrate subcellular structures such as the mitochondria (Li et al. 2003) and nucleus (Chen and von Mücke 2005), causing uncoupling of respiration and increased oxidative stress or interference with the genomic replication or repair.

3.4.3.5. Carcinogenicity

Early studies aimed at understanding the carcinogenicity of low toxicity - low solubility dusts in rats, involving the mechanism of rat lung overload, suggested that the volumetric particle lung burden was the main driver of the effect (Morrow 1988). However, further studies of carbon black and titanium dioxide dusts in ultrafine form showed that the onset of overload could occur at much lower lung mass burden (Oberdörster 1996). It was then found that the tumour formation associated with these low toxicity - low solubility materials was better related to surface area dose than to the lung mass dose (Driscoll 1996). Further studies aimed at investigating the dose metric that best described the onset of 'overload inflammation' following exposure to high levels of these particles, again showed that surface area dose was superior to mass dose (Tran et al. 2000). Since surface reactivity is also known to be a factor that influences inflammation (Duffin et al. 2002), the overall ability of any particle burden to cause chronic inflammation and fibrosis, and therefore potentially to be carcinogenic, will depend on the product of surface area and reactivity. This has important implications for engineered nanoparticles which have very high surface areas per unit mass with the potential to have a reactive surface.

Experimental studies of the carcinogenicity of particles are difficult to interpret with respect to dose responses, the ability to extrapolate between species and the appropriate metrics. In one study, nineteen different particle types were instilled intratracheally at high doses to rats, which were allowed to live for up to 129 weeks. Some materials contained fine and nanoparticulate form, and included TiO₂ and carbon black. Different interpretations of these data have been published (Hohr et al. 2002, Borm et al. 2004, Pott and Roller 2005, Morfeld et al. 2006). The separate consideration of the carcinogenicity of nanoparticles compared to other particles is not supported by the dose-response curves using surface area as a dose metric (Borm et al. 2004). Pott and Roller (2005) also correlated the tumour incidence with mass, surface area, volume, and particle size data and found that the best association was between the volume in connection with particle size. Morfeld et al (2006), in a statistical reanalysis of data, found no better fit when using surface area or volume as dose metrics but found significantly higher tumour prevalence in animals instilled with TiO₂ and carbon black nanoparticles. A dose threshold of about 10 mg mass dose emerged from their calculations.

It is likely that inhaled, non-toxic, nanoparticles can induce lung tumours in rodent models by mechanisms similar to those found with fine particles. These mechanisms include DNA damage and increased cell proliferation, associated with a persistent inflammation in the lung. The metric driving this response is still unclear but surface area has the strongest support from toxicological evidence, so that, based on their higher surface area, nanoparticles have a stronger theoretical potency to induce lung tumours. No increase in extrapulmonary tumours has been seen in inhalation studies, although chronic studies with nanoparticle administration do not appear to have been performed.

3.4.3.6. Diseased lungs and susceptibility to the effects of nanoparticle

There are likely to be individuals who are susceptible to adverse effects of engineered nanoparticles on the basis of existing diseases, particularly since experience with larger particles show that adverse effects are seen predominantly in susceptible populations, especially those with inflammatory airways disease or cardiovascular disease (Pope 2000, Samet et al. 2000). Individuals with asthma and chronic obstructive pulmonary disease (COPD) may experience exacerbation of their disease, when exposure to particles is elevated. These effects will be related to the oxidative and pro-inflammatory effects of nanoparticles, inflamed lungs being more permeable such that nanoparticles may cross the epithelium more readily in these individuals. Deposition of nanoparticles is usually greater in patients with COPD, probably due to their abnormal, inflamed airway. Diabetic patients are also at potential risk as they have endothelial dysfunction, similar to the patients with cardiovascular disease. Although only about one quarter of smokers suffer from COPD, all

will have an increased pulmonary permeability to an inhaled molecular marker. The increased lung permeability is one of the first changes seen on initiation of smoking and one that declines on cessation. Uptake of nanoparticles may be enhanced in smokers.

3.4.4. *Ecotoxicology*

Very few published studies have focused on the fate of nanoparticles in aquatic and terrestrial habitats, their effects on the biota within these habitats or on the mechanisms of any potential ecotoxicity. However, the assessment of the likely environmental impact of nanoparticles is very important since many nanoparticles will enter the environment via wastewater from both domestic and industrial use (Colvin 2003, Nature 2003, Oberdörster et al. 2005). Input through numerous diffuse sources will also be possible as many of the current and intended uses of nanoparticles are environmental. This lack of information also applies to the behaviour of engineered nanoparticles in the environment.

Many nanoparticles tend to form agglomerates, and it is not clear whether nanoparticles within these agglomerates have the same toxic potential and bioavailability as free nanoparticles. As with the other chemicals, the properties of nanoparticles in the environment are likely to change depending on their physico-chemical characteristics and the nature of the local environment. Such characteristics will influence the partition of the nanoparticles between and within various environmental compartments. For example, the release of nanoparticles via wastewater suggests that they will be mixed with significant quantities of household and industrial detergents that may influence the association or dissociation of agglomerates of particles. Furthermore, naturally occurring surfactants, such as humic acids, may also affect their physical and chemical fate, again being consistent with toxicity of substances in general.

In addition to determining the fate and distribution of nanoparticles in the environment, it is essential to assess their potential toxicity to a wide range of species, reports of which are now being published (Oberdörster 2004, Lovern and Klapper 2006, Oberdörster et al. 2006, Stone et al. 2006).

A range of metal oxide and silver nanoparticles have been developed as antibacterial substances. The effect of these nanoparticles on non-target microorganisms involved in biogeochemical cycling in the environment is of concern. Studies assessing the effects of fullerenes on soil microorganisms (*Escherichia coli* and *Bacillus subtilis*) have indicated reductions in growth and respiration (Fortner et al. 2005). Some studies suggest that silver nanoparticles can accumulate in the membrane of *Escherichia coli* bacteria causing the cell walls to pit, so that cell permeability is altered and death ensues (Sondi and Salopek-Sondi 2004). In addition to effects on bacteria, TiO₂-coated hollow glass beads have been shown to inhibit the photosynthetic activity of cyanobacteria and diatoms, suggesting potential useful applications in preventing excessive algal growth (Kim and Lee 2005). The antimicrobial properties of some nanoparticles have been employed in biocides (Koper et al. 2002). The widespread release of such nanoparticles may lead to imbalances within the environmental microbial populations and needs to be addressed appropriately.

Aluminium nanoparticles have been found to inhibit the root growth of plants. Particles at 13 nm size suppressed root growth of five different plant species at 2 mg ml⁻¹ concentration while larger sizes, at 200-300 nm, had no effect. Although the authors suggested that these effects were due to the presence of free hydroxyl groups on the particle surfaces, Murashov (2006) suggested that some of these phytotoxicity effects may have resulted from increased solubility of nanoscale aluminium.

Deleterious effects on crustacean and fish exposed to C₆₀ molecules, and nanosized TiO₂ and carbon black have been published (Oberdörster 2004, Oberdörster et al. 2006, Zhu et al. 2006, Lovern and Klaper 2006, Stone et al. 2006). Although these studies have not yet addressed uptake, bioaccumulation or biomagnification of nanoparticles, and analysis of the

data may be impaired by the exposure medium preparation protocol used, they do suggest some short and mid-term effects such as oxidative stress, and some behavioural and reproductive effects. The effects of nanoparticles such as TiO₂ and carbon black on aquatic crustaceans (*Daphnia magna*, *Artemia salina* and Gammarids) indicate that free nanoparticles are ingested into the gastro-intestinal tract within 30 minutes (Stone et al. 2006). Furthermore, these nanoparticles adhere to crustacean exoskeleton, suggesting multiple routes of exposure and potential impairment (Stone et al. 2006). Exposures of *Daphnia magna* to fluorescent polystyrene nanoparticles resulted in a rapid uptake by neonates and adults into the gastrointestinal tract followed by translocation to lipid storage droplets exposure (Lubick 2006). Oberdörster et al. (2006) observed a delay in moulting and significantly reduced offspring production in *Daphnia magna* exposed for 21 days to 2.5 and 5 ppm C₆₀ concentrations.

Tests conducted on *Daphnia magna* indicate that the acute lethality of the nanoparticles tested is relatively low, possibly with some increased oxidative stress in *Daphnia magna* with increased ultrafine carbon black concentrations, but nevertheless may still be cause for concern (Lovern and Klaper 2006, Oberdörster et al. 2006, Zhu et al. 2006). Published studies on the effects of any nanoparticles on other invertebrates are limited, with some studies on the freshwater amphipod *Hyalella azteca* and on marine benthic harpacticoid copepods (Oberdörster et al. 2006, Zhu et al. 2006).

Oberdörster (2004) published the first non-human, non-rodent vertebrate study on nanoparticle toxicity using juvenile largemouth bass. As with other studies, the C₆₀ was pre-treated with tetrahydrofuran (THF) to aid dispersion. The fish were exposed to 0.5 and 1ppm C₆₀ for 48 hrs and were found to exhibit signs of lipid peroxidation in the brain. Selective transport to the brain has been observed in rodent studies, which, along with the lack of neural antioxidant defence mechanisms, could explain the enhanced brain lipid peroxidation. However, THF is classified as a neurotoxin, and the real significance it not yet clear. In a subsequent study Zhu et al. (2006) demonstrated that THF prepared C₆₀ induced 100% mortality within 6 to 18 hours of exposure in adult fat head minnow (*Pimephales promelas*). Conversely, C₆₀ generated by water stirring had no impact on lethality over the same time period, although lipid peroxidation was observed in the gill, suggestive of oxidative damage, as well as a significantly increased expression of CYP2 family isoenzymes in the liver as compared to control fish. Oberdörster et al. (2006) tried to overcome the preparation-linked problems by stirring the fullerenes in water. The effects of this preparation were assessed on fish species, fathead minnow and Japanese medaka (*Oryzias latipes*) at 0.5ppm concentration for 72 hrs. The results indicated no change in mRNA or protein-expression levels of cytochrome P450 isoenzymes CYP1A, CYP2K1 and CYP2M1. The peroxisomal lipid transport protein PMP70 was found to be significantly reduced in fathead minnow but not medaka which the authors attribute to potential changes in acyl-CoA pathways.

3.5. Risk characterisation of nanomaterials

3.5.1. The difference between nanoparticles and bulk chemicals

On the basis of current knowledge, the risk characterisation of bulk materials as described in the Technical Guidance Documents cannot be directly extrapolated to nanomaterials. The mechanisms of toxic effects of engineered nanoparticles may be dominated by those characteristics specifically introduced in order to meet the intended function of the product of interest, possibly including surface reactivity and quantum effects. Therefore, any unpredicted interactions between nanoparticles and biological systems may depend on their unique physical and chemical properties and their multiple functionalities.

Since key mechanisms for exposure processes and toxicity effects of manufactured nanomaterials are not sufficiently understood, these inherent uncertainties will dominate the estimation of risk. These uncertainties include the following:

1. the persistence of nanoparticles in the atmosphere, which will depend on rates of agglomeration and disagglomeration, and on degradation,
2. the relevance of routes of exposure to individual circumstances,
3. the metrics used for exposure measurements,
4. the mechanisms of translocation to different parts of the body and the possibility of degradation after nanoparticles enter the body,
5. the mechanisms of toxicity of nanoparticles,
6. the phenomenon of transfer between various environmental media.

It should be emphasised that these are not simply uncertainties in the values of some traditional parameters, but rather the uncertainties about the potentially unique or significantly modified causal mechanisms themselves.

3.5.2. Potential risks to human health

The applications of nanomaterials are increasing and it is likely that exposure to manufactured nanoparticles will become more common. The overall potential risks are likely to increase if no control actions are taken, the greater potential risks being associated with the occurrence of free nanoparticles. Among the main factors that underpin this increased potential risk are the ability for nanoparticles to reach tissues that larger particles do not, the unknown effects associated with highly persistent reactive nanoparticles, and the modified toxicokinetics of these nanoparticles compared to conventional bulk materials.

The life-cycle of nanoparticles is clearly of importance. There are different human exposure scenarios during the life cycle of nanoparticles, including those during production, processing and distribution, use and application, storage, and waste disposal and recycling. Humans may also be exposed indirectly through contamination of the food chain by manufactured nanoparticles. If long term stability of a nanoparticle is proven, this may have consequences: for the general public and for potentially vulnerable subpopulations, including the embryo, the very young, and the elderly, beyond that associated with the exposure of workers. Furthermore the role of predisposition factors of individual humans, such as their genetic background and their pre-existing diseases such as allergies, cardiovascular disease and immune diseases needs to be taken into account.

3.5.3. Potential risks to the environment

Release of nanoparticles into the environment may occur from a variety of possible sources and products and is likely to lead to their distribution in various media and to deposition on environmental substrata. Environmental species will then be potentially exposed via the different media, the air, the water and the soil/sediment system. Risk characterisation will involve an assessment of particle concentration in the environment and how this relates to toxic effects and the persistence within the environment and biota. The route of exposure to nanoparticles is likely to have a bearing on the uptake by biota in the environment and on the resulting toxicity. Due to their particulate nature, it is likely that species vulnerability to nanoparticles will relate to their ecology and feeding mode. Species that have well-developed mechanisms for particle uptake or an impaired particle clearance mechanism might be particularly susceptible to the effects of nanoparticles. However, there are no studies that address these scenarios. Some species may be especially vulnerable to nanoparticles that promote active oxygen formation. by virtue of their low anti-oxidant levels

3.6. Measurement methods for characterisation, exposure and effects assessment

The physico-chemical properties of nanoparticles may change with particle size such that a material that consists of nanoparticles does not necessarily behave in a similar manner as the bulk material of the same chemical composition. For example, the melting point, magnetic and electrical properties, reactivity and optical properties will change as the particle size decreases. The nanoparticle composition itself may then change with time depending on the local environment, possibly resulting in agglomeration, degradation or the adsorption of chemicals present in the local environment onto the nanoparticles.

All these factors should be taken into consideration during the characterisation, monitoring and effect assessment of nanoparticles. Accordingly, the particle characteristics should be measured under conditions that mimic all relevant exposure conditions. For example, the particle characteristics should be measured in the relevant media or under the same pH and ionic strength conditions relevant to each specific case.

Current methodologies generally enable nanoparticle characterisation and monitoring in the air. Methodologies for the monitoring of nanoparticles in fluids and other environmental compartments such as soil, which are especially important for the human effects assessment and environmental monitoring, are under development. The full characterisation of nanoparticles is a complex process that is limited to only a small number of laboratories at this time and is not yet routinely available in the field.

3.6.1. Methods for the characterisation of nanoparticles

A range of properties need to be measured in the characterisation of nanoparticles, which may be grouped as follows;

- chemical composition, crystal structure, water solubility, octanol-water partitioning coefficient (K_{ow}). Note that K_{ow} is the ratio of the concentration of a chemical in octanol and in water at equilibrium at a specified temperature. Octanol is an organic solvent that is used here as a surrogate for natural organic matter. K_{ow} also refers to the hydrophilic – hydrophobic balance of substances, but its applicability to nanoparticles is not yet known,
- morphology, particle size and size distribution, shape, aspect ratio (length/thickness), agglomeration state,
- specific surface area,
- surface chemistry, surface charge, surface topography,
- stability (in the appropriate media),
- vapour pressure and boiling point.

Many of the instruments used for the characterisation of larger particles can be used for nanoparticles, although some specialised equipment may also be needed. Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM), equipped with a variety of analysis tools are valuable for the determination of some nanoparticle characteristics, especially morphology and surface chemistry. Normal powder characterisation tools like X-ray diffraction (XRD), BET (Brunauer, Emmett, Teller) surface area, Inductively Coupled Plasma –Mass Spectrometry (ICP-MS) or Atomic Absorption Spectrometry (AAS) may be used for the determination of elemental composition with nanomaterials. Dynamic Light Scattering (DLS) may be used to investigate particle size distribution in suspensions.

Mass size distributions measured by low pressure impactors should not be converted to surface or number size distributions due to their low sensitivity in the nanoscale range and issues related to particle density and agglomerate fractal dimension.

3.6.2. *Measurement methods for exposure monitoring*

There is no national or international consensus on measurement techniques for nanoparticles in the human living environment and at the workplace, nor are there any agreed standards for such techniques. In addition to those general characterisation methods presented above, several methods are available for more detailed measurements at workplaces. The best metric(s) for exposure assessment will eventually be determined by the outcome of series of studies correlating different particle properties with relevant end points.

Traditional industrial hygiene sampling methods collect certain mass fractions of particles with specific sizes, for example the respirable fraction (ICRP 1994). However, an increase in respirable mass fraction does not provide direct information about the number or surface area metrics that are more relevant measures for nanoparticle health effects. An increase in airborne particle mass concentration does not usually have any significant correlation with an increase in particle number concentration.

A few commercially available personal samplers have been designed to measure the particle number, surface area, or mass concentration of nanometre aerosols. However, several methods for stationary detection are available that can be used to estimate surface area, number, or mass concentration for particles smaller than 100 nm. Also, particle number concentration has been correlated with adverse responses to air pollution in some human studies (Timonen et al. 2004, Ruckerl et al. 2005). Although surface area data of ambient air particles are not generally available, the particle surface area has been shown to be a better predictor than either particle number, mass, or volume concentration alone (Oberdörster and Yu 1990, Tran et al. 2000, Duffin et al. 2002, Beck-Speier et al. 2005, Stöger et al. 2006).

An important point to consider is the condensation of environmental (including manufacturing environmental) fluids such as water and lipophilic agents that have been demonstrated to affect the size of the original particles, depending on the saturation conditions in the environment and the properties of the particle surface.

Most commercially available real-time number and/or surface area (so called active surface or Fuchs surface) measurements of aerosol particles are based on electrical charging of the particles. For aerosols less than approximately 100 nm in size, measurement of the Fuchs surface area is probably a good indicator of external surface-area (or geometric surface area). Measurements of active surface-area are generally insensitive to particle porosity and to highly agglomerated chains of very small primary particles. Some nanoparticle health effects correlate best with the surface area measured by the BET nitrogen absorption methodology (Stöger et al. 2006)].

Air borne particle number concentration can be measured relatively easily using condensation particle counters (CPCs). These are available as hand-held static instruments and are generally sensitive to particles greater than 3 to 20 nm in diameter depending on the monitor. However, CPCs designed for the workplace do not generally have discrete size-selective inputs, and so they are typically sensitive to particles up to micrometers in diameter. Under most ambient and nanoparticle exposure conditions the number of micron sized particles are negligible and do not contribute significantly to the total particle number counted. An important issue, however, is the need for distinctive measurement of the engineered nanoparticles separate from the background nanoparticles in the same size range. No monitors currently exist for this specific purpose. Nanoparticles are ubiquitous in many workplaces, from sources such as combustion, vehicle emissions and infiltration of

outside air. Particle counters are generally insensitive to particle source or composition, making it difficult to differentiate between incidental and process-related nanoparticles using number concentration alone. However, background exposure to ambient air nanoparticles may be present and regardless of the metric and method selected for exposure monitoring, it is critical that measurements are conducted before production or processing to obtain background exposure data in order to determine if there has been an increase in exposure above background.

3.6.3. Methods for effects assessment for human health

There are no nationally or internationally agreed reference nanoparticles or nanomaterials which is a major deficiency considering the wide range nanoparticles to be assessed, taking into account differences between monomeric and polymeric nanoparticles, between organic and inorganic nanoparticles and so on, particularly considering the potential addition of multiple functional groups onto nanoparticle surfaces. The selection, establishment and adoption of reference nanomaterials is a very important issue.

There are many measurement methods available to study adverse effects of nanoparticles, including *in vitro* and *in vivo* methods. Both of these are required for a comprehensive understanding of modes of action and underlying mechanisms of these adverse effects. *In vitro* methods usually provide information on modes of actions and the underlying mechanisms at the level of proteins, biomolecules, extracellular matrix, DNA, parenchymal and immuno-competent cells and their compartments. *In vivo* methods are required to identify the biological relevance of the *in vitro* determined modes of actions and the underlying mechanisms in the more complex interplay among multiple cell types, within organs and within the entire organism. The determination of dose-response relationships and of target organs and cells can only be determined *in vivo*. A major problem here is that the definition of dose is not standardised with respect to mass, number, surface area and other metrics and this hinders the objective analysis of the data and comparisons between materials.

In the case of nanoparticles, target organs may not be restricted to the portal of entry, but may include secondary target organs and their cellular constituents, depending on the accessibility of nanoparticles to these sites. Furthermore, interspecies differences need to be considered very carefully in any extrapolation to human effect assessment. Hence, a comprehensive effect assessment usually requires *in vivo* studies for dose-response-relationships at target organs for nanoparticles to supplement the *in vitro* methods for understanding of the modes of actions and underlying mechanisms. This should deliver a time and cost effective risk evaluation.

3.6.3.1. *In vivo* studies

Whilst existing protocols may detect effects on the lungs, liver, spleen and other organs, more specialised techniques may be required to detect more subtle effects on the blood and the nervous system. Particles deposited in the lungs can affect the clotting system, possibly enhancing thrombogenicity. These effects have been observed in models where a thrombus is generated artificially (Nemmar et al. 2003, 2004). In addition, direct effects have so far been seen in the causation of thrombosis in a normal animal (Silva et al. 2005). In the case of the nervous system, there is small but detectable translocation, detected using radiolabelled nanoparticles, and concomitant low level effects on gene expression. These are unlikely to be seen in routine sections and would have to be detected using molecular techniques.

Animal studies can be used for hazard identification related to the properties of nanoparticles compared to conventional particles, especially translocation from the portal of entry into circulation and towards accumulation in secondary target organs. With the

respiratory tract as the portal of entry, both inhalation and instillation can be used, although the latter is problematic for nanoparticles as noted below.

Effects can be anticipated at the site of deposition, including the gut and the skin, as well as effects on other sites following translocation. Consideration of the anatomy of the upper and lower respiratory tracts suggests that these are sites from where translocation to the central nervous system and blood is likely. Subsequent hazard to the blood, nervous system, liver, spleen and bone marrow may be anticipated.

The Technical Guidance Documents identify the relevant OECD test guidelines for the identification of irritating or sensitising substances with respect to skin exposure and it is considered that the assessment for these effects is appropriate for nanoparticles. Carcinogenic effects on the skin are possible, and chronic animal skin exposure studies could be used to investigate this possibility. In the case of the gut, feeding studies and gavage can be used to deliver high doses. Effects can be anticipated on the gut immune response as well pro-inflammatory conditions, and these should be detected by conventional protocols.

Some studies have bypassed the principal portals of entry (lungs, gut, skin) and, on the assumption that translocation to the blood is possible, have involved the injection of nanoparticles into the blood. These have shown some pro-coagulant effects on the liver. Once the endpoint is determined, animals can be used for dose response tests by inhalation, ingestion and dermal uptake in order to define the no observable effects level (NOEL). However, special protocols may be necessary to detect the NOEL for nanoparticle specific effects.

In addition, there have been studies on healthy human subjects and on patients susceptible to the effects of nanoparticles. These tests are designed to determine the initial stages of disease modulation and progression, but, as relevant as they could be, they are of limited value because of strict ethical considerations involved.

3.6.3.2. Instillation versus inhalation

Instillation of particles into rat lungs has often been used in particle toxicology. This mode of exposure is not physiological in the sense that there is usually a very high dose and dose rate and, since the particles are suspended in saline, the lung surface receives particles contained in a liquid, which is likely to affect the defence systems of the lung. The advantage of instillation is that it involves the administration of a more precise nanoparticle dose. Pharyngeal aspiration is a variant of instillation, which still involves a high dose, a high dose rate and the fact that the particles are in suspension, but in this case the exposure is to suspension droplets that disperse in the lung more readily than with simple instillation. However, two side effects may detract from pharyngeal aspiration, involving unusually high doses to bronchioles and the bacterial rinsing induced alveolar inflammation. Results with instillation and pharyngeal aspiration are rather similar in terms of allowing comparison in toxic potency between particle types and can be used for the oropharyngeal region down to the sterile alveolar region in the context of screening purposes and for mechanistic studies. However neither method can be used to determine NOEL.

Inhalation is the physiological process during which nanoparticles are deposited in the respiratory tract, allowing for a slow build up of the dose and for normal clearance processes to occur. This is the only way to determine the NOEL for the airborne concentration of suspended dust. However, determination of the administered nanoparticle dose is difficult and its estimation requires careful monitoring of breathing, of the aerosol parameters and of tissue analysis.

3.6.3.3. *In vitro* studies

Organ culture studies

The isolated perfused lung can be used to study particle translocation.

Cellular studies

The effects of nanoparticles on cells from various target organs can be studied for using various generic (e.g. toxicity) or cell-specific (e.g. the inflammatory response of macrophages) endpoints. These may yield important mechanistic data to support animal – derived hazard identification. For example the selective depletion of dopaminergic brain neurones seen in mice exposed to particles (Veronesi et al. 2005) was confirmed *in vitro*, showing selective death of the same type of neurones when exposed to diesel nanoparticles (Block et al. 2004). Co-cultures of multiple cell types simulating organ compartments apparently provide more relevant output readings than mono-cellular cultures, since they allow for intercellular communication between different cell types, thus resembling a more realistic model. It is desirable that *in vitro* approaches could be used to determine the potential for translocation, but no such assay presently exists. Dose-response studies can be carried out *in vitro* but these needs to be complemented by sound toxicokinetics that allow plausible dosimetry for the cell type in question. It is also unclear whether bacterial or mammalian cell systems are most appropriate to evaluate genotoxicity effects.

Blood

The emerging importance of blood as a target for nanoparticles is based on hazard identification studies showing pro-thrombotic effects following addition of nanoparticles to whole blood. Several studies have shown that various types of nanoparticle added to blood promote platelet activation, with differences between different types of particle. The complement system has evolved to be activated by foreign surfaces; nanoparticles, with their large surface area and activity, are possible activators of this system. Although not demonstrated in animals, it has been found *in vitro* that carbon nanotubes can activate the complement system, with the potential to induce inflammation via this pathway (Salvador-Morales 2006). Care needs to be taken concerning dosimetry, as implausibly high doses might invoke responses not produced *in vivo* at more reasonable doses.

4. OPINION

There are many different attributes of nanoparticles that may influence their human toxicological and ecotoxicological properties. It is widely recognised that in addition to mass concentration, several other parameters, including surface area and number concentration, are required to fully characterise the dose of nanoparticles, thus enabling an assessment to be made of the acute and chronic effects of nanoparticles. Guidance on the evaluation of risks should include reference to all appropriate metrics. In addition, regardless of the characterisation techniques applied, careful consideration has to be given to sample preparation procedures, equipment limitations, and measurement protocols in order to ensure reliable data are obtained. It is important that further understanding be obtained on the relative contributions that each particle property makes to the various toxicological endpoints.

This Opinion on the appropriateness of risk assessment methodologies described in the current Technical Guidance Documents of the chemicals legislation for the risk assessment of nanomaterials covers separately the Chapters of the Documents on Human Health and on the Environment. In each case, the Opinion provides a context commentary and, in bold, recommendations for the subject matter which should be included in the Technical Guidance Documents in order to address the specific issues related to nanomaterials. In view of the fact that the Technical Guidance Documents are lengthy, covering over 1000 pages, several key points are repeated in different sections of the Opinion in order for them to highlight the specific areas where changes are recommended.

4.1. Chapter 2 Human Health

4.1.1. General Introduction (Section 1)

Section 1 of Chapter 2 of the Technical Guidance Documents provides a general introduction to risk assessment for new substances, being comprised of subsection 1.1, Background, and subsection 1.2 General Principles. There is little need to alter the main text of the Background, since this is applicable to all substances and all aspects of effects on human health. It is recommended that specific reference is made here to some special considerations for the evaluation of nanoparticles in order to highlight the issues associated with nanoparticles at an early stage. It should also be noted that the Technical Guidance Documents contain limited information on substances of a particulate nature, but does not specifically mention nanoparticles. However, it is obvious that in view of their physical nature, particles differ from soluble substances, especially with regard to certain exposure scenarios and toxicokinetics. For nanosized formulations, the chemical structure is the same as for the same chemical in bulk formulation, so both formulations have the same chemical identity. However, at certain sizes, the chemical identification with the corresponding bulk chemical cannot be used for interpretation or extrapolation of the hazards involved to nanosized substances. Specific data need to be generated for nanosized formulations. It is proposed that complementary text dealing with nanostructures is provided in Chapter 1.1, indicating the following:

There are substances that may be released in the environment which have dimensions at the nanoscale. It is generally acknowledged that when substances are formulated at the nanoscale the characteristics may differ from the bulk form. These substances, referred to as nanoparticles, are considered to be those that have at least one dimension at nanoscale, defined as 100 nm or less.

Due to the physico-chemical properties of nanoparticles, their behaviour and their potential adverse effects are not solely dependent on exposure in terms of the mass concentration but are more likely to be governed by the

particle number concentration, surface area and size. In addition, the behaviour of nanoparticles is greatly influenced by their specific physical and chemical properties. These physical and chemical properties, including the size distribution, may change during the time spent in the various compartments of both living organisms and the environment, taking into account phenomena such as solubility, agglomeration and disagglomeration. Furthermore, the size distribution of the substance to be evaluated will greatly influence the final outcome, and for persistent particles the toxic properties may be disproportionately affected by the lower end of the size distribution curve.

In Technical Guidance Documents Section 1.2, General Principles, some of the main features of manufactured nanoparticles should be addressed, such as:

- nanoparticles have to be considered both as discrete particles and in different agglomeration states,
- impurities and the surface layer composition may be very significant,
- the size and size – related features, including size distribution; need careful characterisation,

It follows that a more complex risk assessment procedure may be required for nanoparticles than for traditional chemicals. This will allow the assessment of risks with nanoparticles to be distinguished from those associated with bulk chemicals, and for this to be emphasised. It is proposed that a statement to this effect be included, such as;

Special attention should be given to those manufactured nanoparticles, defined for this purpose as particles which have two or more dimensions measured at 100 nm or less, the preparation and use of which will result in exposure of humans to them. Within this size range it is anticipated that the potential for adverse effects on humans is determined by several factors in addition to chemical composition, and that the determination of risks is a more complex process than with conventional bulk materials. Some substances that may be deemed to be of low risk in bulk form may well have significant risk when in nanoparticulate form. Attention should therefore be paid to factors such as:

- **Physical parameters such as number concentration and surface area are likely to be more significant than mass concentration in the determination of exposure,**
- **Nanoparticles may agglomerate and disagglomerate in different environments, such processes affecting their properties,**
- **Impurities within, and adsorbed species on the surface of, nanoparticles may have significant effects on risks,**
- **Biological processes involving nanoparticles, including translocation, cellular uptake and toxicological mechanisms are still largely unknown and depend on the surface layer.**

It should also be noted that reference materials for the evaluation of nanoparticles have not yet been identified.

4.1.2. Exposure Assessment (Section 2)

In the section on exposure assessment, it is considered important to repeat some key general points that relate to nanoparticles.

In section 2.1, in relation to the general comment that external exposure should be defined as the amount of a substance that is ingested, it is necessary to add the caveat that these parameters (such as the amount of a substance in contact with the skin, or the amount inhaled) may not be the most relevant for nanoparticulate formulations, and that alternative parameters such as particle number concentration, together with the characterised size distribution and surface area, should be used, and that a clear justification for the parameters chosen to express exposure should be provided. Due notice should be paid to the processes of agglomeration and disagglomeration. It is recommended that complementary text such as the following be included:

Due to the physico-chemical properties of nanoparticles, their behaviour is not solely dependent on the mass concentration but is more likely to be governed by the number concentration, surface area and size. In addition the dynamics of nanoparticles are greatly influenced by their specific physical and chemical properties. These characteristics, including the size distribution, may change during the time spent in the various compartments of both living organisms and the environment. The size distribution of the substance to be evaluated may greatly influence the final outcome, in particular as the toxic potency is most likely to be greater at the lower end of the size distribution spectrum.

4.1.2.1. Workplace exposure

In sections 2.2.1 and 2.2.2 of the Technical Guidance Documents, it is important to emphasise that particle number concentration and surface area are important parameters, and especially for particles circulating in air, any adsorbed species on these particles may significantly influence their behaviour.

These factors are also relevant to the section on uncertainties (2.2.2.7) where it should be emphasised that the evaluation of exposure of individuals to nanoparticles is impeded by the difficulty of the routine sampling and of counting or measuring particles in this size range. In addition, the background presence of particles at the nanoscale should be distinguished. It should also be noted in this section that there is an almost complete absence of models that address nanomaterials and very little data on this subject is available. When dealing with the assessment of uncertainties, the size, shape and composition are all important, with special reference to the surface composition and the presence of adsorbed species. The possible agglomeration, dissolution and degradation may all be sources of exposure assessment uncertainty with nanoparticle formulations. Furthermore, the modelling needs to be adjusted to the developments in the protection equipment in relation to nanoparticles.

This information should also be provided in the section 2.2.4 dealing with inhalation exposure assessment and modelling, for example as follows:

In the cases of materials in nanoparticulate form, the biological behaviour is likely to be affected by the adsorption of substances onto the particle surface, when there will be corresponding shifts in chemical and physical properties, the associated surface to volume ratio and the particle shape. In addition, agglomeration of nanoparticles may have an effect on their biological behaviour and need to be taken into account in the further development of models. The presence of background concentrations and their nature should be determined together with workplace exposure levels.

The physico-chemical characteristics of nanoparticles, including their size-mass ratio and surface activity may lead to enhanced migration and biological activity, as noted for example with the possibility of olfactory nerve translocation. There may be interactions between different exposure routes; for example the inhalation of particles, including nanoparticles, may

give rise to ingestion exposure if particles are passed from the lung to the gastrointestinal tract through mucociliary transport. The biological relevance of many of these phenomena is still uncertain for nanoparticles.

Similar considerations are applicable for the opening paragraph of section 2.2.7, dealing with exposure levels taken forward to risk characterisation, where it would be a sensible place to emphasise these important issues concerning nanoparticles.

In section 2.2.3, it is recommended that Figure 1 should contain a statement that reflects the need for information on the physical characteristics of nanoparticles when they are present in a product, including number concentration, surface area, size and size distribution.

The influence of respiratory protective equipment on risk assessment is covered in section 2.2.4.4. There is some information about the effectiveness of such equipment with respect to nanoparticles, but it is suggested that attention should be paid to the future developments in relation to this type of equipment.

With respect to dermal exposure assessment (section 2.2.5), this is very much an open question at this time and it is suggested that close attention is paid to emerging data. As noted earlier, section 2.2.7 is a good place to emphasise the potentially unique situation with nanoparticles. Clearly, section 2.2.7.3, which deals with particle size, is also a suitable place to reinforce the fact that if nanoparticles are involved, there is an absolute need for information on the particle size metrics, including number concentration, surface area, size and size distribution. For the section on biological monitoring (2.2.7.5), it is necessary to point to the limitations posed by current methodologies and the need to develop procedures that will allow an understanding of potential biological effects.

With respect to the criteria that determine the worst cases (2.2.7.8), it is recommended that some strong statement be included to the effect that

Extrapolation from data concerned with bulk chemical or chemical analogues to nanoparticles is not straightforward. Moreover, the simple reduction in size of a given material to nanoscale dimensions will change physical characteristics, and hence the potential for biological effects of the substance. Any extrapolation between nanomaterials also has some difficulties. The appropriateness of the extrapolation must be assessed on a case by case basis.

4.1.2.2. Consumer exposure assessment

The Introduction to the section on consumer exposure assessment (2.3) should also include brief statements concerning the lack of information on nanoparticle-containing consumer products and the procedures for their biological monitoring. In the context of the types of consumer exposure (2.3.3) and routes of exposure (2.3.3.1) the following should be noted:

Exposure routes for nanoparticles are the same as for any other substance, but the relative importance of different routes, and the mechanisms by which these routes are associated with entry into the body, may be different in the case of nanoparticle-containing substances. The use of nanoparticles in, for example, food and medical technologies, is leading to exposure scenarios not previously encountered. The physico-chemical characteristics of nanoparticles, including their size-mass ratio and surface activity, may correlate with enhanced migration and biological activity. There may also be interactions between different exposure routes; for example the inhalation of nanoparticles may give rise to ingestion exposure as the particles are passed from the lung to the gastrointestinal tract

through mucociliary transport. The biological relevance of many of these possibilities is, as yet, uncertain.

Dermal exposure to products that contain nanoparticles, and in particular exposure to consumer products such as cosmetics, textiles, household and personal care products, is of concern and needs to be monitored carefully. It should be noted that although nanoparticles bound to a matrix may not pose a specific risk, some risk may be identified after disposal or destruction of the matrix. Some consumer products are used as sprays in the form of aerosols. In this case the exposure to the substance is due to that of the droplets, which needs to be considered specifically in the exposure scenario of nanoparticles.

In section 2.3.4, concerning data needs and sources, it is suggested that sources of exposure data for nanoparticles are included. With respect to the data required for a realistic quantitative exposure assessment (2.3.4.2), it is suggested that additional parameters are included in the lists that determine the data that is ideally required in the case of nanoparticle products. In particular, in the list of concentration parameters it should be stated that where nanoparticles are involved, data on number concentration, particle size and size distribution, known adsorbed species, solubility or degradability and surface activity should be included. Similarly, these characteristics should be included, as appropriate, in section 2.3.6.5 on the outcome of the quantitative exposure assessment.

It is believed that it would be sensible to make reference in section 2.3.9 on improvements to the exposure assessment that there is a significant need for more data on the exposure assessment for nanoparticles.

Potential human exposure via food should be considered. There is at present a lack of knowledge concerning the persistence and fate of certain nanomaterials within the body. Therefore, if environmental species are exposed to nanomaterials and these persist within their tissues, there is a potential of uptake via food and therefore a potential impact on human health as described in Figure 2.

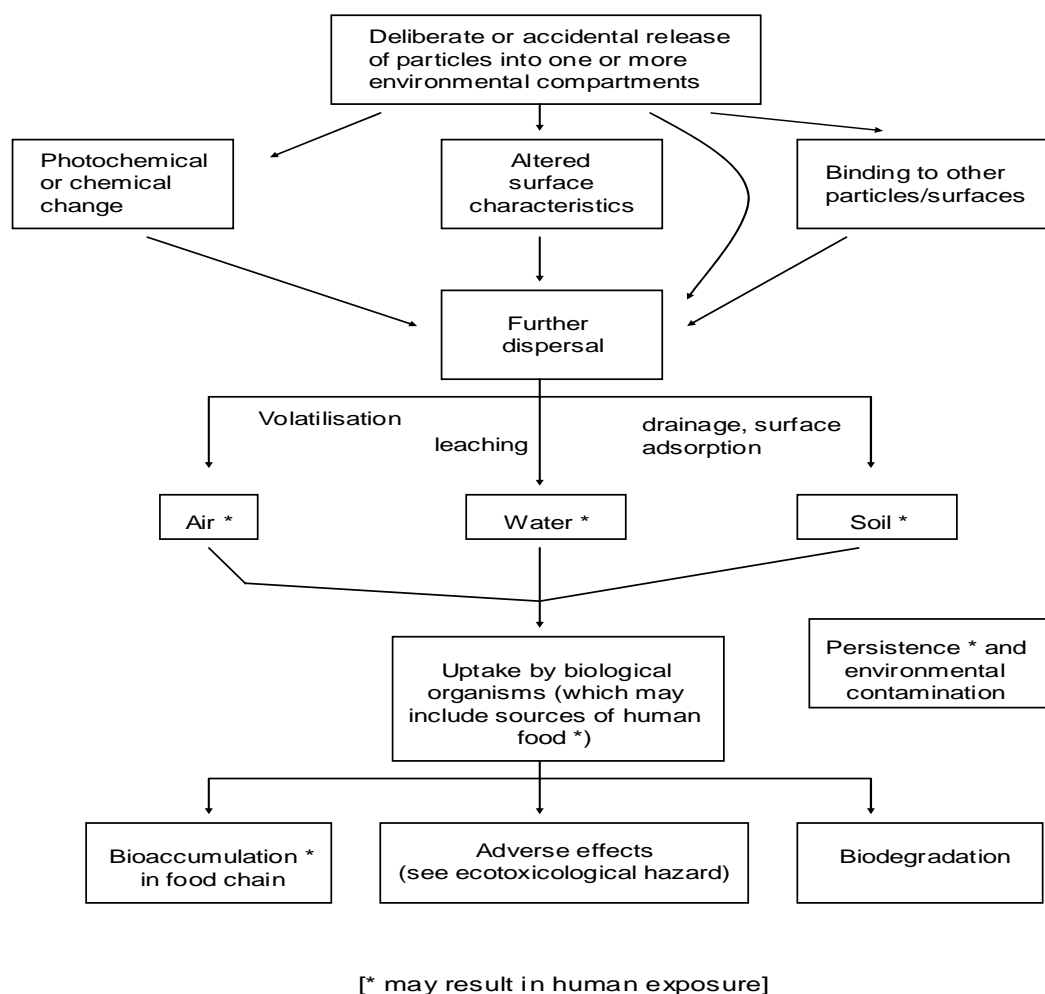


Figure 2. Environmental fate of nanoparticles (SCENIHR 2006)

4.1.3. Effects Assessment (Section 3)

Section 3 of the Technical Guidance Documents deals with the effects assessment. A very important issue arises in relation to the assessment of the effects of nanoparticles in comparison with large particles or bulk chemicals. The essential strategy set out in the Introduction, 3.1, of this section makes it clear that, for chemicals in general, the information to be submitted for registration purposes is dependent on the tonnage produced or imported. In view of the scarcity of data currently available, additional information is needed in order to define the potential risks of nanomaterials. At the moment there is scientific concern and uncertainty regarding the risks from nanomaterials. This may change in the future when more data will become available. Consequently, nanomaterials should be evaluated on a case by case basis.

Furthermore, the characteristics of nanoparticle formulations can change during their life cycle, as the particles pass through the manufacturing or processing phase, during use and subsequent end use, disposal and recycling. These issues should be taken into account in the sampling and testing strategy as well as in the assessment of the reliability of the data (3.1.2.1).

4.1.3.1. Evaluation of data (Section 3.2)

Some general comments about *in vitro* methods may be made.

In vitro testing has not yet been able to obviate the need for animal data in risk assessment. However, *in vitro* testing has provided mechanistic data on particle toxicology and many *in vitro* assays demonstrate convincing differences between low and high toxicity particles. It is therefore considered appropriate that *in vitro* testing is used in situations involving nanoparticles. The large number of nanoparticle variables, related to size, composition and coatings for example, and the ethical pressure against the use of animal experiments, reinforce the desirability that *in-vitro* testing protocols are used with nanoparticles. Short-term *in vitro* testing of nanoparticles has the potential to play an important role in screening procedures and mechanistic studies on nanoparticle toxicology. There is a clear need for validated *in vitro* assays for nanoparticle evaluation, including assays with meaningful endpoints for genotoxicity tests. *In vitro* tests could address key properties of the nanoparticles such as biopersistence, free radical generation, cellular toxicity, cell activation and other generic endpoints. *In vitro* tests could also provide target cell-specific endpoints such as effects on the action potential of nerve cells or the phagocytic capacity of macrophages.

The quantitative structure-activity relationships (QSARS), as applied to certain bulk chemicals, have limitations with respect to nanoparticles and will require specific adaptations for them. The successful application of a QSAR approach necessitates the ability to indicate the toxicity or other properties of a new nanoparticle from its molecular structure and other physico-chemical properties, thus providing information for screening and prioritising. Such QSAR models are plausible, but represent a significant challenge in nanotoxicology. There is general lack of knowledge of which physicochemical properties of nanoparticles are responsible for any specific toxicity. Computational studies need to be supported by appropriate experimental toxicity data, which are still relatively sparse. It should be noted the bulk chemical still provides the ultimate structure activity relationship for the same basic chemical composition; even though difference in toxicity between the larger bulk and nanosized forms have been demonstrated in some cases.

Section 3.2 also refers to the possibility of using route-to-route extrapolation when considering the possible effects of human exposure through one route, where good information exists in relation to another route but no reliable information exists in relation to that route in question. Such extrapolation may be valid under some circumstances, but it should be assumed that they are especially unreliable predictors for nanoparticles and should not be used. Migration of particles is very much dependant on the route of exposure. The possibility of accumulation in organs after prolonged exposure is uncertain and in need of evaluation.

Section 3.2 should reflect these concerns through a commentary such as:

***In vitro* testing has provided mechanistic data on particle toxicology in general and many *in vitro* assays demonstrate convincing differences between low and high toxicity particles; it is therefore considered appropriate that *in vitro* testing is used in situations involving nanoparticles. *In vitro* tests should address key properties of the nanoparticles such as biopersistence, free radical generation, cellular toxicity, cell activation and other generic endpoints and provide target cell-specific endpoints.**

The successful application of a QSAR approach to nanoparticles is dependent on the ability to derive properties of a new nanoparticle from its atomic and molecular structure, thus providing information for screening and prioritising. Such QSAR models are plausible, but represent a

significant challenge in toxicology. In addition, route-to-route extrapolation is particularly difficult with respect to nanoparticles and such extrapolation should be assumed to be poor predictors for nanoparticle toxicology and should not be used.

4.1.3.2. Dose-response assessment

The considerations related to dose response relationship in the toxicology of nanoparticles have been presented in detail in page 32 of the previous SCENIHR Opinion (SCENIHR 2006)). In section 3.4 of the Technical Guidance Documents on dose-response assessment, the basic procedure should be applicable to nanoparticles, but the dose response curve may differ from the one for the conventional chemical due to different toxicological characteristics. The potential for respiratory irritation may be of particular concern in view of the fact that the respiratory route may be the most likely route of exposure.

4.1.3.3. Toxicokinetics

The objectives for investigating toxicokinetics of a substance are given in section 3.5.3 of the Technical Guidance Documents. It is important here to take into account the specific characteristics of nanomaterials that affect the absorption, distribution, metabolism and excretion characteristics (ADME) as well as their half-life and accumulation potential.

In section 3.5.5 concerning the types of studies to be used in risk assessment, it is recommended that the ICRP (International Commission for Radiological Protection) model for nanoparticles is added and that an assessment of the validity of the proposed *in vitro* systems for nanomaterials are added. There are concerns related to cell fractions, purified enzymes, reconstituted systems and recombinant enzymes. Due care must be given to characterising and describing nanoparticles appropriately, as there are likely to be considerable differences in nanotechnology products from one supplier to another and from one batch to another from the same manufacture.

With inhalation studies, consideration should be given to the potential for transfer to the brain and to the blood. Inhalation studies should take into account the fact that nanoparticles with large surface area may rapidly cause saturation of lung clearance. It is generally crucial for risk assessment of nanoparticles to determine the precise tissue distribution profile as there is so little information on translocation,. Also specific comments on nanoparticle metabolism and excretion are required, taking into consideration the limits of detection. However, if particles are rapidly dissolved, data on kinetics of dissolution products can be used.

In section 3.5.8 on physiologically based pharmacokinetic (PBPK) modelling it will be necessary to emphasise that there are no data yet available for PBPK modelling for nanoparticles and consequently, it will be very difficult to carry this out.

4.1.3.4. Acute toxicity (Section 3.5)

A general problem with the existing toxicity testing methods is that the exposure levels at which biological responses to nanomaterials are seen may differ considerably from those of conventional substances. Therefore the dosage triggers recommended in the minimum data requirements of Section 3.6.2.1 may be inappropriate for nanoparticles and need to be specified separately. For example, the current threshold values for LD50 oral should be applied as trigger values for inhalation and dermal testing for nanomaterials. For biocides in nanoscale form, special inhalation studies are needed, as well as a determination of an appropriate new trigger dosage. Furthermore, it is important to evaluate whether the conditions of inhalation toxicity testing are appropriate and whether they should be defined separately for nanoparticles. There may be large species differences in deposition in the

respiratory tract between humans and rodents and inhalation test systems must be appropriate for any species. It also has to be said that the existing animal tests may not be sensitive enough to detect all possible adverse effects of nanoparticles.

Section 3.5 should contain a general statement about the use of conventional acute toxicity tests for nanoparticles to the effect that;

Acute toxicity testing will have a role in the effects assessment of nanoparticles, but the application of such tests in this area should reflect the fact that the exposure levels at which biological responses to nanomaterials may differ considerably from those of conventional substances, such that traditional dosage triggers may be inappropriate. The tests used should also take into account the possibility of large species differences and of inadequate sensitivity to detect all possible adverse effects of nanoparticles.

4.1.3.5. Irritation and corrosivity (Section 3.6)

In the objectives of investigating the potential for substance-induced irritation and corrosion 3.7.1.23, it is realized that for nanoparticles not only the chemical characteristics but the physical characteristics of the powder might be responsible for irritation.

4.1.3.6. Sensitisation (Section 3.7)

Indications for the potential of nanoparticles to induce hypersensitivity may at first be based on the results obtained with the normal chemical formulation. Whether nanoscale powder formulations induce hypersensitisation after skin exposure will depend on the possibility to cross the skin barrier and to interact with proteins. The release of substances from the nanoparticles may also occur. Protein interactions are essential for sensitization, as chemicals generally are not recognised by the immune system. The binding of a substance to a protein may result in the formation of a hapten, whose structure may be recognised by the immune system, resulting in hypersensitivity. Nanoparticles may also have an adjuvant effect in relation to the sensitisation to other substances or proteins (Alessandrini et al, 2006).

4.1.3.7. Repeated dose toxicity (Section 3.8)

With respect to the requirement for repeated dose toxicity testing of chemicals, it is possible that dosages triggers may not apply to nanoparticles. Repeated dose toxicity is potentially an important endpoint in some situations and it will be necessary to develop criteria for the assessment of nanoparticle toxicity under these conditions. Concerning the considerations for initial 28 or 90 days toxicity testing, the potential migration accumulation and clearance of nanoparticles in primary organs of intake and in secondary target organs should be mentioned and also considered under 3.8.6.5 for immediate further testing.

The special characteristics of nanoparticles should be considered when investigating their potential neurotoxicity, immunotoxicity and cardiovascular effects. It will be necessary to evaluate whether the oral 28-day and 90-day tests are adequate for nanoparticles; the potential need for further testing of nanoparticles with respect to neurotoxicity should be mentioned in Table 5 (Methods for investigating neurotoxicity). Increased pro-inflammatory activity and induction of cytokines and other mediators of inflammatory reaction (Donaldson et al. 2005) highlight the need for special studies on nanoparticles in this respect. These should also include the specific effects of nanoparticles on thrombosis and the cardiovascular system (Khandoga et al. 2004)

Overload and pulmonary fibrosis are now known to be significant issues in nanoparticle toxicology, being driven by the surface area dose in the lung. This has major ramifications for nanoparticles, with their high surface area per unit mass. The available data on insoluble dusts indicate that in the workplace overload-related effects can be avoided by maintaining the atmospheric concentration of the substance below the specific gravity value of the substance expressed as $\text{mg}\cdot\text{m}^{-3}$ (i.e. the atmospheric concentration should be $<1.6 \text{ mg}\cdot\text{m}^{-3}$ for a substance with a specific gravity of 1.6).

Bronchiolavage, a sensitive measure of lung inflammation should be used to detect inflammatory effects, and histology should also be used in case there is excessive interstitial inflammation, which is possibly not well measured by bronchiolavage. There is limited evidence that nanoparticles can translocate from the lungs to the blood and the brain, and routine assays for the monitoring of blood and brain transfer of nanoparticles, and their consequences, are required. For blood, markers of thrombosis and atherogenesis need to be considered and potential degenerative effects and oxidative stress on the brain should be assessed.

Section 3.8 should reflect the potential importance of, but uncertainties with, the use of repeated dose toxicity testing of nanoparticles, indicating the potential for such studies to detect migration of particles to organs but also emphasising that the toxicokinetics is the most important factor and should be the main driver of the need for these studies, this need being determined on a case-by-case basis.

4.1.3.8. Mutagenicity (Section 3.10)

There is reason to believe that any mutagenicity or genotoxicity shown by nanoparticles may be detected using currently available protocols. However, there are several uncertainties involved in the testing procedures.

Care needs to be taken in dispersal to mimic in the test systems the state of agglomeration that the lung experiences for that material, and the types of dispersants used. Nanoparticles may themselves prove more mobile within the cell than conventional particles and so might enter 'privileged' compartments such as the nucleus or the mitochondria, and so could enhance mutagenesis. However the final pathways of mutagenicity are likely to involve the production of adduct-forming reactive organic, oxygen or nitrogen species by the particles themselves, or the inflammatory cells induced by them, or interactions between particles and DNA, with or without the involvement of the cytochrome P450 system.

In relation to risk assessment, it is necessary to be very cautious about the interpretation and extrapolation of genotoxicity data obtained with nanoparticles, especially with *in vitro* investigations. Nanoparticles have complex physicochemical properties that can modulate their biological activities in comparison to soluble chemicals. Considerable mechanistic and dose discrepancies exist between *in vitro* studies and *in vivo* genotoxicity or carcinogenicity studies. Typically, most available *in vivo* studies have been performed at high particle concentrations and/or long term exposures, which are associated with marked inflammatory and proliferative responses, and hence may obscure or modify genotoxicity readouts.

It is unknown whether bacterial assays are valid to detect any mutagenic effects of nanoparticles due to uncertainties in uptake. Currently available information suggests that any direct genotoxic effects of nanoparticles would be detected by common mutagenicity and genotoxicity tests using mammalian cells. However, mechanisms that give rise to DNA damage may differ from those of conventional substances. There is some data on the effects of nanoparticles on subcellular function, for example, mitochondrial impairment (Li et al. 2003) and the induction of aberrant clusters of topoisomerase I in the nucleoplasm of cellular systems (Chen and von Mickecz 2005), suggesting that novel pathways might exist for nanoparticles.

In general it should be clarified whether the existing tests are sufficient to detect the mutagenicity of nanoparticles, taking into account that nanoparticles may impact on multiple subcellular systems, which are not always assessed in the tests that address mutagenicity potential.

Section 3.10 should mention the uncertainties involved with the testing for mutagenicity, for example;

Although currently available protocols for the assessment of mutagenicity or genotoxicity should be applicable for nanoparticles, several uncertainties may be identified and have to be taken into account. These include the need for experimental conditions to mimic the conditions of human exposure and the possible dispersion of nanoparticles into privileged compartments, such as the mitochondria and nuclei, and the need to assess effects on multiple subcellular systems.

4.1.3.9. Carcinogenicity (Section 3.11)

Inhaled low toxicity and low solubility particles, including nanoparticles, appear to induce lung tumours in rodent models by mechanisms similar to those found with fine particles. These mechanisms include DNA damage and increased cell proliferation, associated with a persistent irritation and chronic inflammation in the lung. The metric driving this response is still unclear but surface area has the strongest support from toxicological evidence. However, the high surface area dose of nanoparticles may mean that rat lung overload is likely to be a powerful confounding issue in these tests. Simply based on their higher surface area, nanoparticles may have a stronger potency to induce lung tumours. No increase in extrapulmonary tumours has been seen in inhalation studies, but little information is available from chronic nanoparticle administration. Current animal testing methodology is believed to be sufficient to detect the carcinogenic hazard of nanoparticles.

It is suggested that Section 3.11 be modified to include a statement to the effect that;

Inhaled low toxicity and low solubility nanoparticles, appear to induce lung tumours in rodent models by mechanisms similar to those found with fine particles. Simply based on their higher surface area, nanoparticles may have a stronger potency to induce lung tumours than larger particles. Current animal testing methodology is believed to be sufficient to detect the carcinogenic hazard of nanoparticles, but it is unclear whether effects in the rat model are species specific and whether they can be extrapolated to the human situation. It is also unclear whether, or to what extent, it is possible to extrapolate from bulk form of conventional chemicals to nanoparticles of the same material with respect to carcinogenicity. In view of the special characteristics of nanoparticles, a case-by-case approach needs to be applied until appropriate extrapolation methods become available for nanoparticles

4.1.3.10. Reproductive toxicity (Section 3.12)

It can be assumed that the risk assessment methodology for reproductive toxicity as described in the Technical Guidance Documents would apply for nanoparticles, but the OECD guidelines and testing methods used may need to be adapted for this evaluation. The occurrence of developmental toxicity with nanoparticles is dependent on their potential to migrate into the foetus and on placental functioning. So far no specific studies appear to have been published, but the presence of consumer products containing nanoparticles implies that such data should be obtainable.

4.1.4. Risk characterisation (Section 4)

The previous SCENIHR Opinion, p 54 (SCENIHR 2006) noted that in the absence of suitable hazard data, a precautionary approach may need to be adopted for those nanoparticles that are likely to be biopersistent in humans and/or in environmental species. Furthermore, the fact that there is no reliable information on the effect of simultaneous exposure to multiple forms of nanoparticles, where it would be appropriate to assume the effects are additive, or on the interaction between nanoparticles and other stressors, indicates the need for rigorous exposure assessments and also implies that risk characterisation should be considered on a case-by-case basis.

The general aspects of the risk characterisation in section 4.1 need to be extended to cover the whole life-cycle and its phases. The susceptible subpopulations, including the unborn embryo, and those with pre-existing chronic diseases such as cardiovascular and immune diseases need to be taken into account. The threshold values based on mass metrics needs to be complemented by metrics representing more appropriate specific characteristics, including the surface reactivity of nanoparticles. The study design in various testing phases should take these also into account and strive for minimisation of the time required for testing. Moreover, special studies need to be conducted with respect to neurological, immunological and cardiovascular effects of nanoparticles.

4.2. Chapter 3 Environment

4.2.1. Section 1 General Introduction

The Chapter of the Technical Guidance Documents on environmental risk assessment focuses on the release, distribution, fate and hazards of chemicals in the environment. The methods proposed (especially regarding exposure) in this Chapter were initially developed for organic substances, although they have since been adapted to other chemicals. However, these procedures and methods may not be sufficient for the appropriate assessment of potential environmental risks of nanoparticles. Many of the issues mentioned in the comments on human health risk assessment also apply to environmental risk assessment. In particular, these include aspects on characterisation and monitoring of nanoparticles. Nevertheless, it is clear that fate and behaviour in the different environmental media and the food chain will need to be considered and assessed.

Natural, accidentally produced and manufactured nanoparticles, are already present in the environment. They show a range of physico-chemical properties which may be extended by the increasing release of manufactured nanoparticles. However, not all the key physico-chemical characteristics with potential relevance to nanoparticle toxicology are addressed in the Technical Guidance Documents. It is noted that particles in general, although without any indication of a specified size, are mentioned briefly in a few sections of Chapter 3. For example, particles are discussed in the context of waste treatment (by inference only), suspended matter in aqueous media, products of weathering or abrasion, and in the context of the calculation of wet and dry deposition rates from air and substances adsorbed onto particles.

It is recommended that reference is made in section 1.2 of Chapter 3 to nanoparticles and to their specific physico-chemical characteristics, including the importance of the expression of the release, fate, exposure and dose in particle number concentrations and surface area. Nanoparticles may change their status (e.g. agglomerate) when exposed to different environmental conditions such as pH, salinity and the adsorption of organic and inorganic matter, and therefore their uptake and effects could vary. Reference to this must be made within chapter 3. The criteria used for persistence and for PBT (persistence, bioaccumulation and toxicity) assessment applied for substances in soluble form may need to be reassessed. In particular, the persistence or accumulation of non-degradable nanoparticles may result in prolonged exposure, supporting the need for chronic testing.

It is specifically recommended that the following aspect should be included in this Chapter:

There are substances that may be released into the environment which have dimensions at the nanoscale. It is generally acknowledged that when substances are formulated at the nanoscale the characteristics may differ from the bulk form. These substances, referred to as nanoparticles, are considered to be those that have at least two dimensions at nanoscale, defined as 100 nm or less.

Due to the physico-chemical properties of nanoparticles, their behaviour and their potential adverse effects are not solely dependent on exposure in terms of the mass concentration but are more likely to be governed by the particle number concentration, surface area and size. In addition the dynamics of nanoparticles are greatly influenced by their specific physical and chemical properties. These physical and chemical properties, including the size distribution, may change during the time spent in the various compartments of both living organisms and the environment. Furthermore, the size distribution of the substance to be evaluated will greatly influence the final outcome and for persistent particles, the toxic properties may be disproportionately affected by the lower end of the size distribution curve. The criteria used for persistence, bioaccumulation and toxicity (PBT) assessment applied for substances in soluble form should be assessed for applicability to nanoparticles.

4.2.2. Environmental exposure assessment (Section 2)

There have been very few studies focussing on the behaviour of nanoparticles in the environment (SCENIHR 2006). Those studies that have been carried out predominantly involve inorganic substances. The distribution and persistence of nanoparticles in the environment may differ from substances in both the liquid and gas forms, and in the form of larger particles. Due to the lack of systematic studies, no general rules can be identified that govern the dispersal and distribution in the environment of nanoparticles of differing chemical compositions and differing size range.

In section 2.1.1 of the Technical Guidance Documents, it is mentioned that usually no measured environmental concentration will be available for new substances. In the absence of exposure data, model calculations are therefore generally used. In order to perform the model calculations as described in the Documents, estimations for the release of the volume and the number of nanoparticles into the environment should be provided, taking into account the current databases and estimation models.

In relation to section 2.3.3, it is unclear whether existing emission factors for relating production and environmental release, as currently used for bulk substances, will apply to nanoparticles, although it is suggested that they may be used as a rough approximation. It is not clear at this stage how predicted environmental concentrations (PEC) for nanoparticles can be calculated. However, the current emission factors used in the PEC estimations probably would not apply to nanoparticles. It is recommended that a modified or new approach should be developed for this. It is specifically suggested that this section of the Technical Guidance Documents should indicate the following:

For the estimation of the environmental concentrations due to emissions of nanoparticles during production, very limited information is currently available and the applicability of models and emission factors for this estimation to engineered nanoparticles needs to be assessed.

As with the situation of human health exposure, the exposure expressed solely in mass concentration is not appropriate for the environment where the number of particles and/or

surface area may also be more relevant. It is recommended that the following information is included in this section:

Special attention has to be given to substances in the form of nanoparticles that result in exposure of the environment to nanoparticles, which are defined for this purpose as particles which have two or more dimensions measured at 100 nm or less. Within this size range it is anticipated that the potential for adverse effects to the environment is determined by several factors in addition to chemical composition, and that the determination of such risks is a more complex process than with conventional bulk materials. Some substances that may be deemed to be of low risk in bulk form may well have significant risk when in nanoparticulate form and attention should be paid to factors such as:

In the determination of exposure, physical parameters such as number concentration and surface area are likely to be more significant than mass concentration,

Nanoparticles may agglomerate and disagglomerate in different environments, affecting their overall properties,

Impurities within, and adsorbed species on the surface of, nanoparticles may have significant effects,

Biological processes involving nanoparticles within the environment, including translocation, cellular uptake and toxicological mechanisms within relevant species are still largely unknown and depend on the detailed behaviour of the surface layer.

It should also be noted that reference materials for the evaluation of nanoparticles are not yet available.

The commonly used mathematical models of dispersal of vapour and large particulate matter will need adaptation for the assessment of the environmental distribution and dispersal of nanoparticles. This implies incorporation into the models of the key physico-chemical characteristics relevant to nanoparticles such as surface area and morphology; charge, number of particles, size, solubility and potential chemical and physical conversion into other forms, as described earlier. These factors should be considered and introduced in the framework of the calculation of PECs/PNECs for manufactured nanoparticles in the section of 2.3.8. In the event that no data are available to calculate PECs/PNECs, the best possible scientifically based estimates have to be used.

In the section 2.3.7 concerning waste disposal and recycling, some text should be included to point to the fact that the fate (e.g. dissolution, disagglomeration) of nanoparticles in the environment will depend on the actual environmental conditions such as pH, salinity and the adsorption of organic and inorganic matter. For any resuspension of nanoparticles into air, although not expected to occur widely, it should be recognised in this section that their properties will be quite different from that of larger particles.

In the section on biotic and abiotic degradation rates (section 2.3.6) the solubility and the potential for persistence of nanoparticles need to be mentioned for each system.

There may be certain areas where mass weight is appropriate for the expression of the exposure or dose of nanoparticles. However, criteria for when it is appropriate to use concentration or weight data for particulate matter, and when particle numbers should be used instead need to be identified.

4.2.3. Effect assessment (Section 3)

In relating the exposure of nanoparticles to their effects, the traditional use of mass or mass per unit volume parameters alone may not be appropriate. Surface area and/or particle number per volume should be considered in addition to mass. Understanding of behaviour of nanomaterials in the exposure medium is very important in any effect assessments.

As described in the Technical Guidance Documents, the effects assessment comprises two steps, hazard identification and dose/concentration – response/effect assessment. The first of these aims to identify the effects of concern, whereas the second relies on the determination of the predicted no effect concentration (PNEC).

PNEC is defined as a concentration below which an unacceptable effect is unlikely to occur. It is normally calculated by dividing the lowest short term L(E)C₅₀ (lethal/effect median) or long term NOEC (no observed effects concentration) by an appropriate assessment factor. These factors reflect the uncertainty in extrapolation from laboratory toxicity data for a limited range of species to the wider environment. Paucity of data would imply the use of larger safety factors.

Furthermore, so that environmental risk assessments can be conducted, it is important to calculate PNECs for nanoparticles across a variety of environmental compartments, including aquatic and soil compartments. The use of the equilibrium partitioning method for establishing a PNEC for the sediment and soil compartments is probably not suitable for use with nanoparticles, bearing in mind that this method was developed for soluble or solvable organic substances.

In the derivation of PNEC for aquatic systems, section 3.3.1 recommends the use of assessment factors, which are more stringent if only short-term ecotoxicity data are available. Long-term tests are, however, recommended for the derivation of PNEC, following standard methodologies. Nevertheless, it is unclear if these tests would be the most appropriate for delayed toxic effects especially in the context of low reactivity, high persistence inorganic nanoparticles.

It is recognised in this section that no data are available for many substances within the soil / sediment compartment. For sediment/soil -dwelling organisms, it is then recommended that the equilibrium partitioning method is used as a screening method for the derivation of a PNEC_{sed/soil}. Section 3.5.2. refers to the calculation of log K_{oc} or log K_{ow} to provide an indication of effects assessment in the sediment/soil compartment, suggesting that a value above 3 for either of those coefficients may be used as a trigger value for sediment effects assessment.

It is not known whether the extrapolation from laboratory toxicity data to a PNEC value is valid for nanoparticles. Therefore PNECs need to be assessed for a range of nanomaterials. There is little data regarding short and long term exposure effects across a range of species and media, including reproductive toxicity and genotoxicity and it is unclear if the currently used assessment factors would be applicable. Although there is no evidence that nanoparticles will have endocrine disrupting effects, this should also be investigated.

Although the extrapolation between substances (so called analogues) is commonly used in bulk chemical (eco)toxicology (the QSARs), it is premature to apply this to nanoparticles since current knowledge on the main characteristics determining the environmental fate and effects of nanoparticles is too limited to enable a simple classification of nanoparticles to be developed for environmental risk assessment purposes.

Section 3 on the Effects Assessment should contain a paragraph that reflects these general uncertainties, such as;

In order that environmental risk assessments can be conducted, it is important to calculate PNECs for nanoparticles across a variety of environmental compartments. However, the use of the equilibrium partitioning method for establishing a PNEC for the sediment and soil compartments is probably not suitable for use with nanoparticles. It is also unclear whether long-term tests are the most appropriate, nor is it known whether the extrapolation from laboratory toxicity data to a PNEC value is valid for nanoparticles. There is, in fact, little data regarding short and long term exposure effects across a range of species and media, including reproductive toxicity and genotoxicity, and it is impossible to say if the currently used assessment factors would be applicable to nanoparticles in the environment. It is also premature to apply the QSARs approach to nanoparticles since current knowledge on the main characteristics determining the environmental fate and effects of nanoparticles is too limited to enable a simple classification of nanoparticles to be developed for environmental risk assessment purposes.

4.2.4. *Bioavailability*

The uptake, distribution, clearance and elimination of nanoparticles may differ from those of the chemical substances for which the Technical Guidance Documents were initially developed and it is uncertain whether the base set of species selected for ecotoxicity testing is sufficient for the testing of nanoparticles. As there is no information on how nanoparticles behave in the various environmental compartments, it is also unclear what the main exposure and uptake routes may be for different species. Additionally, there is lack of information regarding species sensitivity towards nanoparticles. Therefore, at present, no clear guidance can be given on the appropriateness of the key standard test taxa and recommended procedures to assess adequately the effects of nanoparticles on the various environmental compartments. There may be a need for new standardized ecotoxicity tests for nanoparticles.

Several mechanisms are available to enable organisms to take up particulate matter. For example, many micro-organisms have the ability to carry out pinocytosis and/or phagocytosis and many aquatic multi-cellular organisms are selective or non-selective filter feeders, feeding on particles of various sizes. The upper limits of particles size for these processes have been identified in various organisms. However, the uptake of nanoparticles is not very well understood in relation to these mechanisms and this should be emphasized. Ranges are reasonably well defined for some taxa (e.g. *Daphnia*, bivalve molluscs), although it is important to note that there are both active and passive mechanisms for the uptake of nanoparticles, both of which may be influenced by aggregation.

This section should therefore also reflect the current status of knowledge of bioavailability, such as;

There is no information on how nanoparticles behave in the various environmental compartments, and it is unclear what the main exposure and uptake routes may be for different species. There is lack of information regarding species sensitivity towards nanoparticles. Therefore, no clear guidance can be given on the appropriateness of the key standard test taxa and recommended procedures to assess adequately the effects of nanoparticles on the various environmental compartments.

4.2.5. *Bioconcentration and bioaccumulation*

One way to assess the risk for bioaccumulation of a substance in aquatic species is to measure the Bioconcentration Factor (BCF). The static bioconcentration factor is the ratio between the concentration in the organism and the concentration in water in a steady-state,

or equilibrium, situation. When uptake and depuration kinetics are measured, the dynamic bioconcentration factor can be calculated from the quotient of the uptake and depuration rate constants.

The food chain transfer of nanoparticles should be assessed in the light of the fact that the conventional $\log K_{ow}$ (log of partition coefficient octanol-water) and BCF (bioconcentration factor) assumptions as presently proposed may not hold for nanoparticles. It is suggested in the Technical Guidance Documents that the simplest way to estimate the potential of a substance to bioaccumulate in environmental species is through experimental measurement of BCF. However, these data are not often available, and nor do they provide the full picture, since information on uptake and depuration kinetics, as well as metabolism and other factors, must also be considered in the full assessment of the bioaccumulation potential. These data are even less likely to be available for a wide range of chemicals, so that assessments based on physico-chemical properties and structural parameters are normally used in standard risk assessments.

It is important to stress that the procedures recommended within the Technical Guidance Documents for the assessment of bioaccumulation potential, including the derivation of BMF (biomagnification factors) for top predators (section 4.3), have not been tested for nanoparticles and that adaptations or changes to these procedures will be required.

For surface active substances, the Documents suggests that it may not be advisable to use estimated or measured K_{ow} values as a predictor for K_{oc} (organic carbon partition, soil, sediment, suspended organic matter and sludge) and BCF (fish, worm) because the predictive value of $\log K_{ow}$ for such estimations may be too low. It suggests, for example, that for surfactants it may be appropriate to obtain measured K_p (solid-water partition coefficient) and BCF values. This may also be relevant to nanoparticles.

4.2.6. Environmental risk assessment (Section 4)

The assessment of the risks of substance for marine environments is generally performed using approaches similar to those used for the freshwater compartment, although a number of factors and methods are specific to the marine environment.

The general comments concerning environmental compartments, including the marine environment, are also applicable to nanoparticles. In Section 4 a methodology for the assessment of persistent, bioaccumulative and toxic (PBT) substances is proposed. Although this type of assessment should not be considered a risk assessment, it does provide key information on the fate and effects of the substance. It is not certain if the procedures and thresholds recommended are applicable to nanomaterials, and it is anticipated that criteria for the identification of PBT substances should be assessed for their applicability for nanoparticles. Some comment to this effect should therefore be introduced;

It should be noted that there exists not only the potential for persistence of the nanoparticles themselves in the environment, but also residual persistence of the substance after the degradation of the particle. The methodology should take this into account. The appropriate metrics that best describe the dose response relationship should be also be used to describe PBT

Section 4.4.2. gives the criteria to be used in the assessment of either PBT or vPvB (very persistent and very bioaccumulating substances). These criteria focus on the derivation of half-life, BCF and NOEC, parameters, whose applicability to nanomaterials has already been discussed. In section 4.4.5.1. it is recommended that for persistent and bioaccumulative substances, long-term exposure should be considered and this should cover the whole lifetime of an organism, and even multiple generations where possible. Therefore chronic or long-term ecotoxicity data, ideally covering the reproductive stages, should be used for the assessment of the toxicity criterion. It is recognised that for many chemicals, including

most nanomaterials, the principal data will concern short-term effects, and these would be used in the initial classification. The Technical Guidance Documents also state that mammalian toxicity data must be considered, due to the fact that toxic effects on top predators, including man, may occur through long-term exposure via the food-chain; this principle should also apply to nanomaterials.

The current practice of characterising the risk of a substance with respect to each environmental compartment by comparing the Predicted Environmental Concentration (PEC) to the Predicted No Effect Concentration (PNEC) may be applicable to nanoparticles, but care will need to be taken to ensure that both values are expressed in the same units. These units may not be conventional concentrations (mass/volume) but may need to be expressed in alternative units such as surface area and/or particle number per unit of environmental volume or surface.

Although current regulatory frameworks focus on the risks of individual substances, the fact that nanoparticles have the ability to adsorb other, potentially toxic, substances onto their surface and as such pose an additional risk to the environment should also be considered. This issue is recognised in the current Technical Guidance Documents, which state that 'most of the substances may be associated to particles or aerosol and the real atmospheric half-life could be orders of magnitude higher'. The extent to which the various nanoparticles can adsorb other chemicals and thereby affect the fate and hazard of these chemicals and the environmental risks caused by this association should be considered in the risk assessment procedures. In addition, the uptake of nanoparticles could be enhanced by their adsorption to other chemicals. It should also be noted that when discussing biopersistence of nanoparticles, there exists not only the potential for persistence of the nanoparticles themselves, but also the residual persistence of the substance after the degradation of the particle.

Having conducted the exposure assessment and the dose (concentration) - response (effect) assessment for all environmental compartments, either a quantitative or qualitative risk characterisation has to be carried out. A quantitative risk characterisation is performed by separately comparing PEC with PNEC for each relevant environmental compartment. The full procedure can be followed if PECs and PNECs can be calculated with confidence. As described above, these are not available at present, therefore negating the possibility of a full quantitative risk characterisation as presently defined.

If no quantitative risk characterisation can be pursued, it is recommended that a qualitative risk characterisation be conducted. This would involve PBT assessment, which, as described, cannot be fully followed at present.

Given the lack of information and data on a range of issues, it is suggested that strategies for the assessment of hazard be prioritised following section 6 in the Testing Strategies. Here strategies for PEC and PNEC are given which may be followed when the risk characterisation phase (section 5) concludes that there is a concern and when there is a need to ask for further information to refine the risk assessment. There is no reason why the tests recommended in this section could not be adapted to nanomaterials.

4.3. Conclusions and Recommendations

The nanostructure-dependent physical and chemical properties of many engineered nanoparticles may place them in the category of potential hazards. The direct risk that nanoparticles present to human health and to the environment will depend on the physicochemical characteristics of the surface and core of nanoparticles, on the probability of exposure occurring during each stage of their life cycle, and the extent to which particulate materials exhibit interactions with biological systems associated with their nanostructure. Neither the rate-determining parameters of toxicokinetics for nanoparticles

nor the underlying mechanisms are known. Importantly, these uncertainties mean that methodologies to permit extrapolation between different types of nanoparticles and different species are not available, implying that assessments often have to be made on a case – by – case basis.

In relation to the Technical Guidance Documents, a number of general conclusions and specific recommendations can be made concerning their applicability to nanoparticles and changes that need to be introduced. Specific recommendations for the introduction of new explanatory paragraphs of the various sections in the Technical Guidance Documents have been included in the two previous sections of Chapter 4 of this Opinion. These more general conclusions are summarised in the following two sections on human health and the environment. This is then followed by a detailed recommendation for a staged or tiered approach to the assessment of the human and environmental risks from nanoparticles. Finally the specific answers to the questions posed in the Terms of Reference are provided.

4.3.1. Conclusions on Human Health Chapter

In general, the current methodologies described in the Technical Guidance Documents are likely to be able to identify the hazards to human health associated with the use of nanoparticles. For the determination of dose – response relationships, special attention should be given to the expression of the metrics of the nanoparticle dose. Mass concentration does not seem to be the best description of dose for these materials. Number concentration and surface area are likely to be a better description of the dose response relationship. However, exposure measurement with the use of current methodologies for hazard identification of nanoparticles is limited, the main focus being the lung, and the effects determined after inhalation exposure. During the large scale production of nanoparticles the major risk for exposure is the inhalation route. In this respect the handling procedures of nanoparticles may also pose a high risk for exposure.

Importantly, there is evidence that nanoparticles may cross the blood – brain barrier under some circumstances that they may be associated with long term inflammation in several different types of tissue and organ and may be associated with cardiovascular effects. Although this data is still limited, these possibilities have to be taken into account. Similarly, the available evidence suggests that certain subpopulations, particularly those with pre-existing disease such as asthma and cardiovascular disease may be more susceptible to the adverse effects of nanoparticles, which again should be considered in the assessment of human health hazards

Not all nanoparticle formulations have been found to induce a more pronounced toxicity than the bulk formulations of the same substance. This suggests that the evaluation of nanoparticle formulations should be carried out on a case by case basis. Certain effects of nanoparticles in *in vitro* systems, for example radical oxygen production, have been demonstrated to occur also in *in vivo* inhalation experiments. It is important that it is determined whether such *in vitro* activity will be predictive for human health hazards for all types of nanoparticles.

Special attention should be given to the changes in the nanoparticle physico-chemical characteristics that may occur under local environmental conditions. Such changes may include, but are not limited to agglomeration, dissociation and adsorption of environmental substances, all of which may have an impact on the ultimate toxicity of the nanoparticles. Such alterations to the nanoparticles may be difficult or even impossible to measure under the experimental conditions used. The expression of exposure (dose) would then be based on the description of the nanoparticle as it is produced and initially released.

4.3.2. Conclusions on the Environment Chapter

In the absence of sufficient data on the fate and effect of nanoparticles on the environment it is neither feasible nor appropriate to propose firm rules on how substances in nanoparticle form should be evaluated. Instead, key issues need to be identified for consideration in the risk assessment. The more important issues are as follows.

The validity and appropriateness of the PBT criteria and methodologies for use with nanoparticles needs to be assessed.

Since there is at present no information on the validity of the use of current exposure models, their validity should be assessed and, if necessary, new models and methods should be developed for the prediction of the PEC for nanoparticles in all relevant environmental compartments. One of the main problems encountered in the testing of the ecotoxicity of nanoparticles has been the lack of appropriate standardised protocols. The environmental effects of nanoparticles should be evaluated through the establishment of typical scenarios reflecting their production and use. Appropriate information on the kinetics of the particulate phase and the environmental concentrations, as well as on the effect concentrations, should be obtained in order to follow the traditional approach of environmental risk assessment. This depends on the ratio between predicted environmental concentration (PEC) and predicted no-effect concentration (PNEC).

For certain nanoparticles the exposure and dose-effect models may need to be adapted, taking into account their changing physico-chemical properties over time, including their slow degradation.

In relating exposure dose concentration of nanoparticles to their effects, the traditional use of mass or mass per unit volume alone is unlikely to be appropriate. Surface area and/or particle number per volume in addition to mass should be considered. Additionally, the uptake, distribution, clearance and effects of nanoparticles may differ from those of the substances for which the Technical Guidance Documents were initially developed. From this and the lack of information regarding species sensitivities towards nanoparticles, it is concluded that at present no clear guidance can be given on the appropriateness of the key standard test taxa and recommended procedures to assess adequately the effects of nanoparticles on the various environmental compartments.

Environmental toxicity studies will require both acute and chronic exposures, using standard laboratory species, focussing on the identification of pertinent endpoints specifically relevant for nanoparticles. The route of exposure to nanoparticles is likely to have a bearing on the uptake by biota in the environment and on the resulting toxicity. To perform these ecotoxicological studies, there is a need for a panel of well-characterised reference nanoparticles.

Risk characterisation methodology recommended in the Technical Guidance Documents can be followed for nanoparticles, if and only if PECs and PNECs can be calculated with confidence. These are not generally available at present, negating the possibility of a full quantitative risk characterisation as presently required and defined in the Technical Guidance Document.

4.3.3. A staged approach to the assessment of the human and environmental risks from nanomaterials.

Risk assessment requires data on exposure and hazard. It is noted that irrespective of the exposure, a hazard assessment is needed for the purposes of classification and labelling of a substance, including those that involve engineered nanoparticles.

It is recommended that a tiered approach is developed in order to set out a rationale framework for assessing the potential risks from engineered nanoparticles. The intention is to produce a scientifically valid, cost-effective framework that enables a scientific judgement to be made on the risks to human health and to the environment from nanoparticles. It could also be used as a guide for the safe and sustainable handling of nanoparticles at the various stages of their life cycle. One consequence of this could also be the minimisation of the use of animals.

In the Figure 3. boxes are used to indicate where no further assessment may be needed. It must be emphasised in the application of this framework that where there is any significant doubt, this assignment to any box must not be used.

It is recognised that the full implementation of this framework will require substantial methodological developments. For example only a few of the requisite *in vitro* tests are sufficiently validated to be used in the framework at present. In addition, the further development of analytical methods, including portable equipment for exposure monitoring for nanoparticles, are needed.

The starting point is the adequately characterized nanomaterial. The proposed approach is designed to identify both human and environmental risks from exposure to engineered nanomaterials. The process involves four stages:

Stage 1: to identify whether the manufacture, use and/or end of use disposal or recycling could result in exposure of humans or environmental species and ecosystems,

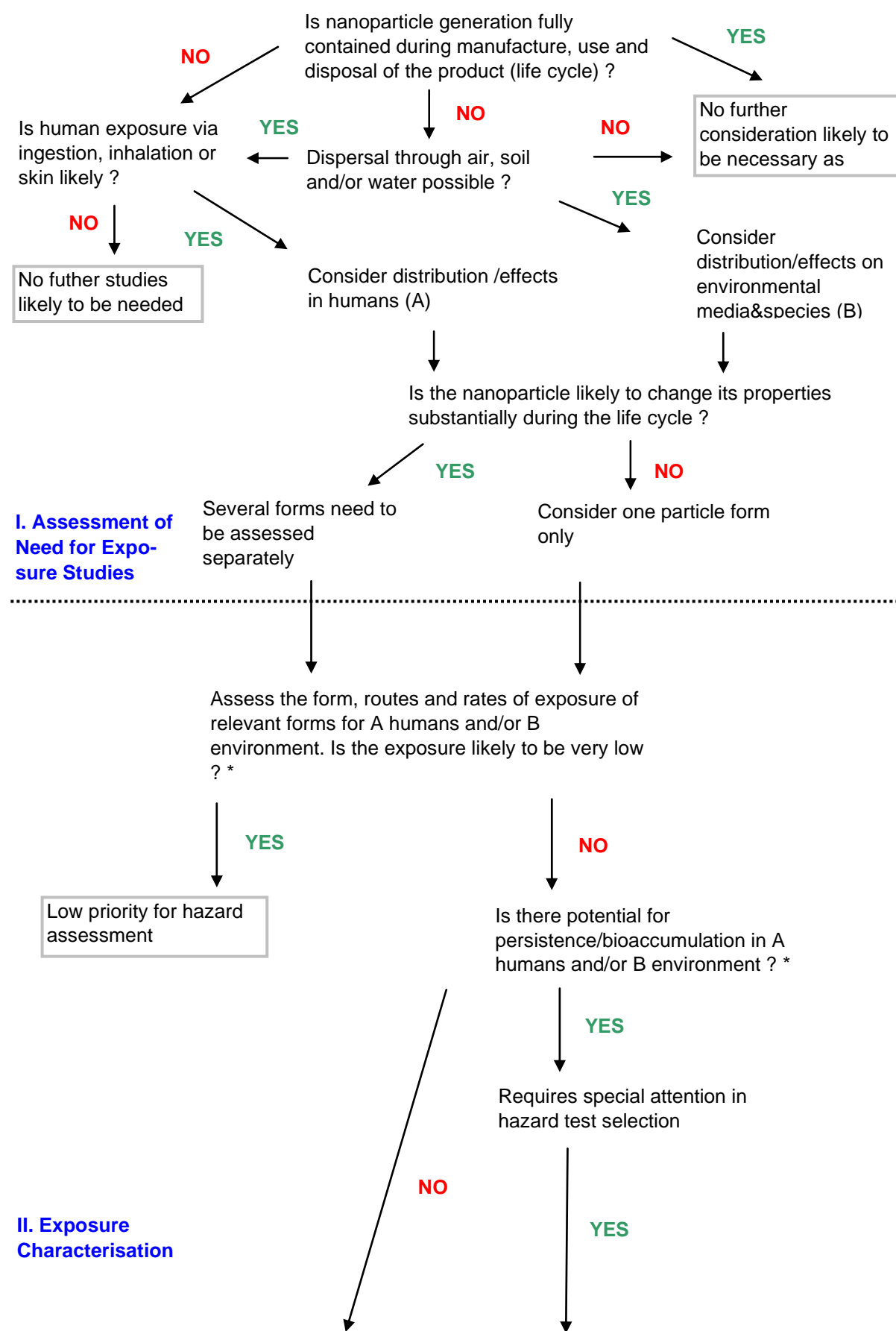
Stage 2: to characterise the nature, level and duration of any exposure,

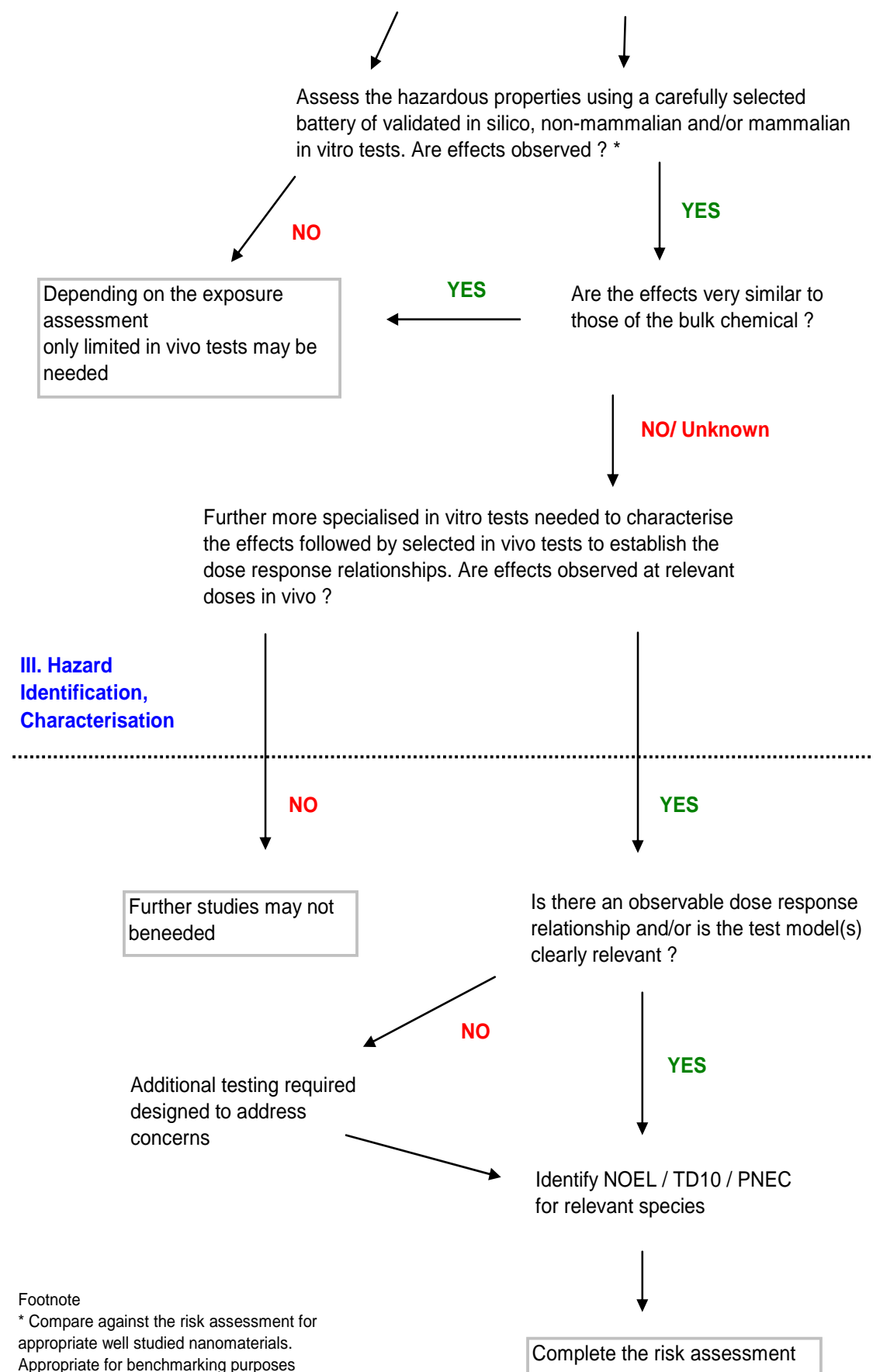
Stage 3: to identify the hazardous properties of any forms of the nanomaterial to which significant exposure is likely,

Stage 4: to characterise of the hazard and the final risk assessment.

These stages are outlined in the following figure

Figure 3. Outline of the staged approach to identifying the human and environmental risks from nanoparticles





IV. Risk Assessment

Stage 1: To identify whether the manufacture, use and/or end of use disposal/recycling could result in exposure of humans and/or environmental species.

This requires a desk top evaluation of the life cycle of the material.

For each stage of the life cycle of the nanomaterial, it is necessary to identify the potential for exposure of humans and/or the environment. For nanomaterials where all the nanoparticles are bound permanently into a much larger three dimensional structure throughout the life cycle, risk assessment methods of conventional chemicals can be applied and no further analysis of the nanoparticles is likely to be needed. Where the complete containment of the nanoparticles is uncertain an assessment of the potential routes that might lead to exposure of humans and/or the environment is necessary. The possibility that the released nanoparticles will change their characteristics during the life cycle needs to be considered in this stage. If it is likely that the nanoparticles will show a substantial change in properties then exposure to each form may need to be assessed.

Stage 2: To characterise the nature, level and duration of any exposure

Where exposure is likely, a more detailed assessment of the routes and rates of exposure of relevant species is required, both for humans and the environment. A stepwise approach to the exposure assessment is appropriate.

The approach is broadly similar for assessing human exposure and for evaluating exposure of the environment, especially when considering the form of the exposure. However, for clarity, the human and environmental scenarios are considered separately.

The Form of Exposure

The physicochemical properties of the nanoparticles can be used to identify the need for further studies by consideration of several critical questions.

First, are the particles homogeneous? If the answer is no, then different particles may need to be assessed separately.

Secondly, are the particles soluble in aqueous media? If they are, then there may be no need for their separate consideration as nanoparticles.

Thirdly, does rapid agglomeration take place? If the answer is yes, then only the assessment of agglomerated particles may be necessary. The possibility of disagglomeration should be also considered.

Fourthly, is it likely that other chemicals will be adsorbed onto the surfaces of nanoparticles? If the answer is yes, then the nature of the adsorbed chemicals will have to be considered as part of the risk assessment.

Routes and Levels of Human Exposure

Step a) Estimating actual exposure

Either measurements or modelling may be used to estimate exposure. The EASE methodology, which is identified in the Technical Guidance Document, is in principle suitable for modelling purposes. However it is likely to require some adaptation for application to nanoparticles.

Step b) Threshold for toxicological concern (TTC)

In principle, a generic exposure level should be identifiable that is gauged as too low to be of concern, similar to general thresholds of toxicological concern discussed by Kroes et al. 2004. However, there is no information as to whether this is applicable to the assessment of manufactured nanoparticles and safe levels cannot currently be identified on this basis. Under some circumstance the currently identified thresholds for conventional substances, but expressed as number of particles, could be considered in identifying priorities for further assessment. If it can be concluded with confidence that the threshold is not exceeded then no further evaluation may be needed. However, since there is a major concern about highly reactive and/or very biopersistent nanoparticles, because of lack of adequate data on such materials, the threshold approach should be applied very cautiously.

Step c) Cellular/ tissue uptake

If it is considered that the nanoparticle exposure could be above any defined threshold, or if the physico-chemical properties indicate any concern at all in relation to reactivity and persistence, then an assessment of the ability of each nanoparticle form to cross biological membranes is required. In principle this can largely be done using *in vitro* techniques. These may be supported by an *in silico* investigation, although in practice the latter methodologies are not reliable for the identification of the possible uptake of nanoparticles by cells or tissue.

It is important to ensure that the exposure conditions used in *in vitro* tests are relevant to predicted actual exposure scenarios. Simple and reliable methods are also needed to monitor the uptake, localisation and fate of nanoparticles within *in vitro* preparations. *In vivo* studies such as PBK modelling may also be needed in the assessment of exposure, but such modelling has not yet been tested sufficiently to assess the validity for nanoparticles.

Routes and Levels of Environmental exposure

Step a) Use of physicochemical data

The Technical Guidance Documents make use of physicochemical data to characterise the exposure scenarios. There is insufficient data in the case of nanoparticles to employ such an approach without considerable modification.

It is also necessary to assess whether the nanoparticle form is likely to be persistent and bio-accumulative in biological systems. Although it is not possible currently to predict persistence and bioaccumulation accurately from physicochemical properties some features have been recognised as critical as discussed above in relation to forms of exposure.

Step b) Use of existing models

Established airborne dispersion models that are applied to emissions from point sources are useful, but may require adaptation in order that they can be applied to nanoparticles. The models described in the Technical Guidance Documents for assessing uptake of substances by environmental species should be appropriate for assessing the uptake of nanoparticles, although this does need to be validated. It may also be necessary to introduce additional bottom feeder species for uptake assessment. The other methodological limitations set out for human exposure assessment also apply to the determination of uptake by environmental species.

Stage 3: To identify the hazardous properties of any forms of the nanomaterial to which significant exposure is likely

Human hazard assessment

Step a) Hazard identification

The identification of the hazard should begin with the judicious use of *in silico*, *in vitro* and non mammalian tests selected on the basis of the physico-chemical properties and any information on the biological properties, including pharmacological properties, of the nanoparticle. If uptake of nanoparticles has been found, or appears likely from the findings from the exposure assessment (stages 2), then it is necessary to identify that each *in vitro* test used is able to take up the nanoparticles.

It is also vital to ensure that each test used is sufficiently sensitive and that appropriate positive and negative reference materials are used in the form of nanoparticles. If biopersistence or biomagnification have been identified as likely, this must be considered in the design and conduct of the tests.

Step b) Mechanisms of toxicity

A combination of *in silico* and acellular and/or cellular tests and/or non-mammalian tests is likely to be required to assess uptake and effects and to provide insights into possible mechanisms of action. Appropriate tests for some endpoints of interest already exist as with cytotoxicity and reactive oxygen species generation, but others will need to be developed.

Step c) Weighting of the evidence.

From a human risk assessment viewpoint the findings can be grouped as follows:

Negative findings in vitro

If no effects are observed in a series of validated *in vitro* tests that have been selected on the basis of the known physicochemical and biological properties, the nanostructured material might be considered non-hazardous. However, before making such a decision the comprehensiveness of the tests should be evaluated, if there is any uncertainty, for example if there are conflicting results from different *in vitro* tests some additional *in vivo* tests need to be conducted. Where there is a considerable body of knowledge on the toxicology of the bulk chemical, including that it has low toxicity, the findings from the *in vitro* tests on the nanoparticle should be compared with that of the bulk chemical. If the properties are very similar it may not be necessary to conduct any *in vivo* tests or use only limited additional *in vivo* tests.

Positive findings in vitro

If the findings of the initial *in vitro* tests are positive, further *in vitro* studies may elucidate mechanisms, but *in vivo* studies should be also be employed to verify the *in vitro* observations. The dose dependence of these parameters must also be identified

Environmental hazard assessment

A tiered approach to hazard identification and characterisation is also appropriate in respect to the environment. The system set out in the Technical Guidance Documents is, in principle appropriate. However some adaptation of the methodology in order to apply it to

nanoparticles is likely to be needed. Where appropriate, integrated testing strategies should be used, including the use of environmental effects data for hazard identification and vice versa.

Stage 4: Characterisation of the hazard and the final risk assessment

The final stage is the risk assessment, which draws on the previous three stages. A critical factor is the need to identify the dose-response relationship for any significant adverse effects and in particular the No Observable Effect Level/ PNEC value (expressed in appropriate units). It is also necessary to extrapolate *in vitro* and *in vivo* data to the species of concern, taking into account where appropriate possible differences between healthy and susceptible individuals. This will require the establishment of suitable criteria for extrapolation between materials and species.

4.3.4. Answers to specific questions asked from SCENIHR

Question 1.

Assess the appropriateness of risk assessment methodologies (effects and exposure assessment) described in the current Technical Guidance Documents of the chemicals legislation, for the risk assessment of nanomaterials;

The Technical Guidance Documents currently make very little reference to substances in particulate form. With respect to human health, the current methodologies described in the Technical Guidance Documents are generally likely to be able to identify the hazards associated with the use of nanoparticles. For the determination of dose – response relationships, special attention should be given to the expression of the metrics of the nanoparticle dose since mass concentration is not necessarily the best description of dose for these materials and number concentration and surface area are likely to be more appropriate. It has to be said, however, that exposure measurement with the use of current methodologies for risk assessment of nanoparticles is rather limited, the main focus being the lung and the effects determined after inhalation exposure.

Not all nanoparticle formulations have been found to induce a more pronounced toxicity than the bulk formulations of the same substance. This suggests that the evaluation of nanoparticle formulations should be carried out on a case by case basis. Certain effects of nanoparticles in *in vitro* systems, for example radical oxygen production, have been demonstrated to occur also in *in vivo* inhalation experiments. It is important therefore that it is determined whether such *in vitro* activity will be predictive for human health hazards for all types of nanoparticles.

In considering the applicability of existing methodologies to nanoparticles, special attention should be given to the changes in the nanoparticle physico-chemical characteristics that may occur under local environmental conditions. Such changes may include, but are not limited to agglomeration, dissociation and adsorption of environmental substances, all of which may have an impact on the ultimate toxicity of the nanoparticles. Depending on the experimental conditions, such alterations to nanoparticles may be difficult or even impossible to measure under the experimental conditions used.

With respect to environmental exposure, the validity and appropriateness of existing technologies are not clear. In the absence of sufficient data on the fate and effect of nanoparticles on the environment it is neither feasible nor appropriate to propose firm rules on how substances in nanoparticle form should be evaluated. Instead the applicability of existing methods for risk assessment of nanoparticles should be evaluated.

One of the main problems encountered in the testing of the ecotoxicity of nanoparticles has been the lack of appropriate standardised protocols. The environmental effects of

nanoparticles need to be evaluated through the establishment of typical scenarios reflecting their production and use. The exposure and dose-effect models may need to be adapted, taking into account their changing physico-chemical properties over time, including their slow degradation.

In relating exposure dose concentration of nanoparticles to their effects, the traditional use of mass or mass per unit volume alone is unlikely to be appropriate. Surface area and/or particle number per volume in addition to mass should be considered. Additionally, the uptake, distribution, clearance and effects of nanoparticles may differ from those of the substances for which the Technical Guidance Documents were initially developed. From this and the lack of information regarding species sensitivities towards nanoparticles, it is concluded that at present no clear guidance can be given on the appropriateness of the key standard test taxa and recommended procedures to assess adequately the effects of nanoparticles on the various environmental compartments.

The risk characterisation methodology recommended in the Technical Guidance Documents can be followed for nanoparticles, if and only if PECs and PNECs can be calculated with confidence. These are not generally available at present, negating the possibility of a full quantitative risk characterisation as presently required and defined in the Technical Guidance Document.

Question 2.

Where current risk assessment methodology may be improved for assessment of nanomaterials, and taking into account the practical limitations of the information available for risk assessments, provide concrete suggestions for improvement of the methodology. Distinctions should be made between improvements that can be made based on current knowledge, improvements that would require specific information on the nanomaterials, and improvements that will require scientific research before they can be implemented;

As a general comment within the size range defined by the nanoscale, it is anticipated that the potential for adverse effects on humans is determined by several factors in addition to chemical composition, and that the determination of risks is a more complex process than with conventional bulk materials. Some substances that may be deemed to be of low risk in bulk form may well have significant risk when in nanoparticulate form. Improvements to the methodologies should therefore take into account factors such as the following.

First, physical parameters such as number concentration and surface area are likely to be more significant than mass concentration in the determination of exposure. Secondly, nanoparticles may agglomerate and disagglomerate in different environments, such processes affecting their properties. Thirdly, impurities within, and adsorbed species on the surface of, nanoparticles may have significant effects on risks and these possibilities should be taken into account. Fourthly, biological processes involving nanoparticles, including translocation, cellular uptake and toxicological mechanisms are still largely unknown and testing methodologies have to address these possibilities.

It should also be noted that reference materials for the evaluation of nanoparticles have not yet been identified.

With respect to specific concrete suggestions, the following points should be noted:

There is a clear need for validated *in vitro* assays for nanoparticle evaluation. *In vitro* tests should address key properties of the nanoparticles such as genotoxicity, biopersistence, free radical generation, cellular toxicity, cell activation and other generic endpoints. *In vitro* tests should also provide target cell-specific endpoints such as effects on the action potential of nerve cells or the phagocytic capacity of macrophages.

The successful application of a QSAR approach to nanoparticles necessitates the ability to indicate the toxicity or other properties of a new nanoparticle from its molecular structure. Such QSAR models are plausible, but represent a significant challenge in nanotoxicology and significant developments are needed here. There is general lack of knowledge of which physicochemical properties of nanoparticles are responsible for any specific toxicity.

Inhalation studies require improvement with respect to nanoparticles. They should take into account the fact that nanoparticles with large surface area may rapidly cause saturation of lung clearance. It is generally crucial for risk assessment of nanoparticles to determine the precise tissue distribution profile as there is so little information on translocation,. Also specific comments on nanoparticle metabolism and excretion are required, taking into consideration the limits of detection.

Similarly, since there is some evidence that nanoparticles can translocate from the lungs to the blood and the brain, assays for the monitoring of blood and brain transfer of nanoparticles, and their consequences, have to be developed. For blood, markers of thrombosis and atherogenesis need to be considered and potential degenerative effects and oxidative stress on the brain should be assessed within these new methods.

With respect to mutagenicity, genotoxicity and carcinogenicity, it is necessary to be very cautious about the interpretation and extrapolation of experimental data obtained with nanoparticles. Since it is not clear whether existing bacterial tests are appropriate to detect the mutagenicity of nanoparticles, further developments are required.

Concerning the environment, it is not clear at this stage how predicted environmental concentrations (PEC) for nanoparticles can be calculated. It is recommended that the validity of the current emission factors and models should be evaluated and, if necessary, a modified or new approach should be then be developed. The commonly used mathematical models of dispersal of vapour and large particulate matter will need adaptation for the assessment of the environmental distribution and dispersal of nanoparticles. This implies incorporation into the models of the key physico-chemical characteristics relevant to nanoparticles such as surface area and morphology; charge, number of particles, size, solubility and potential chemical and physical conversion into other forms, as described earlier. These factors should be considered and introduced in the framework of the calculation of PECs/PNECs for manufactured nanoparticles.

With respect to bioavailability, no clear guidance can be given on the appropriateness of the key standard test taxa and recommended procedures to assess adequately the effects of nanoparticles on the various environmental compartments. There is therefore a need for new standardized ecotoxicity tests for nanoparticles.

Question 3.

Where possible, provide practical examples of how risk assessment of nanomaterials can be performed and of nanomaterials, forms of nanoparticles etc that may cause significantly different adverse effects or different exposure behaviour.

With respect to the performance of the risk assessment of nanomaterials, it is recommended that the staged, or tiered, approach proposed in Chapter 4.3.3 is adopted in order to identify different adverse effects and different exposure data with nanoparticles. It is suggested that due consideration be given to the possibilities now emerging that translocation of nanoparticles away from the portal of entry may occur in humans and other species, and that the passage of nanoparticles across membranes could give rise to adverse effects, for example within the cardiovascular system or following passage across the blood – brain barrier.

5. MINORITY OPINION

None.

6. LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectroscopy
ADME	Absorption, Distribution, Metabolism, Excretion
ASTM	American Society for Testing and Materials
BCF	BioConcentration Factor
BET	Brunauer, Emmett and Teller
BMF	BioMagnification Factor
COPD	Chronic Obstructive Pulmonary Disease
CPC	Condensation Particle Counter
DLS	Dynamic Light Scattering
ICP-MS	Inductively Coupled Plasma – Mass Spectroscopy
ISO	International Standards Organisation
LEV	Local Exhaust Ventilation
MPPS	Most Penetrating Particle Size
MRI	Magnetic Resonance Imaging
NOEL	No Observable Effects Limit
QSARS	Quantitative Structure-Activity Relationships
PBPK	Physiologically – Based Pharmacokinetic
PBT	Persistence, Bioaccumulative and Toxic
PEC	Predicted Environmental Concentration
PNEC	Predicted No Effect Concentration
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
XRD	X-Ray Diffraction

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