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An Assessment of Regulatory Testing Strategies and Methods for Characterizing the Ecotoxicological Hazards of Nanomaterials
Final Report

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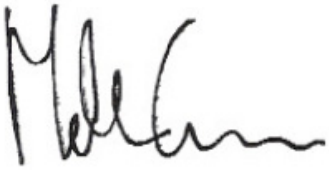
Document Approved by:		3rd August 2007
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EXECUTIVE SUMMARY

This report is a review of where current standard ecotoxicity methods are fit for purpose and where they are not. Standardised aquatic and terrestrial ecotoxicology tests with microbes, algae, higher plants, invertebrates and vertebrates that are routinely used for environmental hazard assessment are assessed for their ability to measure reliably the particular hazards presented by nanomaterials. This assessment includes a review of the relevant white and grey literature, and information from interviews with UK and overseas experts. Gaps identified from this assessment are used to produce recommendations for improving upon current testing strategies that take cost, feasibility and the 3Rs (Reduction, Refinement and Replacement of animals used in experimentation) fully into account.

The specific objectives of this review are:

1. To review critically those studies that have characterised the hazard of nanomaterials, summarising and appraising key issues and challenges arising from these.

2. To describe succinctly the current test strategies and associated methods used within current chemicals regulatory ecohazard (toxicity and bioaccumulation) assessment.

3. To use this information to identify which elements of test strategies and associated methods for hazard assessment are not fit for purpose, giving reasons.

4. To propose pragmatic variants on current tests based on the information gathered.

5. To propose an experimental programme to test variants on the standard methodologies.

The overall conclusions of this review are:

1. Studies on mammals raise the concern that there are toxic effects of nanomaterials (albeit at mg doses), and we should therefore establish whether or not manufactured nanoparticles are also toxic to wildlife. There is little empirical information on the ecotoxicity or bioaccumulation potential of nanoparticles and, depending on exposure pathways, these data need to be generated for aquatic and terrestrial microbes, plants and animals to reduce uncertainties about the hazards posed by these substances. There is an emerging literature on the ecotoxicity of nanomaterials, with most of the studies on aquatic organisms, using a few types of nanoparticles that are commercially available.

2. Current chemicals regulations do not distinguish between the nanoscale and macroscale forms of substances, so ecotoxicity tests performed on the macroscale form may, from a legal point of view, need to be accepted for both macroscale and nanoscale forms by regulatory authorities. This needs to change so that, at the very least, an evidence-based case is presented by manufacturers to show that there is

no difference in the hazards of nanoscale and macroscale forms of the same substance. This evidence may come from the use of rapid tests (see 4 below).

3. Standard methods for testing ecotoxicity are generally preferable because they provide the demographic data on survival, growth and reproduction that are required by environmental (ecological) risk assessors, and for species whose strengths and limitations in toxicity testing are well understood. Additional endpoints could be measured on these species if there is the need to understand mechanisms of toxic effect, which may primarily be of help in the use of read-across from one substance, or form of substance, to another. The main area of considerable uncertainty that applies to all ecotoxicity testing guidelines, *except* those in which dosing is oral, is the way in which the substance is dosed into, and maintained and measured in, the test medium.

4. Rapid test systems (e.g., *in vitro* or genomics tests) for measuring the toxicity of nanoparticles may be a useful addition to the standard suite of ecotoxicity tests, especially if they are used to demonstrate the similarity or dissimilarity of toxicity of macroscale and nanoscale forms, when the mode of action is understood. It is possible to select a suite of rapid tests from existing regulatory tests, although some of these may need to be imported from the clinical regulatory arena. The suite would need to be selected to identify overall toxicity (e.g., cell viability assay or microbial population growth test (e.g., with *Vibrio fischeri*)), and also to check for specific modes of toxicity that may not be detected by a general toxicity screen. This need not be exhaustive, but focus on major toxic mechanisms likely to be relevant for that type of nanomaterial. These might include genotoxicity (abbreviated Ames test or similar), immunotoxicity assays for tiered application), and an oxidative stress assay.

5. However, it is important to understand that it is *only* changes in age-related survival, growth and reproduction that can influence the size of a population of organisms. Ecological risk assessors are rarely interested in the health of individual organisms, so measures of individual effect such as the use of *in vitro* or genomic biomarkers are only useful to them if they can predict effects on survival, growth and reproduction. This differs substantially in both concept and methodology from the practices of human health risk assessment. It is likely that confusion between the aims of human health risk assessment and ecological risk assessment is behind the calls from some researchers for the use of "novel" (often rapid) endpoints to test the ecological risks of nanoparticles. Novel endpoints may throw light on mechanisms of action, and may arguably be quicker and easier to perform than standard ecotoxicity tests, but the measurement of anything other than survival, growth or reproduction must necessarily be translated into these endpoints before it is useful to an ecological risk assessor.

6. In contrast to the call for rapid test systems, there may be a case for requiring manufacturers to generate chronic ecotoxicity data, even in the absence of triggers from short-term tests, because of a lack of information on appropriate assessment factors for extrapolation from acute to chronic toxicity for nanoparticles. There is a recent precedent for this in European guidelines for the testing of human medicines in which manufacturers are required to generate chronic toxicity data.

7. To date there have been no systematic studies of nanoparticle ecotoxicity for the same species in seawater versus freshwater, or during graded salinity change. What little is known implies that toxicity tests in freshwater are unlikely to provide adequate information on toxicity in sea water and a separate series of tests with marine species may be needed.

8. A tiered environmental risk assessment approach similar to that used for macroscale chemicals is likely to be fit for purpose for assessing the risks of nanoparticles. Requirements for further data within such a tiered framework would be triggered by information on use and release patterns, environmental fate, persistence and bioaccumulation. However, there are some concerns about existing physico-chemical test methods, and in particular whether or not the partition coefficient test works for nanomaterials. Clumping of nanoparticles may prevent proper separation of the material in the octanol and water phases. The meniscus between the phases is likely to trap material undermining the founding assumption of free diffusion between the phases. The test may be fundamentally flawed at the nanoscale and this has implications for risk assessment strategies that use the partition coefficient as a trigger for requiring either sediment toxicity tests or bioaccumulation studies.

9. The following factors may be important to the behaviour of nanoparticles in the aqueous phase:
   - Dissolution of the nanoparticle
   - Emulsion or coatings on nanoparticles
   - Dispersion of nanoparticles by interactions with natural organic matter
   - Agglomeration and aggregation
   - Adsorption and precipitation properties of particles on surfaces
   - Effects of impurities on nanoparticle behaviour in solution
   - Effects of solvents, sonication and stirring on nanoparticle solutions

   This is an area where research is particularly lacking on the ecotoxicology of nanoparticles.

10. There are essentially three approaches to achieving a uniform stock solution of a test substance for ecotoxicity testing: dispersion in detergents or similar agents, by sonication, or by prolonged stirring. There are logistical and ecologically-based criticisms of all of these approaches. Nanomaterials discharged to the environment are not likely to occur in the presence of mg quantities of dispersant chemicals or sonication, although natural surfactants and humic substances may have similar effects. It could be argued that the non-dispersed material is likely to more relevant to what will happen in the real environmental, and toxicity test design should reflect that reality.

11. Organisms in ecotoxicity tests should be exposed to nanoparticles in a way that is environmentally relevant. Predicted Exposure Concentrations and Predicted No Effect Concentrations should be based on the same form of nanoparticle (e.g., agglomerated or dispersed) and expressed in the same units so that Risk Characterisation compares like with like. This form may change during the lifecycle of a nanoparticle, and it may therefore be necessary for tests to be repeated on

these different forms to characterise risks adequately. The concentration of nanoparticles in ecotoxicity tests should be expressed as number or surface area per volume and not just as mass per volume.

12. A base set of measurements for characterising nanoparticles in ecotoxicity tests is suggested as a practical approach, given the limitations of the current technology for measuring the chemistry and particle sizes, and also considering what can be reasonably expected of a competent ecotoxicology laboratory. The following are suggested as a minimum base set:
    - Nomenclature information (details provided in Appendix 1 of this report).
    - Concentration of the material (e.g., mg l^{-1}).
    - Electron Microscope Images (or similar) of the material in solution.
    - Measurement of the individual particle size in the stock solutions used for dosing the test system. This would include mean particle size ± standard deviation for at least 6 replicate samples of the stock solution.
    - Measurement of the size of any agglomerated or aggregated material in the solution (means and standard deviation as above).
    - Use of a spectroscopic method, or other similar method, to confirm dispersion of the material at various dilutions of the stock solution within the detection limits of current methods.
    - Some measurements to confirm concentrations of the main expected impurities in the test material, and details of any washing procedures to remove these if this is desired in the experiment.

      Predicted Environmental Concentrations of nanoparticles in the environment must also be characterised in the same way, either through accurate modelling or direct measurement, so that PECs and PNECs can be compared in a meaningful way.

13. It may be desirable when assessing whether current ecotoxicity methods are fit for purpose to make simultaneous measurements with reference materials, especially at the evaluation stage. A starting point for searching for reference materials for aquatic ecotoxicity studies may be to choose some natural particles with appropriate toxic and non-toxic characteristics, such as china clay, that are found in suspension in water. However, this is the subject of another currently funded Defra project, so no specific recommendations on this are made in this report.

There are a limited number of important areas in which current ecotoxicity testing strategies and methods are not wholly fit for purpose. These are:

1. The toxicity of macroscale forms of a substance may not reflect the toxicity of nanoscale forms.

2. The use of solvents or sonication to produce a homogeneous dispersion of nanoparticles at the beginning of ecotoxicity tests may not reflect the behaviour of nanoparticles in the natural environment in which it is possible that agglomerations predominate.

3. The standard reliance in most environmental risk assessment frameworks (including REACH) on acute toxicity data plus large Assessment Factors to predict the chronic effects of chemical substances is not yet supported by sufficient empirical data on acute-to-chronic ratios for nanoparticles. There is little empirical information on the ecotoxicity or bioaccumulation potential of nanoparticles and, depending on exposure pathways, these data need to be generated for aquatic and terrestrial microbes, plants and animals to reduce uncertainties about the hazards posed by these substances.

4. Reporting of ecotoxicity results for nanoparticles as mass concentration does not take account of their unique properties, which are better reflected by measurements of number concentration or surface activity. These parameters need to be measured in test systems - reliance on nominal concentrations is unreliable. These parameters also need to be accurately predicted for, or directly measured in, the environment so that Predicted Environmental Concentrations can be meaningfully compared to Predicted No Effect Concentrations from ecotoxicity tests.

5. There are some concerns about whether or not the partition coefficient test works for nanomaterials. This has implications for risk assessment strategies that use the partition coefficient as a trigger for requiring either sediment toxicity tests or bioaccumulation studies.

In other respects it is likely that existing strategies and methods are sufficiently fit for purpose.

Current ecotoxicity strategies and methods should be amended for testing nanoparticles in the following way.

1. The toxicity of nanoparticles must be measured in separate studies to those used to test the ecotoxicity of other physical forms. Measurement may be by use of appropriate rapid tests to demonstrate that the nanoparticle has similar hazard properties to other physical forms, *so long as the mode of toxic action of the nanoparticle is understood*. When there is uncertainty about the mode of action, a full suite of standard aquatic ecotoxicity tests with algae, invertebrates and fish should be performed with the nanoparticle.

2. Nanoparticles should be tested in ecotoxicity studies in the form in which they occur in the environment. If a reasonable worst case exposure scenario is that a nanoparticle will always be present in agglomerated form, then this should be the form in which it is tested, so that Predicted Environmental Concentrations of the agglomerated form can be compared with Predicted No Effect Concentrations for the same form. There is a lack of knowledge about the fate and behaviour of nanoparticles in the environment - this is the single most important area for research that is identified in this report. Without reliable information on realistic worst case exposure scenarios it is impossible to design appropriate exposure systems in laboratory ecotoxicity tests. As a result of this, many resources and test organisms will be wasted in performing irrelevant ecotoxicity tests that cannot be used in risk assessments.

3. Chronic ecotoxicity data on the effects of nanoparticles need to be generated to determine the potential for establishing reliable acute-to-chronic ratios and standard Assessment Factors. Preliminary information suggests that acute testing may not be a practical way of determining nanoparticle hazards, because of practical difficulties in exposing test organisms to high concentrations of nanoparticles and the slow uptake of these substances by organisms. It may therefore be the case that chronic ecotoxicity testing remains the only practical approach. If this is the case, then a limit test approach, as recommended by RIP 3.3 (EC 2007a) for fish testing under REACH, is likely to be the most cost-effective solution. The results from limit tests with algae (OECD 201), *Daphnia* (OECD 211) and fish early life stages (OECD 212), used in combination with information on the toxicity of other physical forms of a substance should be sufficient to perform an adequate environmental risk assessment for a nanoparticle.

4. Exposure in ecotoxicity tests with nanoparticles must be measured and expressed as number concentration or surface activity, as well as mass concentration. Reporting of nominal concentrations alone is inadequate.

Our main recommendations from this review are that:

1. Research on establishing appropriate ecotoxicity test strategies and methods for nanoparticles should currently focus primarily on defining realistic worst case exposure scenarios for nanoparticles in the environment and then testing the toxicity of nanoparticles under these scenarios. This research programme should consider the fate and behaviour of nanoparticles in the environment, with or without the presence of natural and anthropogenic substances and conditions that may influence aggregation state. The programme should include the following elements:
   - A desk study to characterise fully the environmental parameters that influence the fate and behaviour of the main classes of nanomaterials likely to be released to the environment.
   - Collation of data on these influential parameters for surface waters and terrestrial systems in the UK, and derivation of realistic worst case exposure scenarios for nanomaterials likely to be released to the environment.
   - Chronic laboratory ecotoxicity tests with microbes, algae and fish, which simulate these realistic worst case exposure scenarios. These studies should include sediment ecotoxicity studies designed to test the accuracy and precision for nanoparticles of estimates based on partitioning theory and partition coefficients.
   - Identification of appropriate QA/QC procedures and robust nanoparticle characterisation methods that can be used in long-term laboratory experiments and in field surveys of different media. This should include identification of any requirements for laboratory inter-calibration exercises.

   Ideally such a project would be a joint initiative between the UK Government and the nanotechnology industries, so that funding and the input of appropriate expertise are maximised.

2. A set of rapid, cost-effective tests should be agreed between regulators, industry and other stakeholders that are able to demonstrate that a nanoparticle has similar

hazard properties to other physical forms of a substance. These should include, *inter alia*, tests to identify overall toxicity (e.g., cell viability assay or microbial population growth test (e.g., with *Vibrio fischeri*)), and also to check for specific modes of toxicity that may not be detected by a general toxicity screen, but are relevant for that type of nanomaterial. These might include genotoxicity (abbreviated Ames test or similar), immunotoxicity assays, and an oxidative stress assay.

3. When rapid tests are unable to demonstrate similar hazard properties to other physical forms, the *chronic* effects of nanoparticles should be measured in a limit test design.

GLOSSARY OF CHEMICAL TERMS

A detailed review of particle chemistry is beyond the scope of this report, and the ecotoxicologist is referred to other texts on this topic. For some discussion of how knowledge on natural colloids, and the measurements techniques involved can be transferred to the issue of engineered nanomaterials see the recent review by Lead and Wilkinson (2006) and papers therein. Some recent book chapters in the IUPAC book series are also worth reading on how particles and colloids interact with cell membranes (Taylor and Simkiss 2004), as well as adsorption chemistry and unstirred effects (Handy and Eddy 2004). The following terminology is used in this report.

Adsorption; this is used strictly in the context of surface chemistry to describe the association of materials from the aqueous phase with a solid surface. The latter could be the surface of a nanoparticle, the glass wall of a fish tank, or the surface of an epithelial or cell membrane. The term "adsorption" is used mainly to imply 2-dimensional (2D) interactions at the adsorbing surface. Strictly speaking from a chemistry perspective the more generic term of "sorption" should be used if 3D interactions are involved (e.g., adsorption to the inside surface of a porous structure or with complex organic matter) but we do not make that distinction here. The term "adsorption" is not be confused with the phrase "absorption" which is often used loosely by biologists to describe uptake or accumulation. Biologists need to start using the more precise definitions of adsorption and absorption in this field to avoid confusion in the future.

Agglomeration; Similar to aggregation but involving stronger forces such as ionic bonds, and the process may be more thermodynamically stable. Agglomerates are therefore more difficult to break up than aggregates. The interactions can be particle-particle, but in colloid chemistry binding to other matter in the colloid with the resultant loss of water volume from the agglomerate is considered. In geology, the term agglomeration is used mainly in the context of materials (e.g., pebbles or other discrete items) in a solidified matrix (e.g., muds). So, within the physical sciences the term is used in different ways by different groups of scientists. Note that some scientific papers use the phrases aggregation and agglomeration interchangeably without giving clear definitions of their use in the manuscript. In this report the difference between aggregation and agglomeration is not distinct because the chemistry has not been investigated in sufficient detail in ecotoxicology studies to know which of the processes dominate with engineered nanoparticles (aggregation or agglomeration).

Aggregation; a process in which particles or nanoparticles can associate with themselves. The strict definition is a particle-particle interaction but we also include generally with organic matter or fibrils in the water to form clumps or clusters of nanoparticles in the colloid. The mechanisms involved for aggregation should be fairly weak forces that can be easily broken, such as van der Waals forces or the formation of weak chemical bonds. Whether or not the material stays in the aqueous phase or is precipitated as a result of the aggregation is not considered in this definition.

Aqueous Phase; we use this phrase simply to mean keeping the material in a liquid phase where it is relatively easy to handle. No distinction is made between true solubility, emulsions or colloids in the use of this term here.

Dispersion; to generate a solution containing mostly individual nanoparticles, rather than aggregates or agglomerates. No distinction is made in this report as to how the individual particle remains dispersed (i.e., details of the repelling forces or interactions on the particle surface could be many and varied).

Dissolution; the desorption of atoms or molecules from the surface of a particle into true solution. This may occur with unstable particles that gradually dissolve, e.g., dissolution of metal nanoparticles resulting in free metal ions in solution.

Macroscale material; we use this phrase to differentiate the normal chemical powder form of a substance from the nanoscale material.

Nanomaterial; any materials with at least one dimension between 1 and 100 nm in the fundamental structural unit of the material. Materials that have a distribution of sizes that include a significant proportion between 1 and 100 nm can also be considered as containing nanomaterials.

Nanoparticle; a material that is particulate in nature (not necessarily spherical) with at least one dimension between 1 and 100 nm in the fundamental particle size (e.g., diameter of individual particles).

Particle size; this phrase is used loosely to mean the maximum diameter of a particle. No distinction is made between the diameter of the particle core or the diameter associated with any coatings or capping agents on the particles, unless otherwise stated in the text.

Precipitation; we use this phrase loosely, simply to describe the gravitational loss of material (aggregates or agglomerates) from the aqueous phase to form a precipitate. This may involve **flocculation** in which the various fibrils and organic matter act as "bridging materials" that raise the density of the aggregates to a point where they will drop out of solution.

Solubility; for the ecotoxicology literature review in this report the phrase is used mainly to describe a material in true solution, e.g., a molecule dissolved in water with its associated hydration shell of water molecules. However, in regulatory ecotoxicology protocols the term "in solution" is poorly defined and for practical purposes this often means that the term "in solution" in regulatory toxicology really means just that the sample is in some kind of "aqueous phase" as defined above.

CONTENTS

1. INTRODUCTION

Nanotechnology and the use of nano-scale materials is a relatively new area of science and technology with an estimated global market worth $10.5 billion in 2006 (BCC 2006). While still in its infancy, the development and use of nanomaterials (nanoparticles and nanotubes) is increasing at an ever accelerating rate, yet little is known about the likely inputs, fate, behaviour and effects of nanomaterials in the environment (BAuA 2006, Bergeron and Archambault 2005, Colvin 2003, Dreher 2004, Rickerby and Morrison 2007, Royal Society 2004). Although nano-scale materials have been used in research for more than a decade, there is now wider debate about the risks and benefits of manufactured nanomaterials (Royal Society 2004, USEPA 2007, Handy and Shaw 2007, Owen and Depledge 2007). The UK Government's Nanotechnologies Research Co-ordination Group (NRCG) identified an assessment of the fitness for purpose of current ecotoxicological test methods as one of the areas where further investigation is required. This is, in part, because of disagreement between the NRCG's current working hypothesis that test methods are likely to be adequate, and the suggestion that they are inadequate for some purposes by the European Commission's Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR 2005, 2007).

Nanotechnology is defined as using materials and structures with nanoscale dimensions, usually in the range 1-100 nm (Masciangioli and Zhang 2003, Roco 2003; see Appendix 1 for further discussion of terminology). The potential benefits of these new nanomaterials is considerable, and includes sensors for environmental monitoring, nano-drug delivery, biorobotics, nanoarrays, and nanoscale implants in medicine (Freitas 2005, Rickerby and Morrison 2007, Roco 2003). However, the toxic effects of nanomaterials are poorly understood, and their effects on wildlife are largely unknown. In the absence of such basic toxicological information, it is difficult to set environmental quality standards or perform risk assessments for these materials. The Environment Agency of England and Wales has recommended a voluntary moratorium on the release of engineered nanoparticles into the environment until such information is available (Owen and Depledge 2005).

This report is a review of where current standard ecotoxicity methods are fit for purpose and where they are not. Standardised aquatic and terrestrial ecotoxicology tests with microbes, algae, higher plants, invertebrates and vertebrates that are routinely used for environmental hazard assessment are assessed for their ability to measure reliably the particular hazards presented by nanomaterials. This assessment includes a review of the relevant white and grey literature, information from interviews with overseas experts, and information from a workshop with UK experts. Gaps identified from this assessment are used to produce recommendations for improving upon current testing strategies that take cost, feasibility and the 3Rs (Reduction, Refinement and Replacement of animals used in experimentation) fully into account.

The specific objectives of this review are:

1. To review critically those studies that have characterised the hazard of nanomaterials, summarising and appraising key issues and challenges arising from these.

2. To describe succinctly the current test strategies and associated methods used within current chemicals regulatory ecohazard (toxicity and bioaccumulation) assessment.

3. To use this information to identify which elements of test strategies and associated methods for hazard assessment are not fit for purpose, giving reasons.

4. To propose pragmatic variants on current tests based on the information gathered.

5. To propose an experimental programme to test variants on the standard methodologies.

This report begins with an overall background to ecotoxicity testing for nanoparticles and summarises the conclusions of several recent reports on this issue. We then review available studies on the ecotoxicity of nanoparticles and draw conclusions about the strengths and weaknesses of these studies in identifying ecological hazards. This review of the literature is followed by a brief description of standard ecotoxicity test methods and the tiered frameworks within which they are used to assess environmental risks. The results of interviews with experts are included in this section. Current ecotoxicity methods and frameworks that may not adequately characterise the environmental hazards of nanoparticles are then identified, and possible solutions are proposed.

2. BACKGROUND

Ecotoxicity testing of nanomaterials has been discussed in several recent reports, and we summarise these discussions below to help place the current report within a wider context.

2.1 Royal Society and Royal Academy of Engineering Report

The 2004 Royal Society and Royal Academy of Engineering Report: *Nanoscience and nanotechnologies: opportunities and uncertainties* concluded the following about ecotoxicity testing of nanomaterials:

(i) With the exception of studies on some laboratory mammals related to investigation of human toxicology, almost nothing is known about the effects of nanoparticles on non-human animals, or on plants or micro-organisms.

(ii) It is plausible that organisms in soil or water could take up manufactured nanoparticles that have escaped into the environment which could, depending on their surface activity, interfere with vital functions.

(iii) Simple tests are available for preliminary screening for persistence, bioaccumulation and toxicity of chemical substances. However, it remains to be established whether these tests are suitable for nanoparticles. For example, little is know about whether nanoparticles agglomerate in the environment and whether this affects their toxicity.

(iv) Research is needed to develop internationally agreed protocols and models for investigating the routes of exposure and toxicology to humans and non-human organisms of nanoparticles and nanotubes. This should include investigation of bioaccumulation, particularly of persistent nanoparticles and nanotubes. It is deliberately manufactured nanoparticles and nanotubes that are not immobilised in a matrix that should be the focus of this research.

(v) Current chemicals regulation (Notification of New Substances Regulations) and the forthcoming REACH Regulations (Registration, Evaluation and Authorisation of Chemicals) do not require additional testing of a nanomaterial for which data are available for the macroscale form of the substance. This should change, and substances produced in the form of nanoparticles should be treated as new chemicals.

In February 2005 the UK Government produced a response to the Royal Society and Royal Academy of Engineering Report (HMG 2005). In this response the Government stated that it was *"…strongly committed to filling gaps in knowledge through an immediate programme of research aimed at reducing the uncertainties relating to toxicity and exposure pathways for nanoparticulates."*

2.2 Institute of Occupational Medicine Scoping Report

Tran et al. (2005) at the Institute of Occupational Medicine were commissioned by Defra to identify hazard data needs for addressing the risks presented by nanomaterials. Their main conclusions of relevance to ecotoxicity testing were that:

(i) Standard methodologies should be developed for assessing the toxicity of nanoparticles. Tran et al. (2005) suggest that target test species should be identified on the basis of likely exposure and the existence of standard testing procedures. However, they also recommend the inclusion of biomarkers such as markers of oxidative stress, inflammation, genotoxicity, lysosomal stability, neurological function and stress proteins alongside the more traditional demographic parameters (survival, growth and reproduction) that are measured in ecotoxicity tests. The rationale for this presented by Tran et al. (2005) is that such measurements will allow mechanisms of toxicity to be determined.

(ii) Manufactured nanomaterials should be treated as new chemicals and be tested for ecotoxicity without reliance on any results from macroscale chemical forms. This conclusion is similar to that reached by the Royal Society and Royal Academy of Engineering (2004).

(iii) Ecotoxicity should be considered during the full life-cycle of a substance (i.e., production, use and fate). This conclusion seems to differ from that of the Royal Society and Royal Academy of Engineering, who called for life cycle assessment of the *releases* of nanomaterials so that any differences in exposure could be assessed.

(iv) The significance of endpoints should be assessed by establishing a set of tests, target species and procedures. Tran et al. (2005) explain that the objective of this is to *"…develop procedures which will allow linking and an assessment of significance between different endpoints, from the enzymatic/cellular to the population level."*

(v) The joint toxicity of nanoparticles with metals and organic chemicals should be explored. Tran et al. (2005) do not describe how this could be achieved in practice.

(vi) The potential for nanoparticles to bioaccumulate and biomagnify should be assessed. Tran et al. (2005) do not specifically identify the tests that should be used for this, but their conclusion that standard test systems should be used, if possible, suggests that existing systems should be examined first to determine whether they are fit for purpose.

(vii) Provide manufacturers and developers of nanomaterials with a quick, off-the-shelf assessment methodology. Tran et al. (2005) do not identify which tests should be used within such a methodology, but much of the ecotoxicity section of their report discusses biomarkers and genetically-modified bacteria

tests, so it seems likely that these are the types of rapid tests that they would recommend.

Tran et al. (2005) also interviewed several scientists working in the field of environmental nanoscience. The consensus about ecotoxicity testing amongst these interviewees was that data on the ecotoxicity of nanomaterials are currently so sparse that it is impossible to know how toxic they are to non-human organisms. Interviewees agreed that some nanomaterials have the potential to bioaccumulate and that different nanomaterials will behave in different ways to macroscale chemicals, and also to each other, depending on their type, size and surface chemistry.

2.3 Nanotechnology Research Co-ordination Group

The Nanotechnology Research Co-ordination Group (NRCG) is a cross-departmental and Agency body set up by UK Government in response to the Royal Society and Royal Academy of Engineering Report (2004). The objectives of the NRCG are to:

(i) Develop and oversee the implementation of a cross-Government research programme into the potential human health and environmental risks posed by free manufactured nanoparticles and nanotubes to inform regulation and underpin regulatory standards.

(ii) Establish links in Europe and internationally to promote dialogue and to draw upon and facilitate exchange of information relevant to the Group's research objectives.

(iii) Consider the outputs of dialogue between stakeholders, researchers and the public with a view to enhancing and informing research decisions.

In its first report (NRCG 2005) the NRCG considered the Royal Society and Royal Academy of Engineering (2004) report, the SCENHIR (2005) opinion (see below) and the IOM scoping study (Tran et al. 2005) and concluded that few data are currently available on the ecotoxicity of nanoparticles, or on which environmental compartments and receptors are most likely to be at risk. The NRCG identified the following three main research objectives in this area:

(i) *Research Objective 17. Research to establish the uptake, toxicity and effects of nanoparticles on groundwater and soil microorganisms, animals and plants, especially in the context of remediation.* This objective is based on preliminary information that nanoparticles can be toxic to bacteria (Tran et al. 2005), and the likely use of nanotechnology to remediate contaminated groundwater and soils.

(ii) *Research Objective 18. Research to establish the mechanisms of toxicity, toxicokinetics and* in vivo *effects of nanoparticles to key ecological groups (including invertebrates, vertebrates (e.g. fish) and plants). A key aspect of such work should be the facilitating of knowledge transfer from human toxicological studies to inform ecotoxicology.* This objective is based on the

view that there are few data on the ecotoxicity of nanoparticles, but there may be the potential for useful read-across from mammalian toxicity data.

(iii) *Research Objective 19. Define endpoints to be measured in ecotoxicological studies and assess how fit for purpose current standard tests for persistence, bioaccumulation and toxicity are when considering nanoparticles. This should lead to the defining of a suite of standard PBT protocols for use in environmental hazard assessment.* This objective is based on the view that an understanding of particle physico-chemical properties and mechanisms of toxicity might help to define more appropriate measurement endpoints for assessing persistence, bioaccumulation and toxicity.

The NRCG published a progress report in October 2006 (NRCG 2006) which identified the following activities to meet the three research objectives above:

(i) *Research Objectives 17 and 18.*
   a. Grants awarded by the Natural Environmental Research Council for projects at the Universities of Birmingham, Exeter and Plymouth.
   b. Environmental Nanoscience Initiative launched to address basic research into the fate and behaviour of nanomaterials in the environment.
   c. Support for international conference in London, September 2006 – Environmental Effects of Nanomaterials.
   d. Nanonet - NERC funded network on nanoparticles in the aquatic environment.

(ii) *Research Objective 19*.
   a. Defra-funded project on assessment of how fit for purpose current and proposed regulatory ecotoxicological tests are for nanomaterials (this report is the output from that project).
   b. Co-ordination with European Chemicals Bureau, European Centre for the Validation of Alternative Methods and OECD to assist SCENIHR Nanomaterials Working Group assess the applicability of the Technical Guidance Document for nanoparticles.

2.4 DTI Regulatory Gaps Analysis

The Department of Trade and Industry commissioned a regulatory gaps analysis for nanomaterials (Frater et al. 2006). This analysis identified 33 "environmental" regulations of relevance for the assessment and control of nanomaterials in the environment. The key findings on environmental protection were that the large number of environmental regulations and the narrow scope of many of these *"…may lead to gaps in the regulation of nanomaterials and in the ability of environmental regulation to prevent, restrict or control harm to the environment due to the presence of nanomaterials either prior to or post entry into the environment."* One of the main problems is that if an existing substance has been assessed for hazard or risk, the way that most regulations are written means that the nanoscale form of this substance will not require any additional testing, even if its activity differs substantially from the

existing macroscale chemical. Frater et al. (2006) also mention the lack of information on the toxicity of nanomaterials which adds uncertainty to assessments of risk.

2.5 Environmental Defense – DuPont Nano Partnership

Environmental Defense and Dupont published a draft *Nano Risk Framework* in February 2007. They identify a base set of environmental hazard data for use in assessing nanoparticles:

(i) Aquatic toxicity data for fish (acute tests with fathead minnow or rainbow trout), invertebrates (acute or chronic data for *Daphnia*) and algae.

(ii) Terrestrial toxicity data for earthworms and terrestrial plants.

(iii) Bioaccumulation potential screening test (either a simple octanol-water partition coefficient screen, although its relevance to nanoparticles would need to be demonstrated, or a bioconcentration factor (BCF) or bioaccumulation factor (BAF) test if appropriate analytical methodologies can be developed).

(iv) Additional data to be developed as needed:
   a. ADME (Adsorption, Distribution, Metabolism and Excretion) studies on aquatic organisms;
   b. Chronic toxicity to soil microorganisms (carbon or nitrogen inhibition tests) and sediment- or soil-dwelling organisms;
   c. Further testing for toxicity using additional terrestrial species;
   d. Avian toxicity testing;
   e. Population/ecosystem-level studies; and
   f. Activated sludge respiration inhibition test (if release is to wastewater treatment).

Environmental Defense and Dupont (2007) argue that standard toxicity tests with fish, invertebrates and algae are used to produce toxicity base sets *"…in virtually every voluntary and regulatory program used throughout the world…"* and that there would need to be a compelling justification for use of different test organisms. A chronic *Daphnia* study would be triggered if the nanoparticle was potentially persistent or bioaccumulative. A sediment-dweller test with an organism such as the amphipod *Hyalella azteca* might also be triggered if a nanoparticle is likely to accumulate in sediment.

Additional tests would be triggered if the nanoparticle is,

(i) Produced at high volume;
(ii) Released to the environment in large amounts;
(iii) Detected in the abiotic environment or in wild biota;
(iv) Continuously released;
(v) Used broadly and diversely, or has the potential for widespread environmental dissemination;
(vi) Subject to significant change in its production, processing or use pattern;

(vii) Of uncertain or high inherent hazard potential;
(viii) Highly persistent or bioaccumulative;
(ix) Released to wastewater treatment plants or soils; or
(x) Potentially bioaccumulated or biomagnified up food chains.

2.6 SCENIHR opinions

The European Commission's Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) has published two opinions (SCENIHR 2005, 2007) with relevance to the ecotoxicity testing of nanomaterials.

SCENIHR (2005) concluded that current risk assessment methodologies may require some modification before they are fit for purpose in assessing the hazards and risks of nanomaterials. However, they also state that *"It is assumed that the range and type of adverse effects that could arise from exposure to nanoparticles is likely to be similar to that identified for chemicals in other physical forms. If this assumption is correct, there would be no reason to change existing well established toxicity testing protocols. If it is not, additional endpoints may need to be considered for the toxicological assessment of nanoparticles"* It seems that in the specific case of ecotoxicity tests, the modifications envisaged by SCENIHR (2005) relate to the environmental relevance of the way in which organisms are exposed to nanoparticles, and the way in which the concentration of nanoparticles is expressed (which they, and many others, suggest should be as number concentration or surface area, rather than simply mass concentration).

SCENIHR (2005) recommend a screening assessment approach to determine whether nanoparticles present similar hazards to other physical forms of a substance. This is summarised in Figure 2.1, which shows that selection of a "test battery" of appropriate tests for demonstrating equivalent hazard is an essential step in implementing this approach. SCENIHR (2005) suggests that in vitro and chemical tests could play an important role in such a screen, but *"…no appropriate tests are currently available."*

SCENIHR (2007) recently published another opinion on the appropriateness of risk assessment methodologies for dealing with nanomaterials. They conclude the following:

(i) Exposure and dose-effect models may need to be adapted to take into account the changing physico-chemical properties of nanoparticles over time, including their slow degradation. For example, if rapid agglomeration occurs then only the assessment of agglomerated particles may be necessary.

(ii) Nanoparticle surface area or particle number per volume should be measured and reported in exposure and effects assessments in addition to mass or mass per volume.

(iii) The uptake, distribution, clearance and effects of nanoparticles may differ from those of substances for which the Technical Guidance Document (EC 2003) was initially developed. This and the lack of information on different species sensitivities to nanoparticles means that no clear guidance can be given on the appropriateness of test species and methods used to assess

ecotoxicity and bioaccumulation. However, SCENIHR does state that *"A tiered approach to hazard identification and characterisation is…appropriate in respect to the environment. The system set out in the Technical Guidance Document is, in principle, appropriate. However some adaptation of the methodology in order to apply it to nanoparticles is likely to be needed."*

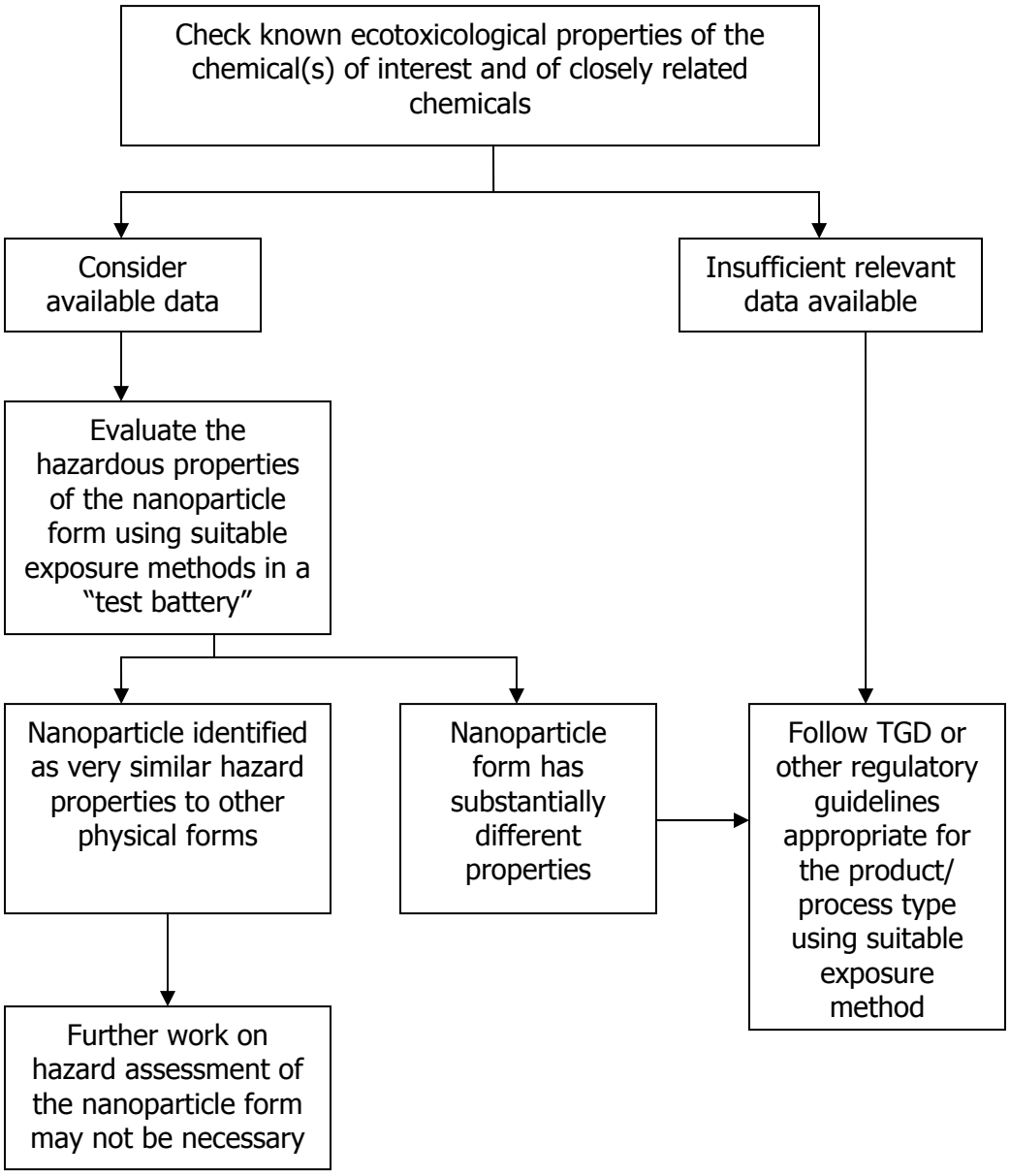
(iv) Environmental toxicity studies will require both acute and chronic exposures, using standard laboratory species, focussing on the identification of pertinent endpoints specifically relevant for nanoparticles.

The SCENIHR (2007) opinion also states that *"…particle size and shape characteristics should be measured in the most relevant dispersed state"* and that *"Ideally, the nanoparticle characteristics should be measured under conditions that mimic those of the potential human and environmental exposure."* They also state that *"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."*

2.7 United States Environmental Protection Agency

USEPA (2007) produced a *Nanotechnology White Paper* in February 2007. This report reaches the same conclusion as others summarised in this section about the need for standard ecotoxicity data within a tiered risk assessment framework to fill our absence of knowledge. However, it also points out that nanomaterials are engineered to have very specific properties and as a result of this may have unusual toxicological effects. The report then states that *"Experiences with conventional chemicals suggest that in these cases, chronic effects of exposure are often a more important component of understanding ecological risk than acute lethality. As such, initial studies should include longer-term exposures measuring multiple, sub-lethal endpoints."*

Figure 2.1 Approach to determine whether the hazards of nanoparticles differ from other physical forms of a substance (SCENIHR 2005)



2.8 Summary of reported opinions on the ecotoxicity of nanoparticles

The consensus of views expressed in the reports discussed above can be summarised as follows:

- There is little empirical information on the ecotoxicity or bioaccumulation potential of nanoparticles and, depending on exposure pathways, these data need to be generated for aquatic and terrestrial microbes, plants and animals to reduce uncertainties about the hazards posed by these substances.

- Current chemicals regulations do not distinguish between the nanoscale and macroscale forms of substances, so ecotoxicity tests performed on the macroscale form may from a legal point of view need to be accepted for both macroscale and nanoscale forms by regulatory authorities. This needs to change so that, at the very least, an evidence-based case is presented by manufacturers to show that there is no difference in the hazards of nanoscale and macroscale forms of the same substance.

- A tiered environmental risk assessment approach similar to that used for macroscale chemicals is likely to be fit for purpose for assessing the risks of nanoparticles. Requirements for further data within such a tiered framework would be triggered by information on use and release patterns, environmental fate, persistence and bioaccumulation.

- Standard methods for testing ecotoxicity are preferable because they provide the demographic data on survival, growth and reproduction that are required by environmental risk assessors, and for species whose strengths and limitations in toxicity testing are well understood. Additional endpoints could be measured on these species if there is the need to understand mechanisms of toxic effect, but only Tran et al. (2005) argue strongly for extensive use of additional toxicity endpoints. Others (e.g., NRCG 2005, Environmental Defense and Dupont 2007) suggest that understanding of mechanisms of action may primarily be of help in the use of read-across from one substance, or form of substance, to another.

- Organisms in ecotoxicity tests should be exposed to nanoparticles in a way that is environmentally relevant. Predicted Exposure Concentrations and Predicted No Effect Concentrations should be based on the same form of nanoparticle (e.g., agglomerated or dispersed) and expressed in the same units so that Risk Characterisation compares like with like. This form may change during the lifecycle of a nanoparticle, and it may therefore be necessary for tests to be repeated on these different forms to characterise risks adequately. The concentration of nanoparticles in ecotoxicity tests should be expressed as number or surface area per volume and not just as mass per volume.

- Rapid test systems (e.g., in vitro or genomics tests) for measuring the toxicity of nanoparticles may also be a useful addition to the standard suite of ecotoxicity tests, especially if they are used to demonstrate the similarity or dissimilarity of toxicity of macroscale and nanoscale forms.

- In contrast to the call for rapid test systems, there may also be a case for requiring manufacturers to generate chronic ecotoxicity data even in the absence of triggers from short-term tests, as suggested by USEPA (2007). There is a recent precedent for this in European guidelines for the testing of human medicines (EMEA 2006) in which manufacturers are required to generate chronic toxicity data.

There is also mention in several of the reports of the potential for joint toxicity caused by mixtures of nanoparticles with other substances, such as heavy metals. This may be a cause for concern, but none of the reports provides a solution to this potential problem, which is widely acknowledged as a general issue by environmental risk assessors when considering mixtures of chemicals in the environment.

3. REVIEW OF ECOTOXICITY STUDIES TO CHARACTERISE NANOMATERIAL HAZARDS

3.1 Introduction

The implications of nanoparticles for humans have been discussed in the context of routes of exposure and public health (Handy and Shaw 2007a), occupational exposure to nanomaterials in the work place (HSE 2004), and respiratory toxicity in rodent models (Warheit et al. 2005). However, until recently there has been less investigation and debate about the ecotoxicity of nanoparticles (Moore 2006). In this section we aim to:

(i) summarise current data on the toxicity and ecotoxicity of manufactured nanomaterials from the peer-reviewed published literature;

(ii) rationalise this ecotoxicity data in the context of the chemistry of nanoparticles; and

(iii) consider whether current ecotoxicity tests are "fit for purpose" and what end points we should select to measure the biological effects of nanomaterials.

3.2 Issues of concern raised by mammalian studies

The literature on mammalian models has recently been reviewed (Handy and Shaw 2007a&b). Table 3.1 gives some examples of the respiratory toxicity of nanoparticles in small mammals. Carbon nanotubes (CNTs) can cause significant lung damage to mammals when exposed to intratracheal (i.t) doses. For example, mice exposed to a dose of 0.5 mg CNT showed 56% mortality within 7 days of exposure: macrophage granulomas formed beneath the bronchial epithelium, along with necrosis and inflammation of interstitial and peribronchial tissues during the 90-day post-exposure follow-up (Lam et al. 2004). Metal oxides also produce lung injury during respiratory exposure. Rats exposed to cadmium oxide nanoparticles for 6 hours showed increased numbers of neutrophils and multifocal alveolar inflammation. In 50% of the rats exposed to 550 μm m^{-3}, an elevated blood cadmium level was also measured, suggesting movement of the particle throughout body systems (Takenaka et al. 2004).

These reports raise a number of concerns from the perspective of ecotoxicology. First, the lung is representative of typical mucous epithelial tissue and it is possible that similar epithelia in aquatic organisms could also show toxic effects. The epithelia of concern would be the gills and gut tissue of fish/invertebrates, as well as specialised epithelial tissue like the mantle of shellfish or the body surface of organisms such as earthworms. Smith et al. (2007) recently showed epithelial injury to the gill and the intestine during CNT exposure in trout. Second, the latent effects of acute respiratory exposure and the inflammation reactions in rat lung raise concerns about the long term health of organisms after even quite short exposures. However, it could also be argued that the typically milligram doses used in rodent studies are not likely to occur routinely in the environment, except during accidental spills of nanomaterials.

Table 3.1. Selected examples of the toxic effect of nanoparticles on small mammals (modified from Handy and Shaw 2007a).

Nanomaterial/chemical characteristics	Dose and exposure time	Exposure route	Species	Toxic Effects	Authors
SWCNT. 1.4 nm particles, predicted to agglomerate into 30 nm diameter ropes. Impurities include 5 % nickel and 5 % cobalt. Dispersed in Tween 80 and PBS using polytronic dispersion method.	1 or 5 kg^{-1}. Observed post exposure for 24 hrs then 1,4 or 12 weeks	Intratracheal instillation	Rats	Multifocal macrophage containing granulomas in lung	Warheit et al. (2004)
SWCNT. Experiments with non-purified ("raw") nanoparticles (impurities: 26.9 % Fe, 0.78 % Ni and trace Cu, Mo and Zn) and more purified product (main impurity: 2.14 % Fe. Dispersed by sonication in heat inactivated mouse serum.	0.1 or 0.5 mg /mouse, observed post-exposure for 7-90 days	Intratracheal Instillations	Mice	56 % mort in 0.5 mg dose. Weight loss, lung lesions in 0.5 mg group - necrosis, macrophages and granulomas, interstitial & peribronchial inflammation.	Lam et al. (2004)
MWCNT. Products reported to be 80 and 90 % pure. The 80 % purity preparation contained <0.01 mg l^{-1} Fe. Nanotubes dispersed in PBS with 1 % Tween.	12.5 mg. Exposed for 3 months	Intratracheal instillation	Guinea pigs	Caused 'organising pneumonitis' pulmonary lesions.	Grubek-Jaworski et al. (2006)

Nanomaterial/chemical characteristics	Dose and exposure time	Exposure route	Species	Toxic Effects	Authors
MWCNT. Size range 7.6-15.2 nm diameter, length 5.9 µm. MWCNT were ground up (length, 0.7 µm). Preparations were 97.8 - 98 % pure, but impurities were not stated.	0.5,2.0 or 5.0 mg /rat, observed post-exposure for 3 and 15 days (inflammatory response) and 60 days (histopathology, biopersistence, fibrotic response)	Intratracheal instillation	Rats	Intact MWCNT produced collagen-rich granulomas and surrounding alveolitis. Ground MWCNT induced inflammatory and fibrotic responses.	Muller et al. (2005)
SWCNT Size, 0.8 - 2.2 nm diameter, length not given Impurities include 1-1.5 % Fe. No dispersion, implanted in neat form.	2 mg implant for 3 months	Subcutaneous implantation	Mice	SWCNT produced some activation of the histocapability complex, in CD4+/CD8+ T-cells.	Koyama et al.(2006)
Ultrafine TiO_2 Particle diameter 21 nm. No dispersion, inhaled in neat form.	0.5, 2.0 or 10.0 mg m^{-3} for 6 hrs per day for 5 days a week over 13 weeks - with recovery up to 52 weeks	Aerosol inhalation	Mice, Rats and Hamsters	Pulmonary particles overload and inflammation in rats and mice exposed to 10 mg m^{-3}. Inflammation included increased number of macrophages and neutrophils, progressive fibrosis in rats, elevated protein and lactate dehydrogenase levels.	Bermudez et al. (2004)
TiO_2 particles rods or dots. Mostly anatase crystal structure, size approximately 200 nm x 35 nm. Added to PBS and then subjected to polytronic dispersion.	1 or 5 mg dose exposure, followed recovery for up to 3 months.	Intratracheal instillations	Rats	Exposures to nanoscale TiO_2 rods or nanoscale TiO_2 dots produced transient inflammatory and cell injury effects at 24 h post exposure and were not	Warheit (2006)

Nanomaterial/chemical characteristics	Dose and exposure time	Exposure route	Species	Toxic Effects	Authors
				different from the effects of TiO_2 particles. In contrast, pulmonary exposures to quartz particles in rats produced lung inflammation and fibrosis.	
Ultrafine cadmium oxide. 40 nm diameter.	70 µg m^{-3} for 6 h, observed post-exposure for 0,1,4 and 7 days post exposure, or 550 µg m^{-3} for 6 h, observed post exposure for 0 and 1 days	Inhalation	Rats	Increased percentage of neutrophils at day 0, and multifocal alveolar inflammation on days 0 and 1, of the higher dose group, 2 of 4 rats also showed elevated blood Cd.	Takenaka et al. (2004)
Ultrafine metallic nickel	0.15, 1.14 or 2.54 mg m^{-3} for 5 h, observed post exposure at 1,3,7,14 and 21 days	Inhalation	Rats	Dose-dependant increase in pulmonary nickel, increase in lung weight over time (mid and high doses), accumulation of foamy alveolar macrophages (AM) degenerated AM indicating alveolar lipoproteinosis (aggravated up to 4 weeks in high dose group), acute calcification of the degenerated AM.	Serita et al. (1999)

PBS, phosphate buffered saline; SWCNT, single walled carbon nanotube; MWCNT, multi-walled carbon nanotube.

With few exceptions, the work carried out using live mammals has focused on the effects of intratracheal instillations or inhalation in rodents. *In vitro* studies with mammalian cells have looked at a wider range of effects in different cells from animals and humans (Table 3.2). Some common themes are emerging in terms of toxic effects. For example, most of the studies with carbon-based nanomaterials show increased cell permeability (e.g., lactate dehydrogenase leak) or cell death. Studies on cells with metal-based nanoparticles raise the issue of oxidative stress. Studies on both types of nanomaterials suggest the release of factors involved in inflammation from cells (Table 3.2). Microscopy studies have shown an alteration in the appearance of the nucleus, mitochondria, tonofilaments and other cytoplasmic organelles and the morphology of the cell membrane itself (Shvedova et al. 2003, Tian et al. 2006). Clearly, if aquatic organisms and other wildlife could absorb μg ml^{-1} levels of nanoparticles into the blood (or other extracellular fluid) then we might expect to see some of these toxic effects in a range of cells in different tissues. If enough cells are injured, this could lead to organ dysfunction and ultimately poor health of the wildlife

3.3 Laboratory studies on the ecotoxicity of nanomaterials

The studies on mammals described above raise the concern that there are some toxic effects of nanomaterials (albeit at mg doses), and we should therefore establish whether or not manufactured nanoparticles are toxic to wildlife. There is an emerging literature on the ecotoxicity of nanomaterials, with most of the studies on aquatic organisms, using a few types of nanoparticles that are commercially available.

Lethal toxicity of nanoparticles

There are few lethal dose values published on the ecotoxicity of nanoparticles (Table 3.3), although new studies are being published at an increasing rate. Studies on fish and invertebrates (Lovern and Klaper 2006, Zhu et al. 2006) suggest that C_{60} fullerenes are toxic in the milligram per litre range, but the LC50 values obtained are very dependent on the method of preparation of the material and the addition of dispersants (Table 3.4). It is possible that dispersed C_{60} nanoparticles are more toxic than "non-dispersed" material, or that the solvents used have some effects, or somehow change the toxicity of the dispersed nanoparticles themselves. The general lack of LC50 values for fish may also be for technical reasons. Maintaining the high mg l^{-1} concentrations needed to achieve acute lethal toxicity is difficult. At concentrations above 10 mg l^{-1} there is significant aggregation with many types of nanoparticles, and even with prolonged sonication plus the addition of dispersants it remains difficult to achieve reproducible solutions (Handy, personal observations). Interestingly, there are no lethal toxicity values for *in vitro* assays using non-mammalian cells. Work on acute toxicity to fish and invertebrate cell lines is therefore required if rapid tests with these models is required for risk assessment.

Table 3.2. Selected examples of in vitro studies on nanoparticles using mammalian cells (modified from Handy and Shaw 2007a).

Nanomaterial/chemical characteristics	Dose and exposure time	Exposure route	Toxic Effects	Authors
Silver nanoparticles. Size, 15 or 100 mn. Dispersed in deionised water.	5.0 - 50.0 µg ml^{-1} for 24 hrs.	Rat liver derived cell line.	Lactate dehydrogenase (LDH) leakage from cells was observed. Loss of mitochondrial function and cell glutathione levels decreased. Oxidative stress and the production of reactive oxygen species (free radicals) were suggested.	Hussain et al. (2005)
Silver nanoparticles. Ag particles ~15 nm diameter dispersed in PBS.	5 - 100 µg ml^{-1}	Mouse Spermatogonial stem cell lines	Mitochondrial function was adversely affected, cells showed increased LDH leakage.	Braydich-Stolle et al. (2005)
SWCNT Size and purity not specified.	Serial dilutions from 0.78 - 200 µg ml^{-1}	Human HEK293 (human embryo kidney) cells	Inhibition of HEK293 cell proliferation, induced cell apoptosis, and decreased cellular adhesive ability.	Cui et al. (2005)
MWCNT Sonicated in Keratinocyte growth medium before harvesting. Nanotubes grown in a monolayer and scrapped manually in to the dishes containing cells.	0.1, 0.2 and 0.4 mg ml^{-1} for 1, 2, 4, 8, 12, 24 and 48 hrs	Human epidermal keratinocytes (HEK)	MWCNT present within the cytoplasmic vacuoles of the HEK. Exposure initiated irritation response including the release of the pro-inflammatory cytokine interleukin 8.	Monteiro-Riviere et al. (2005)
SWCNT and MWCNT. SWCNT, size 2 x 500 nm and surface area of 3.15 μm^2. MWCNT,size 50 nm x 5 µm and surface area - 789 μm^2 Sonicated in Keratinocyte growth medium	0.8-100 µg ml^{-1}	Human fibroblast cells	SWCNT and MWCNT showed a decrease in surviving cell numbers to 58 and 78 % respectively. SWCNT affected cell morphology by creating membrane "ruffles" and rounding of the cell. Refined (catalytic metals removed) SWCNT caused increased cell mortality (apoptosis/necrosis) when compared to unrefined.	Tian et al. (2006)
Ultrafine carbon black or nanoparticle carbon black.	Exposure to foetal bovine	Macrophage migration assay	Ultrafine carbon caused a 1.8-fold increase in macrophage migration over the control rate;	Barlow et al. (2005)

Nanomaterial/chemical characteristics	Dose and exposure time	Exposure route	Toxic Effects	Authors
Ultrafine particles: 260 nm diameter, surface area 7.9 m^2 g^{-1}. Nanoparticles: 14 nm diameter, surface area 254 m^2 g^{-1}.	serum that was pre-treated with 5 or 10 mg ml^{-1} of the particle.	in foetal bovine blood serum previously exposed to particles	addition of antioxidants abolished the effect. Nanoparticles did not increase macrophage migration rates compared to the ultrafine carbon.	
SWCNT 30 % iron content by weight. Size not specified.	0.06-0.24 mg ml^{-1} for up to 18 hrs	HEK cells in culture	Cells showed a dose-dependant decrease in viability and a depletion of antioxidants including vitamin E and glutathione. Oxidative stress is indicated be an increase in thiobarbituric acid reactive substances (TBARS assay).	Shvedova et al. (2003)
TiO_2 nanoparticles. Anatase form, 10 or 20 nm diameter. Rutile form, 200 nm diameter.	10 µg ml^{-1} final concentration in the culture dish for 1 hr exposures.	Human bronchial Epithelial cells (BEAS 2B)	Anastase form caused DNA damage, lipid peroxidation and the appearance of hydrogen peroxide in the medium, micro nuclei formation indicating damage to nucleus. Rutile form was generally much less toxic.	Gurr et al. (2005)
TiO_2 nanoparticles. Anatase and rutile forms, size varies.	A range of dilutions from 3 to 30 mg ml^{-1} added to culture dishes for up to 48 hrs.	Human lung epithelial cells (A549 cells)	Cells show a dose-dependant decrease in viability, some lactate dehydrogenase release from cells at the highest doses. Inflammatory mediators (interleukin 8) induced at highest doses.	Sayes et al. (2006)
TiO_2 nanoparticles Size; 20-30 nm, sonicated for 2 minutes.	5 µg ml^{-1} suspensions added to culture dish, exposure 4-24 hrs.	Human red blood cells	Electron microscope studies a show aggregates of nanoparticles appear in the cells, indicating they are readily taken up by blood cells. Red cell morphology is not generally affected suggesting the red cells are not damaged.	Rothen-Rutishauser et al. (2006)

Table 3.3. Toxic effects of aqueous nanoparticle exposures to fish and invertebrates.

Nanomaterial/chemical characteristics	Concentration and exposure time	Species	Toxic Effects	Authors
SWCNT Size, 1.1 nm mean outside diameter, 5–30 µm length. Dispersed in sodium dodecyl sulphate (SDS) and with sonication. Impurities in mnaufacture: Al 0.08, Cl 0.41, Co 2.91, and S 0.29 %; but not detected in solutions.	0.1 – 0.5 mg l^{-1} for 10 days	Rainbow trout (*Oncorhynchus mykiss*)	Increased ventilation rates, gill pathology and mucus secretion. Disturbances to Zn and Cu levels in the brain and gill. Up regulation of Na^+K^+-ATPase activity in gill and intestine. TBARS decline in gill, brain and liver. Total glutathione increased in gill and liver. Liver and brain pathology reported.	Smith et al. (2007)
C_{60} Fullerenes Dispersed in tetrahydrofuran (THF), but not sonicated. Aggregates of 30-100 µm. Uncoated. 99.5 % Carbon, 0.5 % impurities not specified.	0.5 or 1.0 mg l^{-1} for 48 hours	Large mouth bass (*Micropterus salmoides)* Juveniles	Reduced lipid peroxidation in liver and gill tissues (0.5 mg l^{-1} treatment) and increased lipid peroxidation in brain tissue (0.5 and 1.0 mg l^{-1} treatment). GSH activity reduced in the gill tissue (0.5 mg l^{-1} treatment).	Oberdörster (2004)
C_{60} fullerenes. Dispersed by stirring in water for a minimum of 2 months, 10-200 nm aggregates formed. 99.5 % pure, 0.5 % impurities not quantified.	0.5 mg l^{-1} for 96 hours	Fat head minnow (*Pimephales promelas)*	Down regulation in the expression of PMP70 by 42 %. No alteration in the expression of CYP iso-enzymes. Japanese medaka show no response over 48 h in the same experiment.	Oberdörster et al. (2006)
C_{60} fullerenes. Dispersed by stirring in water for a minimum of 2 months, 10-200 nm aggregates formed. 99.5 % pure, 0.5 % impurities not	0.5 mg l^{-1} for 48 hours	Fat head minnow (*Pimephales promelas)*	Elevated lipid peroxidation indicated in the gill, possibly the brain. Increases in some hepatic CYP2-like iso-enzymes resulting in changes in P450 protein	Zhu et al. (2006)

Nanomaterial/chemical characteristics	Concentration and exposure time	Species	Toxic Effects	Authors
quantified.			expression.	
C_{60} Fullerenes Dispersed in THF and filtered (not sonicated). Particle size was 0.72 nm diameter which formed aggregates of 10-20 µm. Uncoated. Impurities not specified.	40 - 880 µg l^{-1} for 48 hours	*Daphnia magna*	Positive correlation between mortality and concentration. 100 % mortality shown at 880 µg l^{-1}.	Lovern and Klaper (2006)
C_{60} Fullerenes As above but sonicated instead of using solvent and unfiltered. Formed aggregates of 20-100 µm.	0.2 - 9 mg l^{-1} for 48 hours	*Daphnia magna*	Highest mortality rates at 9 mg l^{-1}.	Lovern and Klaper (2006)
C_{60} fullerenes. Dispersed by stirring in water for a minimum of 2 months, 10-200 nm aggregates formed. 99.5 % pure, 0.5 % impurities not quantified.	0.5 - 5.0 mg l^{-1} for 21 days	*Daphnia magna*	Decreased offspring production over the 21 day period, reduction contributed to by increased mortality (<40 %) and delayed moulting. Initial brood stock significantly reduced by 0.5 mg l^{-1} exposure.	Oberdörster et al. (2006)
C_{60} fullerenes. Dispersed by stirring in water for a minimum of 2 months, 10-200 nm aggregates formed. 99.5 % pure, 0.5 % impurities not quantified.	7 mg l^{-1} or less for 96 hours	*Hyalella azteca*	No effects seen on locomotion, moulting or feeding behaviour.	Oberdörster et al. (2006)
C_{60} fullerenes. Dispersed by stirring in water for a minimum of 2 months, 10-200 nm aggregates formed. 99.5 % pure, 0.5 % impurities not quantified.	22.5 mg l^{-1} or less for 96 hours	Marine Harpacticoid copepod (species not identified)	No toxic effects seen.	Oberdörster et al. (2006)

Nanomaterial/chemical characteristics	Concentration and exposure time	Species	Toxic Effects	Authors
Fluorescent mono-dispersed nanoparticles made of latex (polystyrene). A range o of diameters used in the experiments: 39.4, 474, 932, 18600, 42000 nm diameter Nanomaterial exposure was performed in an embryo rearing solution (a physiological saline*).	1 -30 mg l^{-1}, depending on the experiment	See-through medaka (*Oryzias latipes)* eggs and juveniles.	Exposure of eggs to 1 mg l^{-1} for 3 days did not cause egg mortality and fluorescence was detected in whole egg, especially the chorion. Particle size affected fluorescence, and increasing salinity precipitated higher doses of the nanomaterial. In the juvenile fish, fluorescence was detected especially in the gills and gut, the blood, and possibly other internal organs.	Kashiwada (2006)
TiO_2 nanoparticles. Particles of 10-20 nm diameter were dispersed in THF and filtered (but not sonicated). 30 nm aggregates were formed.	0.2 -10 mg l^{-1} for 48 hours	*Daphnia magna*	Positive correlation between mortality and concentration. 100 % mortality shown at 10 mg l^{-1}.	Lovern and Klaper (2006)
TiO_2 nanoparticles. Particles of 10-20 nm diameter as above, but dispersed by sonication (no THF and unfiltered. 100-500 nm aggregates were formed.	50 -500 mg l^{-1} for 48 hours	*Daphnia magna*	No significant mortalities reported.	Lovern and Klaper (2006)
TiO_2 nanoparticles.	0 – 1.0 mg l^{-1} for up to 14 days	Rainbow trout (*Oncorhynchus mykiss*)	No major haematological or iono-regulatory disturbances although changes in tissue Na^+K^+-ATPase are reported. Oxidative stress occurred as evidenced by changes in tissue TBARS and total glutathione. Pathologies are reported in the gill and other internal organs.	Federici et al. (2007)

SWCNT, Single walled carbon nanotubes; MWCNT, Multi walled carbon nanotubes; THF, tetrahydrofuran; SDS, sodium dodecyl sulphate; TBARS; thiobarbituric acid reactive substances; *, embryo rearing medium (g l^{-1}); 1 NaCl, 0.03 KCl, 0.04 $CaCl_2$, 0.163 $MgSO_4$, adjusted to pH 7.2 with 1.25 % sodium bicarbonate.

Table 3.4. The effect of nanoparticle preparation method on lethal toxicity estimates.

Nanomaterial	Preparation method	LC50 (48 hr)	NOEC (48 hr)	LOEC (48 hr)	Concentration causing 100 % mortality (48 hr)	Author
TiO_2	Filtered	5.5 mg l^{-1}	NM	2 mg l^{-1}	10 mg l^{-1}	Lovern and Klaper (2006)
C_{60} fullerenes	Dispersion attempted by sonication	7.9 mg l^{-1}	0.2 mg l^{-1}	0.5 mg l^{-1}	NM	
C_{60} fullerenes	Dispersion attempted with THF	0.46 mg l^{-1}	0.18 mg l^{-1}	0.26 mg l^{-1}	0.88 mg l^{-1}	
C_{60} fullerenes	Dispersion attempted by stirring.	>35 mg l^{-1}	NM	NM	NM	Zhu et al. (2006)
C_{60} fullerenes	Dispersion attempted with THF	0.8 mg l^{-1}	NM	NM	NM	

THF, tetrahydrofuran, used to disperse C_{60} fullerenes. LC_{50}, median lethal effect concentration for 50 % of organisms after 48 hrs. NOEC, no observable effect concentration. LOEC, lowest observable effect concentration. NM, not measured.

Sub-lethal effects of aqueous exposure to nanoparticles in fish

Several authors have exposed teleost fish to nanomaterials (Table 3.3). These include the effects of C_{60} fullerenes on large mouth bass (*Micropterus salmoides,* Oberdörster 2004), fathead minnow (*Pimephales promelas*, Oberdörster et al. 2006, Zhu et al. 2006), and Japanese medaka (*Oryzias latipes*, Oberdörster et al. 2006). The effects of CNT's (Smith et al. 2007) and TiO_2 nanoparticles (Federici et al. 2007) on rainbow trout (*Oncorhynchus mykiss*,) have also been studied. These studies have identified potential target organs for nanoparticles. Kashiwada (2006) examined the body distribution of fluorescently labelled nanoparticles in a transparent colour morph of the medaka (*Oryzias latipes*). This experiment used measurements of fluorescence in the organs of this "see-through" fish to infer the location of the nanoparticles. The fish were exposed to mono-dispersed, non-ionised, fluorescent polystyrene microspheres with a diameter of 39.4 nm at an aqueous concentration of 10 mg l^{-1} for 7 days. The gills, as expected, showed the greatest increase in fluorescence, shortly followed by the intestine. This suggests that nanoparticles can at least attach to the gill surface or may enter the epithelial cells. Similar observations have been made in trout where CNT precipitate on the gill mucus during aqueous exposure (Smith et al. 2007). However, in the medaka study the increases in fluorescence in the internal organs were relatively small and, apart from the gall bladder, there were no statistically significant increases in fluorescence in other tissues (brain, liver, kidney or testis; Kashiwada 2006). Of course, this does not mean that the fluorescent nanoparticles were not absorbed into the blood and circulated to the internal organs; it could simply be that exposure time needed to be longer or that excretion rate matched uptake rate (with no net accumulation). However, some caution is also needed when interpreting the results of any experiment with labelled nanoparticles. For example, unequivocal evidence that the fluorescent label remains attached to the nanoparticles inside the tissues is often unavailable.

Both the study on medaka (Kashiwada 2006) and rainbow trout (Smith et al. 2007) demonstrated the presence of nanoparticles in the gut, despite the fact that the delivery route was via aqueous exposure. Freshwater fish do drink a few ml of water kg^{-1} body mass h^{-1} (Eddy 1982) and Smith et al. (2007) argue that this could explain the appearance of nanoparticles in the gut. This would be especially important during toxicity, since a stress-induced drinking response could greatly increase the amount of water imbibed (Smith et al. 2007).

Other target organs for nanomaterials in fish are inferred from observations of toxic effects in those organs, rather than demonstration of nanoparticles within the organ/tissue of concern. This is partly because the techniques for extracting and measuring nanoparticles in tissues are not routinely available. However, we should not exclude the possibility that toxic effects occur at doses that are difficult to detect in individual organs (i.e., there is high potency). For CNT's at least, the liver appears to be an important target organ. Smith et al. (2007) demonstrated pathology in the livers of trout exposed to up to 0.5 mg l^{-1} CNT for 10 days. Histological changes included altered nuclear morphology with condensed nuclear bodies that had the appearance of apoptotic bodies, and cells with diffuse nuclei in the early stage of cellular necrosis. Biochemical change is also observed in the liver. Smith et al. (2007) found a statistically

significant reduction in thiobarbituric acid reactive substances (TBARS) in the livers of trout exposed to CNT. In contrast, Oberdörster (2004) did not find such an increase in largemouth bass exposed to C_{60} for a much shorter time. Oberdörster et al. (2006) also studied the expression of mono-oxygenases (CYP family proteins) in the liver of fathead minnows exposed to 0.5 mg l^{-1} C_{60} fullerenes for 96 h. They found no effects on either hepatic CYP mRNA levels or the proteins themselves. However, they did report a statistically significant reduction in PMP70 protein, an isoenzyme involved in hepatic lipid metabolism (Oberdörster et al. 2006). Interestingly, the reduction in PMP70 may be species or exposure-time dependent, since measurements of PMP70 in medaka exposed to the same material for a shorter time (48 h) showed no effect on protein levels (Oberdörster et al. 2006).

The brain is also a potential target organ, at least for carbon-based nanomaterials (Oberdörster 2004, Smith et al. 2007). Oberdörster (2004) first suggested the possibility of brain injury in fish when a significant elevation in lipid peroxidation was seen in the brains of juvenile largemouth bass exposed to 0.5 mg l^{-1} C_{60} fullerenes. There is some logic to this concern given that several biomedical studies are attempting to exploit the permeability of the blood-brain barrier (BBB) to natural lipid micelles and particulates (endocytosis), to develop nano-drug delivery techniques for the brain (Cui et al. 2005, Dobson 2001, Kreuter 2005). Smith et al. (2007) demonstrated brain pathology in rainbow trout exposed to 0.5 mg l^{-1} CNT for 10 days. These pathologies included injury to the cerebral vasculature (suspected aneurisms) and more minor cellular injuries such as individual necrotic bodies and small foci of vacuolisation in parts of the brain (Smith et al. 2007). However, brain injury was not a feature of TiO_2 exposure in rainbow trout (Federici et al. 2007), and it seems likely that other types of nanoparticles will have different target organs.

The Smith et al. (2007) study used a body systems approach to investigate organ effects, and was able to show that CNTs did not cause major haematological disturbances, but was a respiratory toxicant. However, there are many organ systems for which data are not available, including skeletal muscle and locomotion effects, and effects on spleen, kidney or bone functions. Much more data are needed before a definitive list of all the target organs for different nanomaterials can be produced. Nonetheless, the sub-lethal exposure studies do at least identify some common themes in toxic mechanisms, which are similar to those found in mammalian studies. The mechanisms may include oxidative stress, disturbances to trace element metabolism, and vascular injury, as well as the expected injury to the gill during aqueous exposure (see Smith et al. 2007). For example, several studies report lipid peroxidation or changes in TBARS in tissues (Oberdörster et al. 2004, Zhu et al. 2006, Smith et al. 2007). Changes in anti-oxidant defences in tissues are also implicated. Smith et al. (2007) observed a compensatory increase in total glutathione in the gill and intestine during CNT exposure over 10 days, while much shorter exposures to C_{60} fullerenes might deplete tissue glutathione (Oberdörster et al. 2004).

Sub-lethal aqueous toxicity of nanoparticles to *Daphnia* and other invertebrates

There are a few reports on the effects of nanoparticles on invertebrates (Table 3.3), although there is an early report on the effects of "metal particles" coated with Telflon on *Daphnia magna* which resulted in a 48 h EC_{50} of about 20 µg l^{-1} for brass particles (Johnson et al. 1986). The use of *Daphnia* as a standard ecotoxicology test species is well known, and it is perhaps no surprise that studies have focused on this organism. Several studies have used *Daphnia* to estimate LC50 values (Table 3.3). Oberdörster et al. (2006) have also investigated chronic effects in *Daphnia* and other invertebrates. *Daphnia* were exposed to up to 5 mg l^{-1} C_{60} fullerenes over 21 days. At the highest C_{60} concentration (5.0 mg l^{-1}), mortality of 40% was observed, along with fewer offspring and a delay in moulting. In the same study, exposure of the freshwater invertebrate, *Hyallela azteca*, to 7 mg l^{-1} C_{60} fulleres for 96 h was reported to have no effect on mobility, moulting or feeding behaviour (Oberdörster et al. 2006). Roberts et al. (2007) recently reported that *Daphnia* were able to ingest and feed on lysophosphatidltycholine-coated single walled nanotubes, suggesting that the type of coatings on nanomaterials can have a profound effect on the biological response. Overall, the lack of published literature on invertebrate species, not just for aquatic organisms but also for earthworms and other soil/terrestrial invertebrates, is a significant knowledge gap. However, abstracts of work in progress are appearing in the grey literature.

Sub-lethal aqueous toxicity of nanoparticles to bacteria, algae and other aquatic plants

Similar to the situation for aquatic invertebrates, there are very few published studies on the ecotoxicity of nanoparticles to bacteria, algae and aquatic plants. Hund-Rinke and Simon (2006) exposed algae (*Desmodesmus subspicatus*) in a growth inhibition test to titanium nanoparticles and, depending on the preparation of the material, found EC_{50} values of 44 mg l^{-1} to no effects at the highest concentration used (50 mg l^{-1}). However, it has been noted that the surface structure or matrix of plant cell walls can act as a surface to grow nanoparticles. Scarano and Morelli (2003) noted that stable nano-crystals form on marine phytoplankton when exposed to Cd. This raises the possibility that metal nanoparticle exposure in marine algae could simply arise from existence of suitable conditions for crystal formation at the surface of the organism during aqueous metal exposures. Plant viruses are also used as scaffolds for nanoparticle construction (Barnhill et al. 2007). These observations raise the concern that organisms do not necessarily need to be exposed to nanomaterials added directly to the water, and the possibility that viruses could act as vectors for the movement or growth of nanoparticles. Plant cells have also been used as tools during the development of imaging technology for nanomaterials, and this has generated some incidental information on uptake. Wu et al. (2007) found that zinc oxide nanoparticles aggregate on the surface of plant cells. Published studies on the ecotoxicity of nanomaterials to bacterial species which are relevant to the aquatic environment or ecotoxicology is limited. However, the bacterium *Shewanella algae*, has been demonstrated to deposit platinum nanoparticles, and this is suggested as a biotechnology application to recover platinum (Konishi et al. 2007). This at least indicates that environmentally relevant

bacteria could accumulate nanoparticles, and may even have useful applications in the bioremediation of environmental media contaminated with nanomaterials in the future.

3.4 Physico-chemical properties of manufactured nanoparticles and implications for ecotoxicity

General considerations for ecotoxicity experiments

Nanomaterials are manufactured for a particular application and therefore the physico-chemical properties and reactivity of each material will vary. We should therefore be cautious about making general statements about the chemistry of nanomaterials. This is reflected in the ecotoxicological reports (Table 3.3) in which a range of nanomaterials of different sizes, shapes, and solubility have been used. Some of these studies attempt to disperse the material so that organisms are exposed to individual particles or small aggregates of particles; but others report experiments with untreated (raw) nanomaterials. However, there are some key features that are likely to be relevant to ecotoxicity. These include particle size, shape, surface area, solubility and the types of coatings or reactive groups added to the basic nanostructure during manufacture. It would therefore be useful to report as much of the above information as possible in the methodology of experiments on biological effects. Smith et al. (2007) give a good example, in which the authors confirm the size of the CNTs being used along with information on impurities in the products. They also use optical methods (spectrophotometry and the electron microscope) to confirm the chemical form of the nanotubes (dispersed CNTs) in the exposure medium.

However, there are almost no ecotoxicological data that have systematically investigated the physico-chemical parameters above. One assumption is that nanomaterials are made because the small size contributes some novel physico-chemical property to the manufactured product. It is therefore logical that we should expect some size/shape effects on ecotoxicity. Kashiwada (2006) showed a particle-size effect on the accumulation of fluorescent nanoparticles in the Japanese medaka (*Oryzias latipes*), with the smaller particles accumulating more quickly. Alternatively, studies on mammalian immune cells show no difference in toxicity of ultrafine carbon dust compared to much smaller carbon nanoparticles (Barlow et al. 2005). There are also reports of differences in the acute toxicity of dispersed nanoparticles compared to larger aggregates of the same material in invertebrates (Lovern and Klaper 2006; Table 3.4). So, the presence or absence of aggregates of nanoparticles is likely to be important when assessing the ecological hazards of nanoparticles.

Minimum set of measurements for chemical characterisation

Given the general discussion above, there needs to be some agreement in the scientific community on the minimum requirements for the characterisation of nanoparticles. This will not only enable journal editors to set standards for peer reviewed publications in this new field, but could also be used to define a base set of measurements needed to characterise the exposure in a regulatory test. The following are suggested as a minimum base set of data:

- The nomenclature information and manufacturer's information indicated in Appendix 1 of this report.
- Concentration of the material (e.g. mg l^{-1}).
- Electron Microscope Images (or similar) of the material in solution.
- Measurement of the individual particle size in the stock solutions used for dosing the test system. This would include mean particle size ± standard deviation for at least 6 replicate samples of the stock solution.
- Measurement of the size of any agglomerated or aggregated material in the solution (means and standard deviation as above).
- Use of a spectroscopic method, or other similar method, to confirm dispersion of the material at various dilutions of the stock solution within the detection limits of current methods.
- Some measurements to confirm concentrations of the main expected impurities in the test material, and details of any washing procedures to remove these if this is desired in the experiment.

This base set of measurements is suggested as a practical approach given the limitations of the current technology for measuring the chemistry and particle sizes (see Appendix 2 for a summary of these methods) and also considering what can be reasonably expected of a competent ecotoxicology laboratory. Therefore the minimum equipment needed would be access to an electron microscope to take photographs and measure particle size using the standard image tools on such microscopes. In addition, a scanning spectrophometer should be available to show the characteristic signature of the material, or more simply a bench-top spectrophotometer or fluorimeter for materials where absorption peaks have already been identified for the material. The issue of impurities could produce an endless list of chemical analysis, but this is not the intention here. The logic is to identify the key chemicals of concern by considering knowledge on the manufacturing process and the manufacturer's information and whether or not these chemicalss are likely to be toxic to the test organism at the dilutions used.

It is possible to suggest a subsequent tier of additional information. This might include (i) detailed particle size distribution plots, (ii) measurements of particle surface reactivity, and (iii) detailed repeated characterisations of all the above for each dilution of the test media where aggregation/agglomeration is known to change with concentration of the material. However, there are concerns that the ecotoxicologist could be easily overburdened with excessive and unnecessary demands for test substance characterisation, when only a practical minimum is required. We should also consider achieving a "level playing field" across the ecotoxicology disciplines from the view point of publishing experimental work. For example, how many ecotoxicology studies on chemicals such as pesticides report detailed measured concentrations of the chemical, its structure in solution (isoforms) or chemical impurities measured in the formulation for each test solution? We do not advocate a situation where important data on biological effects are not published just because the supporting chemistry is limited by expertise or current technology. Equally, it is important that mistakes are not made in the biological interpretation of data because the chemistry has not been given enough attention.

Particle surface area issues and ecotoxicity

The surface area of the material (e.g. m^2 g^{-1} of material) may be more important than size/shape, especially if the reactive properties or chemical groups are on the external surface. There is some speculation that the ability of nanoparticles to generate reactive oxygen species (ROS) at the surface of the nanoparticles, and adjacent to the cell membrane, might initiate inflammation reactions or immune responses (Barlow et al. 2005). Oxidative stress has been reported in the tissues of aquatic organisms during nanoparticle exposure (CNT, Smith et al. 2007; TiO_2, Federici et al. 2007). There is also some logic to explanations of why ROS may be generated in aquatic tests. Consider the arguments for TiO_2 nanoparticles which are well known for their catalytic activity (Watanabe et al. 1999, Hirano et al. 2005). Federici et al. (2007) argue that ROS generation is from the catalytic chemical properties of TiO_2 nanoparticles, which in the presence of light, can transfer electrons from substrates such as hydrogen peroxide or chlorinated organic compounds to generate the hydroxyl radical (Hirano et al. 2005). Since these substrates are not normally present in the aquarium water of ecotoxicity tests, then these reactions are most likely to occur in contact with test organism tissue, e.g., by using the normal trace endogeneous hydrogen peroxide production from cells, or in the case of the gut any residue organic matter in the gut lumen. In any event this ROS production can be relatively slow, unless the material is also sonicated (see below).

Whatever the mechanisms involved, there is some evidence of particle size effects. The mammalian literature contains many reports of particle size effects. For example, Oberdörster et al. (1992) showed that the level of lung inflammation in rats was associated with particle size, with the smaller ultrafine TiO_2 causing greater adverse effects. Tian et al. (2006) studied several different carbon particles (SWCNT, MWCNT, active carbon, carbon black and graphite carbon) and were able to demonstrate that surface area was the property that best predicted the potential toxicity of carbon particles to cell lines. SWCNT had the smallest surface area (3.15 μm^2) of the five particles tested and caused the largest decrease in surviving cells (- 65 %) over a 5 day exposure (25 μg ml^{-1}). Oberdörster et al. (2007) recently explored the issue of surface area in the context of dose-response metrics, and found that surface area rather than the number of particles gave a better description of an inflammatory dose-response relationship. An equivalent literature on particle size effects on aquatic species is lacking at present, but these mammalian studies highlight the importance of attempting to identify particle size and surface area. "Apparent" surface area may also be important, as aggregates of nanoparticles will have a lower apparent surface area than a dispersed solution of individual nanoparticles, and will therefore, perhaps, cause lower ecotoxicity.

Nanoparticles in aqueous solution

The "solubility" of nanomaterials also has some implications for ecotoxicology. Some of these are practical considerations for getting the material into an aqueous phase for experiments (see below), but there are more fundamental concerns. First, the ability of nanomaterials to remain in an aqueous phase (as an emulsion, agglomerate, or as a soluble chemical) will alter the fate and transport of the material in the environment. Second, the chemical behaviour in solution will alter toxicity, and consideration must be given to water quality as part of the interpretation of any toxic effects. A detailed review

of particle chemistry is beyond the scope of this report, and the reader is referred to other texts on this topic (for a review see Lead and Wilkinson (2006)). However, the following issues may be important to the behaviour of nanoparticles in the aqueous phase:

- Dissolution of the nanoparticle
- Emulsion or coatings on nanoparticles
- Dispersion of nanoparticles by interactions with natural organic matter
- Agglomeration and aggregation
- Adsorption and precipitation properties of particles on surfaces
- Effects of impurities on nanoparticle behaviour in solution
- Effects of solvents, sonication and stirring on nanoparticle solutions
- General abiotic factors such as temperature, salinity or ionic strength, light intensity, hardness and pH

Dissolution of the nanoparticle could be a particular issue for metal and metal oxide particles. Values for solubility are based on using the standard form of a chemical. However, a powder of sparing solubility may give a misleading impression about the solubility of the nanoscale form. For example, a larger surface area presumably implies more rapid dissolution of atoms from the particle surface to the aqueous phase. This raises several practical issues for toxicity testing, such as the need to have new solubility data on the nanoscale form rather than relying on existing data for the chemical. There is also the risk that toxicity may not be due to the particle itself but the free ion chemistry, and that the particle may dissolve completely during the life of the test. For example, a 1 mg l^{-1} solution of metal particles, even with only 1-2% dissolution would result in toxic concentrations of free metal ions. Metals are needed in the manufacturing process, for example to grow "bamboos" of carbon nanotubes on artificial surfaces, and so some impurities in the raw product are inevitable.

It is also possible that manufacturers supply extracted or dried powder products that have been in contact with a solvent during the extraction process. However organic solvent impurities are rarely reported, partly because the expense of organic analysis (HPLC methods, etc.) is not cost effective for the manufacturer at present, or the purity issue is not a big concern in their product application. Nonetheless, from the viewpoint of protecting the environment, it suggests that we should examine the whole product to measure the true biological effect. Alternatively if the main experimental objective is to look at toxic mechanisms, then there is some justification for washing the nanomaterial so that only the effects of that substance are examined.

Nanomaterials may also be composite materials, with a core structure covered with a coating or capping agent to improve dispersion in the aqueous phase. This is typical of materials such as quantum dots. Arguably, the initial behaviour of the material in solution will depend on the ligands in the capping agent, and whether or not the capping agent remains intact over time. Part of the characterisation of these materials may include demonstration that the material remains intact for the duration of the test. Similar arguments apply to natural coatings that might be used to disperse material such as humic substances. However, the presence of potential coating materials in natural waters is also an issue for data interpretation. For example, in a natural water rich in organic matter would low toxicity of a nanomaterial be attributed to the fact that it has a

protective coating or to the chemistry of the particle itself? Tests with and without organic matter would be needed to determine this.

The issue of agglomeration and aggregation is also fundamental, and this has already been discussed in the context of surface area (above). However, if the chemistry predicts that a nanoparticle will exist mainly in the aggregated or agglomerated forms in the environment, then dispersion to single particles may not be relevant, or may only be relevant to particular types of water (e.g., those with high levels of humic substances). Alternatively, the same materials may adsorb or precipitate onto organic matter or other surfaces (sediment or soil), and even onto the organisms themselves (Smith et al. 2007). This ability of particles to stick to organisms may arguably be considered as a mode of toxicity, and is at the very least a physical mechanism for causing adverse biological effects. We might also expect environmental transport of nanomaterials with particulate matter in river water and in soil runoff. This also raises the possibility of nanomaterials accumulating in fine sediments and in the biofilm of sediment or organism surfaces. The ecotoxicology of these interphases and micro-environments may therefore be of fundamental importance to environmental exposure (Handy and Eddy 2004).

Solvents, sonication and other methods of dispersing nanomaterials are discussed in section 4.2

Abiotic factors in water chemistry

For many chemicals the ability to remain in an aqueous phase, and their consequent toxicity, are a function of abiotic factors such as pH, salinity, water hardness, temperature, and the presence of dissolved organic matter in the water, amongst other factors. This is an area where research is particularly lacking on the ecotoxicology of nanoparticles. For most materials we do not know how these factors will alter toxicity. To date, there have been no systematic studies on the effects on toxicity of changes in pH, hardness, and ionic strength, using aquatic organisms. However, we can derive some information by examining the available data for natural colloids (Lyvén et al. 2003, Lead and Wilkinson 2006, Stolpe and Hassellöv 2007). From the viewpoint of chemistry, additions of humic substances can help disperse and stabilise nanoparticles in the aqueous phase (Lead and Wilkinson 2006), but the consequences of this for ecotoxicity are less clear. For example, if humic substances form a hydrophilic coat around the particle, then presumably this will increase apparent solubility without increasing toxicity or surface reactivity. Alternatively, this could theoretically increase bioavailability for endocytosis into the organism, and promote accumulation or toxic effects inside the tissues instead. Similarly for metals: do the same rules that apply to dissolved metal ions concerning the protective effects of increased water hardness or altered pH apply to nano-metals? We might argue that they should if the functional material on the surface of the metal particle is also a metal ion.

Toxicity in seawater and brackish water

To date there have been no systematic studies of the same species of organism in seawater versus freshwater, or during graded salinity change. However, some recent data on particle chemistry in seawater is emerging. Stolpe and Hassellöv (2007) found

that iron-rich freshwater colloids showed a dramatic 50% decrease in colloid particle concentration and a shift in size distribution towards smaller diameters, even with very modest increases in salinity from freshwater to 2.5 ppt. For many estuarine organisms such a small salinity change alone would have little biological effect, but this study predicts the rapid loss of colloid from the freshwater as soon as it enters the estuarine zone. This implies that toxicity tests in freshwater are unlikely to provide adequate information on toxicity in sea water and a separate series of tests with marine species may be needed. One might argue that the freshwater tests would encompass material in the aqueous phase and therefore a freshwater test would protect both types of ecosystems in a risk analysis. On the other hand, the mechanisms of toxicity may be different with particles "sticking" to the organisms in seawater instead.

There is some literature on natural and biologically generated particles (granules) in some marine species. There is evidence that salinity alters these processes (Prevodnik et al. 2007, Blanchard and Grosell 2006), and therefore the possibility arises that nanoparticles will act in a similar manner. Kashiwada (2006) exposed medaka (*O. latipes*) eggs to fluorescent nanoparticles (30 mg l^{-1}) at a variety of different salinities. An increase in toxicity was seen with increasing salinity, along with a greater tendency for the particles to form aggregates. At a salinity of approximately 18.5 ppt the greatest fluorescence in the tissue (accumulation) was reached and 100% of the eggs were dead within 24 hours. At higher salinities, approaching that of normal seawater, accumulation decreased but the egg mortality rate remained high. This appears to be the only study so far to explore the effects of salinity on the ecotoxicity of nanomaterials.

3.5 Grey literature and databases

All the above information on ecotoxicity was derived from peer-reviewed published literature found in scientific journals. Of course, in any new field there is also a body of "grey literature" which may have appeared on the internet, or in databases and conference abstracts, that have not yet been peer reviewed. This type of data has not been used in this report. Nonetheless, some useful links are listed below:

International Council on Nanotechnology (ICON database hosted at Rice University)
http://icon.rice.edu/research.cfm

Woodrow Wilson Centre International Scholars (database on emerging research)
http://www.nanotechproject.com/index.php?id=18

National Information Library (NIL) in the USA
http://www2a.cdc.gov/niosh-nil/index.asp

4. CURRENT TEST STRATEGIES AND ASSOCIATED METHODS USED WITHIN CHEMICALS REGULATORY ECOHAZARD ASSESSMENT

4.1 Introduction

Ecotoxicity tests are tools used within hazard assessment frameworks to answer questions about the intrinsic dangers of chemical substances which may be released into the environment. When such hazard assessments are compared with exposure assessments the likely risk of adverse effects can be characterised. Hazard assessments usually follow a tiered approach. Short-term tests are generally used first, with observation of organism survival the most common measurement of effect. Longer-term tests, with observation of sublethal effects on organism growth or reproduction the most common measurements of effect, are generally used when results from short-term tests require further refinement. In the literature short-term tests are often referred to as "acute tests" and longer-term tests are often referred to as "chronic tests."

The use of acute and chronic tests in an ecotoxicity test strategy is illustrated by the sequence of ecotoxicity testing activities proposed in draft reports from REACH Implementation Project 3.3 for aquatic toxicity (EC2007a, Figure 4.2) and terrestrial toxicity (EC 2007b, Figure 4.2 and Table 4.1). These schemes are typical of tiered ecotoxicity testing strategies worldwide. Several important points emerge from study of Figures 4.1 and 4.2, and Table 4.1:

(i) Aquatic toxicity data are likely to be the primary, and often only, information on toxicity for assessing risks to both aquatic and terrestrial environments.

(ii) Standard short-term aquatic toxicity tests with algae, waterfleas and fish will be performed first, with longer-term tests performed only if these short-term tests suggest that there may be risks. A standard Assessment Factor approach will be used to produce Predicted No Effect Concentrations based on short-term data for used in risk characterisation. This process is likely to be over-ridden only if there are indications that a substance is PBT (Persistent, Bioaccumulative and Toxic) or vPvB (very Persistent and very Bioaccumulative).

(iii) There is a desire to minimise vertebrate testing, with several points in the Figure 4.1 scheme at which it is possible to use alternative test approaches, if these are available, or to provide evidence that fish are likely to be less sensitive. Even if these conditions cannot be fulfilled, a fish limit test (i.e., using only one high concentration versus a control) should be performed first to establish the relative sensitivity of fish compared with algae and *Daphnia*, before a full fish test is performed.

It is therefore conceivable that short-term tests with just algae (e.g., OECD 201) and *Daphnia* (e.g., OECD 202) might be used to assess the ecohazard of a substance in aquatic and terrestrial systems.

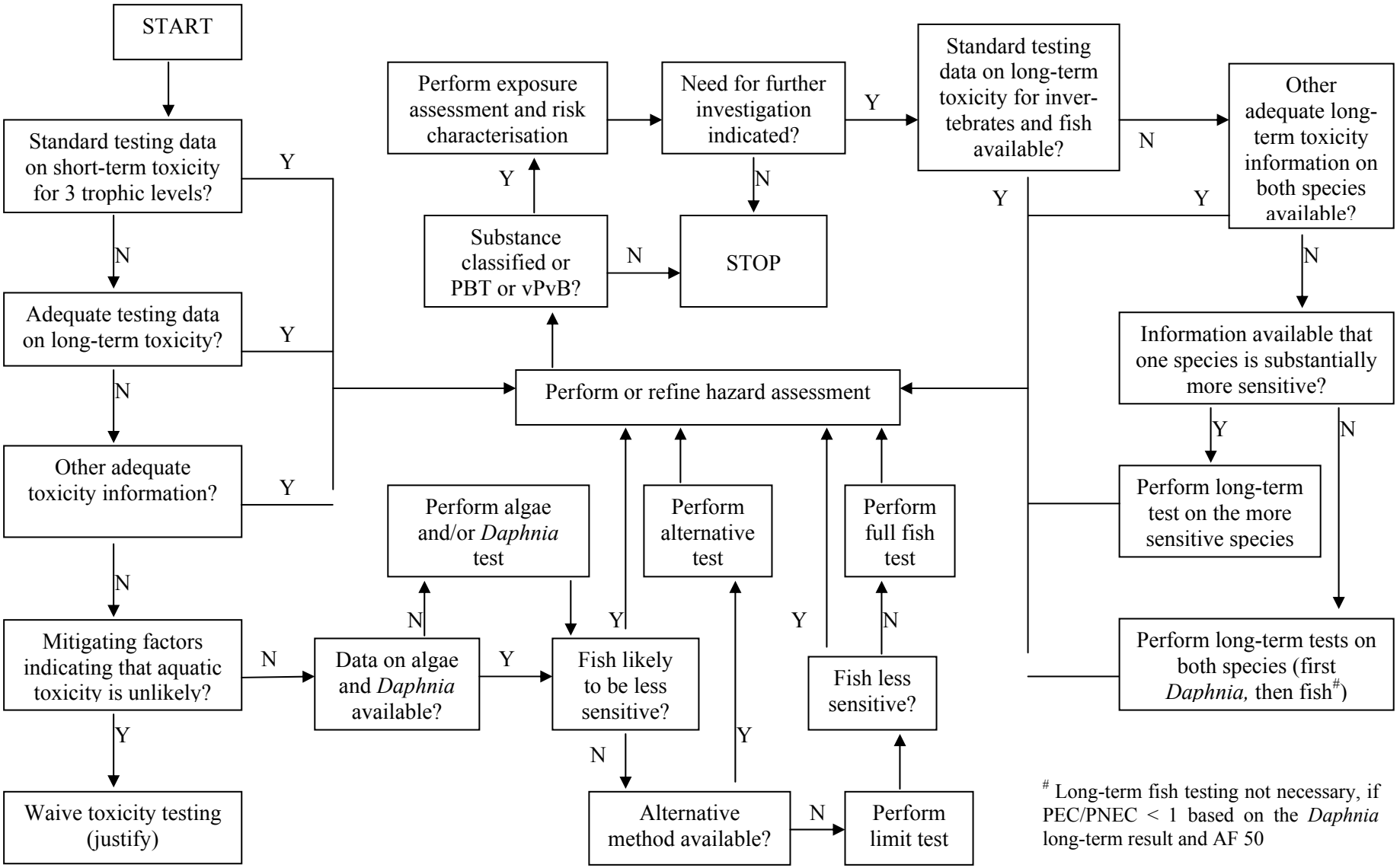


Figure 4.1 Proposed RIP 3.3 scheme for assessing aquatic toxicity under REACH

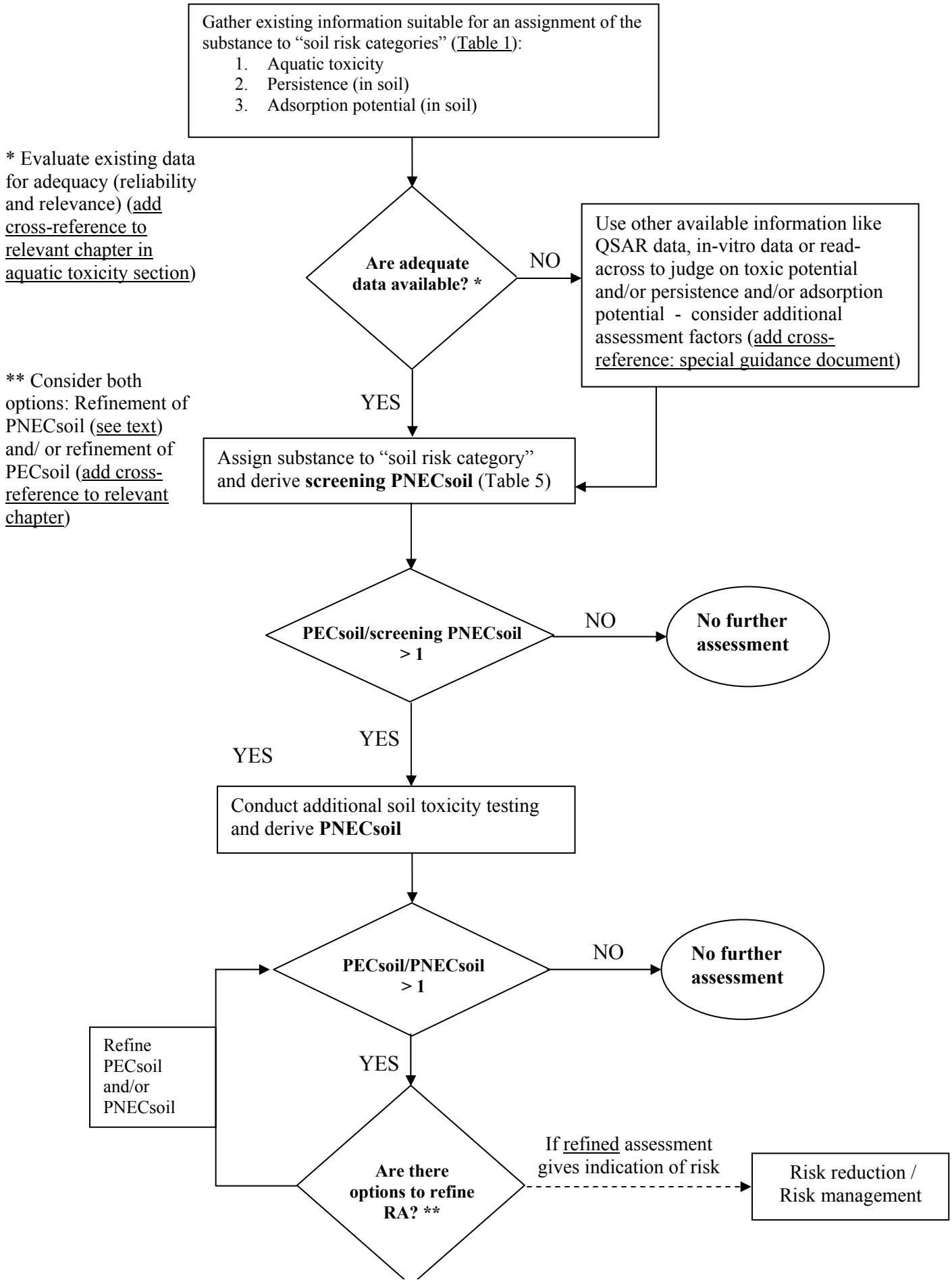


Figure 4.2 Proposed RIP 3.3 scheme for assessing terrestrial toxicity under REACH

Table 4.1 Soil risk categories and derivation of screening PNECsoil for initial risk assessment

	Risk category 1	Risk category 2	Risk category 3	Risk category 4
Is there indication for a **low toxic potential**[1] of the substance from aquatic toxicity data?	YES	NO	YES or NO	YES or NO
Is there indication that the substance will **persistent**[2] in soil?	YES	NO	YES	YES or NO
Is there indication for a **high adsorption potential**[3] of the substance in soil?	YES	NO	NO	YES
Screening PNECsoil	No soil toxicity testing required, no risk assessment for soil organisms required.	Derive screening PNECsoil from PNECwater according to EPM **and** from one short-term soil toxicity test. Choose lower value for initial risk assessment.	Derive screening PNECsoil from PNECwater according to EPM **and** from one long-term soil toxicity test. Choose lower value for initial risk assessment.	Derive screening PNECsoil from PNECwater according to EPM (if applicable, consider additional AF: 10) **and** from one long-term soil toxicity test. Choose lower value for initial risk assessment.

[1] e.g. EC/LC50 > 100 mg/L for algae, daphnia and fish or no toxicity up to the water solubility limit

[2] e.g. classified as "readily biodegradable"

[3] e.g. logKow < 5 (log Koc < 4)

AF: Assessment factor, EPM: Equilibrium partitioning method

Table 4.2 summarises the more extensive information on standard ecotoxicity tests found in Appendix 3. It focuses on the species and measurement endpoints that are used in ecotoxicity tests, and the methods of test substance dosing and exposure, as these are likely to be the most important aspects of the tests for understanding whether they are suitable for assessing the toxicity of nanomaterials. In all but the avian tests, the test substance is dosed into the test medium: either water or soil. Solvents and other dispersants can be used to achieve this in most cases if a substance is sparingly soluble. The endpoints that are measured in these tests are usually Lethal Concentrations (LC), Effective Concentrations (EC) or No Observed Effect Concentrations (NOECs). In most cases these are for survival, growth or reproduction, but for microbes and algae the endpoint is population growth because of the rapid generation time of these simple organisms. These are widely accepted endpoints for use in risk assessment and there is no reason to believe that they are inappropriate for assessing the hazards of nanoparticles.

An important point to grasp here is that it is *only* changes in age-related survival, growth and reproduction that can influence the size of a population of organisms. Ecological risk assessors are rarely interested in the health of individual organisms, so measures of individual effect such as the use of biomarkers are only useful to them if they can predict effects on survival, growth and reproduction. This differs substantially in both concept and methodology from the practices of human health risk assessment. It is likely that confusion between the aims of human health risk assessment and ecological risk assessment is behind the calls from some for the use of "novel" endpoints to test the ecological risks of nanoparticles. Novel endpoints may throw light on mechanisms of action, but the measurement of anything other than survival, growth or reproduction must necessarily be translated into these endpoints before it is useful to an ecological risk assessor.

The main area of considerable uncertainty that applies to all of the guidelines, *except* those in which dosing is oral, is the way in which the substance is dosed into, and maintained and measured in, the test medium. This is discussed further in the next section.

Table 4.2 Summary of standard ecotoxicity tests

Guideline number	Test Species	Test medium	Dosing method	Test Duration	Test endpoints
OECD 201.	*Pseudokirchneriella subcapitata.* *Desmodesmus subspicatus.* *Navicula pelliculosa.* *Anabaena flos-aquae.* *Synechococcus leopoliensis.*	OECD and AAP (US EPA) growth medium.	Dosed into medium. Solvents can be used.	72 hours.	ErC10/20/50, LOEC, NOEC.
OECD 202.	*Daphnia magna.*	Natural water.	Dosed into water. Solvents, emulsifiers and dispersants should be avoided.	48 hours.	EC50.
OECD 203.	*Brachydanio rerio* (Zebrafish). *Pimephales promelas* (Fathead minnow). *Cyprinus carpio* (Carp). *Oryzias latipes* (Medaka). *Poecilia reticulata* (Guppy). *Lepomis macrochirus* (Bluegill sunfish). *Oncorhynchus mykiss* (Rainbow trout).	Natural water.	Dosed into water. Solvents, emulsifiers and dispersants can be used.	96 hours.	LC50.
OECD 204.	*Brachydanio rerio* (Zebrafish). *Pimephales promelas* (Fathead minnow). *Cyprinus carpio* (Carp). *Oryzias latipes* (Medaka). *Poecilia reticulata* (Guppy). *Lepomis macrochirus* (Bluegill sunfish). *Oncorhynchus mykiss* (Rainbow trout).	Natural water.	Dosed into water. Solvents, emulsifiers and dispersants can be used.	14 days.	EC/LC50 and NOEC.
OECD 205.	*Anas platyrhynchos* (mallard duck). *Colinus virginianus* (bobwhite quail). *Columba livia* (pigeon). *Coturnix coturnix japonica* (quail). *Phasianus colchicus* (pheasant).	Basal diet.	Mixed with diet. Water is preferred as a carrier, but other carriers can be used.	8 days.	LC50.

	Alectoris rufa (redlegged partridge).				
OECD 206.	*Anas platyrhynchos* (mallard duck). *Colinus virginianus* (bobwhite quail). *Coturnix coturnix japonica* (quail).	Basal diet.	Mixed with diet. Water is preferred as a carrier, but other carriers can be used.	20 weeks.	NOEC.
OECD 207.	*Eisenia. foetida foetida.* *Eisenia foetida andrei.*	Filter paper. Artificial soil.	Mixed into water and then into test soil. Solvents, emulsifiers and dispersants can be used. Test substance can also be mixed with sand and then into the soil or directly into soil.	14 days.	LC50.
OECD 208.	Up to 28 test species (monocotyledonous and dicotyledonous).	Commercial potting soil, artificial soil or inert media such as sand or glass beads.	Mixed into water and then into test soil. Solvents, emulsifiers and dispersants can be used. Test substance can also be mixed with sand and then into the soil or directly into soil.	14-21 days post emergence.	ECx NOEC and LOEC.
OECD 209.	Dependant on bacterial inoculum.	Activated sludge.	Dosed directly into sludge. No mention of solvents.	30 mins and 3 hours.	LC20/50/80.
OECD 210.	*Brachydanio rerio* (Zebrafish).	Water.	Dosed directly into	Until control	NOEC and

	Pimephales promelas (fathead minnow). *Oryzias latipes* (Medaka). *Oncorhynchus mykiss* (Rainbow trout). *Cyprinodon variegatus* (Sheepshead minnow).		water. Solvents, emulsifiers and dispersants can be used.	fish are free-feeding.	LOEC.
OECD 211.	*Daphnia magna.*	Elendt M4 and M7 media.	Dosed directly into water. Solvents, emulsifiers and dispersants can be used.	21 days.	ECx NOEC and LOEC.
OECD 212.	*Brachydanio rerio* (Zebrafish). *Pimephales promelas* (Fathead minnow). *Oryzias latipes* (Medaka). *Cyprinus carpio* (Common carp). *Oncorhynchus mykiss* (Rainbow trout). *Lepomis macrochirus* (Bluegill). *Carassius auratus* (Goldfish). *Gadus morhua* (Cod). *Clupea harengus* (Herring). *Menidia peninsulae* (Silverside). *Cyprinodon variegatus* (Sheepshead minnow).	Water.	Dosed directly into water. Solvents, emulsifiers and dispersants can be used.	The test is terminated just before the yolk-sac of any larvae in any of the test chambers has been completely absorbed or before mortalities by starvation start in controls.	EC/LCx NOEC and LOEC.
OECD 213.	*Apis mellifera.*	Food (sucrose solution)..	Dosed directly into food. Solvents, emulsifiers and dispersants can be used.	48-96 hours.	LD50.
OECD 214.	*Apis mellifera.*	Organic solvent	Applied directly to	48-96 hours.	LD50.

		or carrier.	bee using solvent or carrier.		
OECD 215.	*Oncorhynchus mykiss* (Rainbow trout). *Oryzias latipes* (Medaka). *Danio rerio* (Zebrafish).	Water.	Dosed directly into water. Solvents, emulsifiers and dispersants can be used.	28 days.	ECx NOEC and LOEC.
OECD 216.	Natural microorganism community.	Field soil.	Chemical is mixed with water or sand and mixed with test soil.	28 – 100 days.	ECx.
OECD 217.	Natural microorganism community.	Field soil.	Chemical is mixed with water or sand and mixed with test soil.	28 – 100 days.	ECx.
OECD 218.	*Chironomus riparius.* *Chironomus yoshimatsu.i* *Chironomus tentans.*	Sediment and water.	Dosed directly into sediment. Solvents, emulsifiers and dispersants can be used.	28 – 65 days.	ECx NOEC and LOEC.
OECD 219.	*Chironomus riparius.* *Chironomus yoshimatsui* *Chironomus tentans.*	Sediment and water.	Dosed directly into water. Solvents, emulsifiers and dispersants can be used.	28 – 65 days.	ECx NOEC and LOEC.
OECD 220.	*Enchytraeus albidus.* *Enchytraeus buchholzi.* *Enchytraeus crypticus.*	Artificial soil.	Mixed into water and then into test soil. Solvents, emulsifiers and dispersants can be	42 days.	EC/LCx NOEC and LOEC.

			used. Test substance can also be mixed with sand and then into the soil or directly into soil.		
OECD 221.	*Lemna gibba.* *Lemna minor.*	A modification of the Swedish standard (SIS) or 20X – AAP *Lemna* growth medium.	Dosed directly into water. Solvents, emulsifiers and dispersants can be used.	7 days.	ECx NOEC and LOEC.
OECD 222.	*Eisenia foetida.* *Eisenia andrei.*	Artificial soil.	Mixed into water and then into test soil. Solvents, emulsifiers and dispersants can be used. Test substance can also be mixed with sand and then into the soil or directly into soil.	56 days.	EC/LCx NOEC and LOEC.
OECD 224.	Dependant on bacterial inoculum.	Activated sludge and nutrient broth.	Dosed directly into water/sludge. Solvents, emulsifiers and dispersants can be used.	3 days.	EC50.
OECD 227.	Up to 28 test species (monocotyledonous and dicotyledonous).	Commercial potting soil, artificial soil or	Sprayed onto plant surface. Solvents, emulsifiers and	21-28 days.	ECx NOEC and LOEC.

		inert media such as sand or glass beads.	dispersants can be used.		
OECD 301 A.	Dependant on bacterial inoculum.	Distilled water and mineral media.	Dosed directly into water/sludge. Not suitable for low solubility substances.	28 days.	D_t.
OECD 301 B.	Dependant on bacterial inoculum.	Distilled water and mineral media.	Dosed directly into water/sludge. Solvents, emulsifiers and dispersants can be used.	28 days.	D_t (%ThCO_2).
OECD 301 C.	Dependant on bacterial inoculum.	Distilled water and mineral media.	Dosed directly into water/sludge. Not suitable for low solubility substances.	28 days.	D_t (%ThOD).
OECD 301 D.	Dependant on bacterial inoculum.	Distilled water and mineral media.	Dosed directly into water/sludge. Solvents, emulsifiers and dispersants can be used.	28 days.	D_t (%ThOD or COD).
OECD 301 E.	Dependant on bacterial inoculum.	Distilled water and mineral media.	Dosed directly into water/sludge.	28 days.	D_t.
OECD 301 F.	Dependant on bacterial inoculum.	Distilled water and mineral media.	Dosed directly into water/sludge. Solvents, emulsifiers and	28 days.	D_t (%ThOD or COD).

			dispersants can be used.		
OECD 302 A.	Dependant on bacterial inoculum.	Activated sludge.	Dosed directly into water/sludge.	Months.	% biodegradation.
OECD 302 B.	Dependant on bacterial inoculum.	Distilled water and mineral media.	Dosed directly into water/sludge.	28 days.	D_t.
OECD 302 C.	Dependant on bacterial inoculum.	Distilled water and mineral media.	Dosed directly into water/sludge.	28 days.	% biodegradation.
OECD 304 A.	Dependant on bacterial inoculum.	Various field soils.	^{14}C labelled compound mixed with water or acetone and dosed directly onto soils.	64 days.	50% mineralization/ Volatilisation.
OECD 305.	*Danio rerio* (Zebrafish). *Pimephales promelas* (Fathead minnow). *Cyprinus carpio* (Common carp). *Oryzias latipes* (Ricefish). *Poecilia reticulata* (Guppy). *Lepomis macrochirus* (Bluegill). *Oncorhynchus mykiss* (Rainbow trout). *Gasterosteus aculeatus* (Three-spined Stickleback).	Water.	Dosed directly into water. Solvents, emulsifiers and dispersants can be used.	Up to 90 days.	BCF.
OECD 306.	Dependant on bacterial inoculum.	Field collected sea water.	Dosed directly into water.	60 days.	D_t.
OECD 308.	Dependant on bacterial inoculum.	Sediment.	Dosed into sediments using water. Solvents, emulsifiers and dispersants can be	<100 days.	DT50/75/90.

			used.		
OPPTS 850.2100.	*Colinus virginianus* (Northern bobwhite). *Anas platyrhynchos* (Mallard).	Basal diet.	By gavage or gelatine capsule. Water is preferred as a carrier, but other carriers can be used.	14-21 days.	LD50.
TGD Mesocosms	Several taxonomic groups , in more or less natural ecosystem.	Not stated.	Not stated.	Not stated.	NOEC.

4.2 Dispersion of nanoparticles in test medium

Ideally in most aquatic ecotoxicity studies, test substances should be in an aqueous phase, and the stock solution should be homogenous so that representative sub-samples of the stock are easily pipetted into the test system. There are essentially three approaches to achieving a uniform stock solution of the test substance: dispersion in detergents or similar agents, dispersion by sonication, or by prolonged stirring. The advantages and disadvantages of each approach are summarised in Table 4.3. Details of the dispersion method should be given very careful consideration. The aggregation process may influence the dose-response relationship, and could lead to fundamental flaws in ecotoxicological test design. Aggregates form more easily when particle concentration is increased. Thus when a concentrated stock solution of nanomaterial is diluted, the number of aggregates in the solution may decline. If toxicity is a function of the number of aggregates, or size of the aggregates in the test solution, then inevitably each solution in a dilution series will have a different toxicity regardless of what the "total concentration" is in the solution. One way to resolve this problem is to make test solutions of different concentrations with a consistent ratio of individual particles: aggregates in each solution. This can be done by adding dispersants (e.g., Smith et al. 2007), or by filtering out the aggregates so that only smaller particle sizes remain (Lovern and Klaper 2006). Of course the latter will also change the total concentration of the material since some of it will be lost during filtering, and there is no guarantee that aggregates will not reform in the filtered solution. The use of dispersants will prevent aggregation, but different concentrations of dispersant/solvent will be needed across the dose range, which has implications for the number of solvent controls in the test design. Smith et al. (2007) also point out that one cannot simply add "extra" solvent so that all the test concentrations have the same amount of solvent. This is because excess dispersant/solvent can deform nanoparticles (Ham et al. 2005) and subsequent changes in particle shape/size might alter toxicity.

For CNTs and carbon fullerenes there is a general consensus that they have poor aqueous solubility (Ham et al. 2005), and that some use of chemical dispersants, stirring, and/or sonication is needed to maintain them in aqueous solution. The choice of dispersant is problematic, since some of the best dispersants from the view point of chemistry (e.g., amides and furans (Ham et al. 2005)) are also likely to be toxic to organisms. For example, chemistry predicts that tetrahydrofuran (THF) is a good dispersant of C_{60} fullerenes, and it has been used in ecotoxicity studies (e.g., Oberdörster 2004), but there are concerns about the toxic effects of THF. Zhu et al. (2006) exposed two populations of *Daphnia magna* to C_{60} fullerenes, one dispersed in THF, the other stirred into water for a minimum of 2 months. The 48h LC50 values were 0.8 mg l^{-1} and >35 mg l^{-1} for THF and water-stirred particles respectively. These findings suggest that the toxicity of C_{60} particles was increased by the THF. Fortner (2005) argues this could be due to residual THF being trapped in the centre of C_{60} aggregates, or some other unpredicted effect of THF on particle shape/size. A compromise is possible in which a less powerful dispersant is used that will still give reasonably good dispersion, but with less risk of solvent toxicity to the test organism. This approach was used by Smith et al. (2007) who used the detergent sodium dodecyl sulphate (SDS) to disperse carbon nanotubes.

There is no simple answer to the dispersion issue. Sonication or prolonged stirring can be used instead of solvents, or combinations of these techniques can be used. Stirring at least does not add any extra chemicals, but the time-frames for solution preparation can be long (e.g., 2 months stirring (Oberdörster et al. 2006)), and there is little firm evidence that nanoparticles will stay in suspension after stirring has stopped. Sonication has been used in several studies (Smith et al. 2007, Lovern and Klaper 2006, Monteiro-Riviere et al. 2005), sometimes in combination with solvents, but sonication also has some problems. Carbon nanotubes are cut and structurally damaged by prolonged sonication, which affects the aspect ratio of the tubes and therefore their chemical properties (Yu et al. 2007). There are also concerns, as with stirring, that the particles will re-aggregate after sonication is stopped.

There are ecologically-based criticisms of all of the above approaches. Nanomaterials discharged to the environment are not likely to occur in the presence of mg quantities of dispersant chemicals or sonication. It could be argued that the non-dispersed material is likely to more relevant to what will happen in the real environmental, and toxicity test design should reflect that reality. Alternatively, perhaps there is a case for using naturally occurring dispersants that can be found in river water. Natural organic matter (NOM) is a possible dispersant. Hyung et al. (2007) found that adding CNT to samples of natural river water high in NOM, and to pure water containing added NOM (commercially available NOM, both from the Suwannee River) kept the matter suspended for considerably longer than a 1% solution of SDS. Transmission electron microscope (TEM) analysis of the suspensions showed the CNT in both the NOM samples were suspended as individual particles rather than as aggregates.

Humic acids are known to make up a significant part of the NOM of aquatic systems. Adding C_{60} fullerenes to low humic acid concentrations (1 mg l^{-1} total organic carbon) results in rapid adsorption of the nanoparticles with the humic acids. Furthermore, aggregation rates are significantly reduced compared to solutions of C_{60} not containing humic acids, and further additions of humic acids can further slow the rate of aggregation of the C_{60} particles (Chen and Elimelech 2007). Clearly, using natural humic substances may resolve the practical problem of dispersion, while also being environmentally relevant.

Other natural substances have also been suggested. Karajanagi et al. (2006) used a variety of proteins to disperse CNTs in aqueous solution. They achieved suspensions of individual nanotubes that were non-covalently bound to the protein molecules. Carbon nanotubes have a natural affinity to protein molecules and will conjugate to form CNT-protein molecules (Asuri et al. 2006, Lin et al. 2004, Pantarotto et al. 2003). However, there are concerns that CNT-protein conjugates are immunotoxic (Pantarotto et al. 2003). There is also the issue of bioavailability and bioreactivity, both of which are presumably reduced by conjugation. Protein conjugation may not be the answer, but this chemistry has interesting implications for the possible rapid flocculation of nanomaterials in sewage treatment works where protein levels and other organic matter are high.

Table 4.3 Advantages and disadvantages of test substance dispersion methods, and of not dispersing at all.

Approach	Advantage	Disadvantage
No attempt at dispersion	• Not adding any extra chemicals, no solvents. • Very short preparation time for solutions. • No solvent or sonication controls needed in the test design. • May be more environmentally relevant for some types of hard water quality, or seawater.	• Aggregation state will inevitably change with dilution, and therefore could also change toxicity. • No control over particle size or aggregation in the test system. • May not be the best test design for natural waters that are high in humic substances or other natural dispersants.
Solvents or chemical dispersants	• Such chemicals can disperse nanoparticles into aqueous phase without stirring or sonication. • Preparation method is fast. • It may be possible to manage ratios of individual particle numbers: aggregates so that each test concentration has a similar profile of particle types. • Nanoparticles more likely to stay dispersed while the solvent or dispersing agent is present.	• Adding an extra chemical, the solvent may also be toxic. • Excess solvent might deform the shape of nanotubes and change toxicity. • Could the residue solvent or dispersant trapped inside nanotubes or in aggregates be internalised by the organism to cause toxicity? • Need to keep a consistent ratio of solvent: test material to avoid problems above but this would require more solvent controls in a regulatory test.
Sonication	• Not adding any extra chemicals, no solvents. • Relatively short preparation time, from a few hours to a day. • No solvent control needed in the test design. • No sonication control needed, but only if the material is sonicated in ultrapure water and there is no risk of sonication-induced reactive oxygen	• Sonication times will change with total concentration. • Sonication may break nanotubes or damage the surface of nanoparticles. • The material may not remain dispersed for long once the sonication is stopped. • Sonication in natural waters or in the presence of any electron donors could result

Approach	Advantage	Disadvantage
	species generation.	in reactive oxygen species being generated in the solution. In such cases, a sonication control is needed in the test design.
Stirring	• Not adding any extra chemicals, no solvents. • No solvent control needed in the test design.	• Similar problems to the sonication approach. • Very long stirring times may be needed (several weeks). • Stirring times will change with total concentration. • Stirring may break nanotubes, or polish/scratch the surface of nanoparticles. • The material may not remain dispersed for long once the stirring is stopped. • A non-stirred control is not likely to contain the same quality of nanomaterial as the stirred solution. Difficult to devise a true control for stirring in the test design.

4.3 Reference materials for ecotoxicity testing

The issue of reference nanomaterials is being reported by another project team funded by Defra (Aitken et al. 2007), and only some key points that relate to ecotoxicity testing are discussed here. The first issue for the ecotoxicology community is to decide what they want to use the "reference material" for. Even in the respiratory health scientific community there is considerable debate about the purpose of these materials in toxicity tests (Aitken et al. 2007). The concept of a "reference material" is to provide a benchmark using a well characterised material of known toxicity, and preferably where the toxic mechanism associated with the reference material is also well documented. Clearly, there also needs to be batch consistency in the reference material, and a good shelf life, so that it can be used confidently by many researchers in different laboratories over time. In mammalian respiratory toxicology reference materials are available, and these have been used mainly for dual purposes, as a "reference material" and as a "positive control", with particles of known toxicity to rodents in the test design (e.g., carbon black or quartz particles are often used, (Oberdörster et al. 1992)). However, ecotoxicologists have noted that there is a major knowledge gap in the ecotoxicology literature on reference materials for aquatic species, and we are not yet in a position to select a reference material that fits the criteria above (Federici et al. 2007, Handy and Shaw 2007b). It is unclear which reference materials (if any) should be selected for fish or other aquatic species, and some materials used in mammalian studies (e.g., quartz

(sand) particles) may not be good positive controls given that many aquatic species are probably adapted to living on substrates containing large quantities of these materials.

There are also other uses of reference materials in nanotoxicology. One of these is to act as a "negative control", that is a particle of the same size/shape as the test substance, but which has no toxic effect. There is also the possibility of having a reference material of matching particle size/shape to the test substance, but which is also toxic so that chemical toxicity can be discerned from particle size (a "chemistry only positive control"). However this concept also has many disadvantages. For example, it would be imperative to choose a reference particle with exactly the same mechanism of toxicity, so that the chemical toxicity alone (not particle size effects) are truly reflected in the results. Finally, if we choose reference materials that have neither the same shape/size or chemical makeup as the test substance, then no useful information can be obtained about mechanisms of toxicity; only a relative toxicity of the test substance compared to the reference material can be obtained.

Perhaps a starting point for searching for reference materials for aquatic studies is to choose some natural particles with appropriate toxic and non-toxic characteristics that are found in suspension in water. There is a large literature on effluents and aquaculture wastes containing suspended matter, but almost no studies have been performed on the ecotoxicity of the individual particulate components. There are a few reports of particulate matter increasing the toxicity of chemicals (e.g., Weltens et al. 2000). Historic reports on individual particle types are rare (e.g., Jonhnson et al. 1986), although some of the historic studies on effluents did examine wastes dominated by one type of material (e.g., china clay (Herbert et al. 1961)). Perhaps a defined clay particle could be used as a reference material for aquatic species. Clearly, existing reference materials like carbon black may not be relevant to the aquatic environment.

4.4 Critique of and additions to physico-chemical tests

1. Although the current list of methods for the determination of physico-chemical properties are not strictly part of a regulatory ecotoxicity test, it is worth considering if any of the characterisation approaches for nanoparticles should become mandatory and therefore listed as one of these methods. We should reflect on the suitability of using existing methods for physico-chemical properties with nanoparticles (Table 4.4). For the former, it is suggested that the minimum base set of characterisation (discussed earlier in section 3) could be adopted in some revised form. This of course would require some method development to obtain standardised protocols, and the usual inter-laboratory testing. There are also some concerns about existing physico-chemical test methods, and in particular whether or not the partition coefficient test will work with nanomaterials. This has implications for risk assessment strategies that use the partition coefficient as a trigger for requiring sediment toxicity tests or bioaccumulation studies.

Table 4.4 Critique of current methods for physico-chemical properties

Method	Issues for nanomaterials
Melting point	The substance is already pulverised, so this step could be omitted from the test. How can the final steps in melting be visualised if the particles are nm scale?
Boiling temperature	No obvious concerns.
Relative density	Relative density will be a function of particle size when using any of the methods based on buoyancy; test must be particle size specific.
Vapour pressure	Vapour pressure is likely to be a function of particle size, which will at least affect the stability of the measurement. Measurements that rely on changes in resistance may not work well with nanomaterials that have very high conductivity (e.g., carbon nanotubes).
Surface tension	All the issues about dispersion and the use of solvents will impact on how nanoparticles are prepared for this test. Clearly, solvents will alter any surface tension measurement.
Water solubility	The test protocol states that the final aqueous solution "must not contain any undissolved particles". Methods would need adaptation to verify this at the nanoscale.
Partition coefficient	Clumping or the sticky nature of nanoparticles may prevent proper separation of the material in the octanol and water phases. The meniscus between the phases is likely to trap material (rheology) undermining the founding assumption of free diffusion between the phases. The test may be fundamentally flawed at the nanoscale.
Flash point	For liquid substances, may be relevant to products sold as a liquid phase.
Flammability tests	For powders, the burning time in the test may be a function of particle size or surface area.
Pyrophoric properties	No obvious concerns.
Explosive properties	Dusts are well known for this property, and the results will be a function of particle size as well as chemical properties.
Ignition tests	Unclear whether dusts should be tested as a "vapour" or a "solid". The cube holder for the solid test may not hold the material (cakes, powders).
Oxidising properties	The test specifies a particle size of <125 mm, the reference material in the test (barium nitrate) will be much larger than the nanoparticles.

Additional physico-chemical tests for nanoparticles should include measurements of the individual particle size, the size of any aggregates, and size distributions in the sample. However, there may also be unique chemical properties as well. For example, nanoparticles are known to adsorb onto surfaces, clump or agglomerate and it may be useful to include a rheology test to describe this, e.g., the ability of the material to migrate in a viscous solution. No doubt nanotubes and spherical particles will have very

different rheological properties, and these factors are likely to be of direct relevance to toxic mechanisms in organisms (particle migration in gill mucus for example).

4.5 Rapid tests

Previous reports on nanomaterials discussed in Section 2, and interviews with practitioners (below) suggest the need for rapid tests or an initial tier of screening tests which would help to decide whether or not the nanomaterial is sufficiently different from the existing normal substance to warrant further ecotoxicity testing. However, it should not be necessary to invent new tests. It is possible to select a suite of rapid tests from existing regulatory tests, although some of these may need to be imported from the clinical regulatory arena. The suite would need to be selected to identify overall toxicity (e.g., cell viability assay or microbial population growth test (e.g., with *Vibrio fischeri*), and also to check for specific modes of toxicity that may not be detected by a general toxicity screen. This need not be exhaustive, but focus on major toxic mechanisms likely to be relevant for that type of nanomaterial. These might include genotoxicity (abbreviated Ames test or similar), immunotoxicity assays (see Galloway and Handy, 2003 for tiered application), and an oxidative stress assay.

4.6 Interviews with practitioners

The individuals listed in Table 4.5 were identified as possessing relevant expertise for this project in different areas of nanomaterials science, and were interviewed by telephone or email in early 2007.

Table 4.5 Individuals interviewed during this project

Interviewee	**Affiliation**
Anders Baun	Technical University of Denmark
Christian Blaise	Environment Canada
Samantha Dozier	People for the Ethical Treatment of Animals, USA
Alex Ford	UHI Millenium Institute
Laurie Hughes	Croda International
Jamie Lead	University of Birmingham
Andrew Nelson	University of Leeds
Willie Peijnenburg	RIVM, the Netherlands
Paul Reip	QinetiQ Nanomaterials Ltd
Steve Robertson	Environment Agency of England & Wales
Janeck Scott-Fordsmand	NERI, Denmark
Vicki Stone	Napier University
Kevin Thomas	NIVA, Norway
Charles Tyler	University of Exeter
Stefano Zuin	Consorzio Venezia Richerche, Italy

The questions they were asked and a summary of the range of answers are provided below.

What experience have you had in examining the environmental toxicology of nanomaterials?

Respondents had experience with a range of nanoparticles including C_{60}, titanium dioxide and zinc oxide. Several had performed hands-on ecotoxicity experiments with these substances, while others had commissioned such studies.

Respondents also had experience in testing several aquatic and terrestrial species with nanoparticles. Aquatic species used by them included bacteria, algae, crustaceans (e.g., *Daphnia* and *Artemia*), molluscs, and fish in both freshwater and saltwater systems; tests included short-term and long term toxicity and bioaccumulation studies. Terrestrial species tested included earthworms and springtails.

Research questions of interest to respondents included interactions of nanoparticles such as C_{60} with organic pollutants, and development of biomarkers of exposure (e.g., measures of oxidative stress, DNA damage and metalliothionein induction). Many respondents stated that they were at the stage of "playing" with nanoparticles and test systems to explore the best way forward.

In what ways, if any, did your experiences differ from those that you might have when examining the environmental toxicology of other (non-nano) substances?

Many respondents commented that preparation of appropriate test solutions is the main challenge in running aquatic toxicity tests because of low solubility and the tendency for nanoparticles to aggregate and settle to the bottom of test vessels, or be ingested by test organisms. This uncertainty about exposure in tests leads to *"…uncertainty on the x-axis of dose response curves."*

Another issue mentioned by several respondents was that short-term tests seem to be of limited value because very high concentrations are often necessary before any acute effects can be measured. This could be because uptake mechanisms are slow for substances of the size range of nanomaterials: the point was made that nanoparticles are really very large when compared with the dissolved chemical molecules that are usually tested in ecotoxicity studies. Also, at the high concentrations required to produce effects in acute tests, apparent effects on small invertebrates such as *Daphnia* may be caused by physical processes – adsorption of nanoparticles can lead to test organisms "sticking together". Finally, there may also be animal welfare issues in running short-term tests with fish, as respiratory distress ("coughing") has been observed when fish are exposed to nanoparticles.

The absence of toxic effects in short term tests in which the bioavailability of nanoparticles is maximised would provide reassurance *if* there was confidence that the mode of action remained the same between short-term and long-term exposure. However, there is currently no empirical evidence to support or refute this assumption, so most respondents were of the view that long-term studies should be performed even

in the absence of toxicity triggers from short-term tests until evidence is available to help set reliable Assessment Factors.

In this project we are interested in how to measure the environmental effects of nanomaterials in aquatic and terrestrial toxicity tests with microbes, algae, plants, invertebrates, fish and other vertebrates. How do you think the biological effects of nanomaterials on these organisms differ from those of other substances?

Most respondents felt that the biological effects of nanoparticles on endpoints such as survival, growth and reproduction are unlikely to differ substantially from other chemicals with similar potencies, as long as differences in number concentration and activity are taken into account. However, interactions with membranes may be different because some nanoparticles seem to move directly to the cell membrane without diffusion in the macroscale solution. It is therefore the *way* in which organisms become exposed to nanoparticles that differs from macroscale chemicals, and not the ultimate effects on survival, growth and reproduction.

Can these different effects be measured effectively using standard environmental toxicity tests?

Most respondents believed that standard toxicity tests are adequate for measuring the effects of nanoparticles on survival, growth and reproduction, so long as exposure concentrations can be maintained and measured appropriately. At high concentrations, shading effects can occur in algal tests, for which the usual colour control or additional lighting are appropriate responses.

The "stickiness" of nanoparticles was a recurring theme amongst respondents with practical experience of running tests with these substances. This can lead to test organisms sticky together, or the "furring" of test systems. This could be a major practical problem when running tests without solvents.

What are the strengths and weaknesses of the environmental toxicity tests on nanomaterials performed to date that you are aware of?

Most respondents believed that the main strength of standard ecotoxicity tests are their ease of interpretation by risk assessors and managers, while their main weakness is the need to use solvents to get nanoparticles into solution *if* an homogeneous solution or suspension is required throughout a test exposure.

How should exposure concentrations be measured in aquatic and terrestrial environmental toxicity tests with nanomaterials?

There was general agreement that analytical methods for accurately measuring number concentration and surface area in test systems are more useful than traditional measurement of mass concentration alone. Measurement of body burdens might obviate the need to measure solution chemistry, but methods are not yet sufficiently developed to do this with high confidence.

Real time measurements using appropriate sensors or probes may also be important if nanoparticles change their degree of aggregation or other physical or chemical properties during the course of an ecotoxicity test.

Is the concept of dose-response or concentration-response meaningful when testing nanomaterials?

Most respondents believed that the concept remains theoretically sound, but dose uncertainty in ecotoxicity tests with nanoparticles remains a practical problem. Aggregation state can change with dose. If this is the case then the dose-response will not necessarily be monotonic, which can make interpretation difficult.

How should concentrations of nanomaterials be prepared for use in environmental toxicity tests? For example, what should the role of solvents be?

Some respondents were of the view that OECD approaches for testing poorly soluble substances might provide useful advice on the acceptable use of solvents and other methods for getting substances into solution. Other respondents felt that experiences with solvents in some published studies suggest that either solvent toxicity, or changes in nanoparticle toxicity caused by interactions with solvents, can produce un-interpretable results, with limited relevance for the natural environment.

Problems with the use of solvents have led some respondents to rely on sonication to produce homogeneous suspensions before addition of these to ecotoxicity test systems. However, as soon as sonication ends, most nanoparticles will begin to aggregate and exposure conditions will become uncertain. Because of this, several respondents were of the view that nanoparticles should be added to ecotoxicity systems without the use of solvents or sonication and allowed to behave "naturally" – i.e., to aggregate and settle or adsorb to solid surfaces. However, the disadvantage of this is that test systems can become coated with nanoparticle aggregates.

What meaningful information, if any, can be provided by bioaccumulation studies with nanomaterials?

Respondents felt that bioaccumulation studies might be a very useful means of understanding the relationship between true dose (as revealed by tissue burden) and biological effect, and that there is no reason to believe that standard fish bioaccumulation tests are not fit for purpose, so long as exposure concentrations can be maintained, measured and interpreted.

What additional tests, endpoints or analyses should be performed in order to assess the environmental toxicity and bioaccumulation of nanomaterials?

Respondents recommended a range of additional endpoints that could be measured. Those interested in biomarkers of exposure mentioned oxidative stress, DNA damage, metallothionein induction and measurement of inflammation. Other respondents

mentioned the need to measure genotoxicity and endocrine disrupting potential, as would be the case for macroscale chemicals.

However, most agreed that the central focus of standard ecotoxicity tests should be the measurement of survival, growth or reproduction.

What resources do you think might be needed to i) test the usefulness of your proposed additions, and ii) include these additions in routine testing of nanomaterials?

Respondents believed that funding of appropriately focused PhD studies could be a cost-effective means of developing information in key areas, but that standardisation would be necessary across initiatives, and that this would also require funding.

Adequate measurement and characterisation of nanoparticles in test systems is of fundamental importance, but the instruments necessary to achieve this are expensive. Funding of central facilities to provide this analytical support would be very useful.

To what extent are laboratory tests on nanomaterial hazards able to simulate likely hazards in the real environment?

Respondents generally felt that laboratory studies are unlikely to simulate the real environment particularly well, but there is insufficient knowledge to determine how large the gap might be between laboratory tests and the environmental behaviour and effects of nanoparticles. For example, it is conceivable that natural surfactants in surface waters, or anthropogenic surfactants in wastewaters, might help to disaggregate nanoparticles in the natural environment. Most respondents believed that this is rather unlikely, but a lack of empirical evidence to either support or refute this possibility means that it is difficult to define realistic worst case exposure scenarios.

Currently, most chemical substance hazard testing strategies are tiered, with short, inexpensive tests on endpoints such as survival used first of all in the lower tiers, with large safety factors applied to the results to derive Predicted No Effect Concentrations. Longer-term, more expensive, sublethal tests are then used if these earlier tests suggest high hazard (or high risk when compared with predicted exposure concentrations). To what extent is this approach transferable to the hazard and risk assessment of nanomaterials, and where is it not fit for purpose?

Respondents agreed that a tiered hazard and risk assessment framework is the most cost-effective approach, but some expressed reservations about whether there is currently enough knowledge to be confident that short-term tests are able to predict long-term effects. In the absence of this knowledge it is difficult to have confidence in the use of standard Assessment Factors for extrapolating from short-term studies to Predicted No Effect Concentrations.

Are there any Quality Control or Quality Assurance issues for environmental toxicity or bioaccumulation studies that differ for nanomaterials when compared to other chemical substances?

Characterisation of particles in real test solutions emerged as the primary issue that can reduce the quality of ecotoxicity tests with nanoparticles when compared with other types of chemical substance. Uncertainty about the degree of nanoparticle aggregation and true exposure experienced by organisms during a test means that it is difficult to interpret results.

There are also difficulties in ensuring that different batches (e.g., of CNTs) are similar, and even within the same batch, some respondents reported that they have experienced problems with the repeatability of ecotoxicity test results, probably because of difficulties in diluting test solutions consistently.

To summarise, what would you say are the most important weaknesses of environmental toxicity and bioaccumulation tests when examining the hazards of nanomaterials?

Respondents agreed that there are two main weaknesses in current ecotoxicity testing of nanoparticles:

(i) Poor characterisation of nanoparticles in test systems and, as a result of this, a poor understanding of the dose to which test organisms are exposed.

(ii) Little understanding of how laboratory tests with nanoparticles relate to exposures that might realistically occur in the natural environment.

5. CONCLUSIONS: SHOULD ECOTOXICITY TEST STRATEGIES AND METHODS BE AMENDED WHEN TESTING NANOPARTICLES?

5.1 Overall conclusions

1. Studies on mammals raise the concern that there are toxic effects of nanomaterials (albeit at mg doses), and we should therefore establish whether or not manufactured nanoparticles are also toxic to wildlife. There is little empirical information on the ecotoxicity or bioaccumulation potential of nanoparticles and, depending on exposure pathways, these data need to be generated for aquatic and terrestrial microbes, plants and animals to reduce uncertainties about the hazards posed by these substances. There is an emerging literature on the ecotoxicity of nanomaterials, with most of the studies on aquatic organisms, using a few types of nanoparticles that are commercially available.

2. Current chemicals regulations do not distinguish between the nanoscale and macroscale forms of substances, so ecotoxicity tests performed on the macroscale form may, from a legal point of view, need to be accepted for both macroscale and nanoscale forms by regulatory authorities. This needs to change so that, at the very least, an evidence-based case is presented by manufacturers to show that there is no difference in the hazards of nanoscale and macroscale forms of the same substance. This evidence may come from the use of rapid tests (see 4 below).

3. Standard methods for testing ecotoxicity are generally preferable because they provide the demographic data on survival, growth and reproduction that are required by environmental (ecological) risk assessors, and for species whose strengths and limitations in toxicity testing are well understood. Additional endpoints could be measured on these species if there is the need to understand mechanisms of toxic effect, which may primarily be of help in the use of read-across from one substance, or form of substance, to another. The main area of considerable uncertainty that applies to all ecotoxicity testing guidelines, *except* those in which dosing is oral, is the way in which the substance is dosed into, and maintained and measured in, the test medium.

4. Rapid test systems (e.g., *in vitro* or genomics tests) for measuring the toxicity of nanoparticles may be a useful addition to the standard suite of ecotoxicity tests, especially if they are used to demonstrate the similarity or dissimilarity of toxicity of macroscale and nanoscale forms, when the mode of action is understood. It is possible to select a suite of rapid tests from existing regulatory tests, although some of these may need to be imported from the clinical regulatory arena. The suite would need to be selected to identify overall toxicity (e.g., cell viability assay or microbial population growth test (e.g., with *Vibrio fischeri*)), and also to check for specific modes of toxicity that may not be detected by a general toxicity screen. This need not be exhaustive, but focus on major toxic mechanisms likely to be relevant for that type of nanomaterial. These might include genotoxicity (abbreviated Ames test or similar), immunotoxicity assays for tiered application), and an oxidative stress assay.

5. However, it is important to understand that it is *only* changes in age-related survival, growth and reproduction that can influence the size of a population of organisms. Ecological risk assessors are rarely interested in the health of individual organisms, so measures of individual effect such as the use of *in vitro* or genomic biomarkers are only useful to them if they can predict effects on survival, growth and reproduction. This differs substantially in both concept and methodology from the practices of human health risk assessment. It is likely that confusion between the aims of human health risk assessment and ecological risk assessment is behind the calls from some researchers for the use of "novel" (often rapid) endpoints to test the ecological risks of nanoparticles. Novel endpoints may throw light on mechanisms of action, and may arguably be quicker and easier to perform than standard ecotoxicity tests, but the measurement of anything other than survival, growth or reproduction must necessarily be translated into these endpoints before it is useful to an ecological risk assessor.

6. In contrast to the call for rapid test systems, there may be a case for requiring manufacturers to generate chronic ecotoxicity data, even in the absence of triggers from short-term tests, because of a lack of information on appropriate assessment factors for extrapolation from acute to chronic toxicity for nanoparticles. There is a recent precedent for this in European guidelines for the testing of human medicines in which manufacturers are required to generate chronic toxicity data.

7. To date there have been no systematic studies of nanoparticle ecotoxicity for the same species in seawater versus freshwater, or during graded salinity change. What little is known implies that toxicity tests in freshwater are unlikely to provide adequate information on toxicity in sea water and a separate series of tests with marine species may be needed.

8. A tiered environmental risk assessment approach similar to that used for macroscale chemicals is likely to be fit for purpose for assessing the risks of nanoparticles. Requirements for further data within such a tiered framework would be triggered by information on use and release patterns, environmental fate, persistence and bioaccumulation. However, there are some concerns about existing physico-chemical test methods, and in particular whether or not the partition coefficient test works for nanomaterials. Clumping of nanoparticles may prevent proper separation of the material in the octanol and water phases. The meniscus between the phases is likely to trap material undermining the founding assumption of free diffusion between the phases. The test may be fundamentally flawed at the nanoscale and this has implications for risk assessment strategies that use the partition coefficient as a trigger for requiring either sediment toxicity tests or bioaccumulation studies.

9. The following factors may be important to the behaviour of nanoparticles in the aqueous phase:
   - Dissolution of the nanoparticle
   - Emulsion or coatings on nanoparticles
   - Dispersion of nanoparticles by interactions with natural organic matter
   - Agglomeration and aggregation
   - Adsorption and precipitation properties of particles on surfaces
   - Effects of impurities on nanoparticle behaviour in solution

- Effects of solvents, sonication and stirring on nanoparticle solutions

This is an area where research is particularly lacking on the ecotoxicology of nanoparticles.

10. There are essentially three approaches to achieving a uniform stock solution of a test substance for ecotoxicity testing: dispersion in detergents or similar agents, by sonication, or by prolonged stirring. There are logistical and ecologically-based criticisms of all of these approaches. Nanomaterials discharged to the environment are not likely to occur in the presence of mg quantities of dispersant chemicals or sonication, although natural surfactants and humic substances may have similar effects. It could be argued that the non-dispersed material is likely to more relevant to what will happen in the real environmental, and toxicity test design should reflect that reality.

11. Organisms in ecotoxicity tests should be exposed to nanoparticles in a way that is environmentally relevant. Predicted Exposure Concentrations and Predicted No Effect Concentrations should be based on the same form of nanoparticle (e.g., agglomerated or dispersed) and expressed in the same units so that Risk Characterisation compares like with like. This form may change during the lifecycle of a nanoparticle, and it may therefore be necessary for tests to be repeated on these different forms to characterise risks adequately. The concentration of nanoparticles in ecotoxicity tests should be expressed as number or surface area per volume and not just as mass per volume.

12. A base set of measurements for characterising nanoparticles in ecotoxicity tests is suggested as a practical approach, given the limitations of the current technology for measuring the chemistry and particle sizes, and also considering what can be reasonably expected of a competent ecotoxicology laboratory. The following are suggested as a minimum base set:
    - Nomenclature information (details provided in Appendix 1 of this report).
    - Concentration of the material (e.g., mg l^{-1}).
    - Electron Microscope Images (or similar) of the material in solution.
    - Measurement of the individual particle size in the stock solutions used for dosing the test system. This would include mean particle size ± standard deviation for at least 6 replicate samples of the stock solution.
    - Measurement of the size of any agglomerated or aggregated material in the solution (means and standard deviation as above).
    - Use of a spectroscopic method, or other similar method, to confirm dispersion of the material at various dilutions of the stock solution within the detection limits of current methods.
    - Some measurements to confirm concentrations of the main expected impurities in the test material, and details of any washing procedures to remove these if this is desired in the experiment.

    Predicted Environmental Concentrations of nanoparticles in the environment must also be characterised in the same way, either through accurate modelling or direct measurement, so that PECs and PNECs can be compared in a meaningful way.

13. It may be desirable when assessing whether current ecotoxicity methods are fit for purpose to make simultaneous measurements with reference materials, especially at the evaluation stage. A starting point for searching for reference materials for aquatic ecotoxicity studies may be to choose some natural particles

with appropriate toxic and non-toxic characteristics, such as china clay, that are found in suspension in water. However, this is the subject of another currently funded Defra project, so no specific recommendations on this are made in this report.

5.2 When are current ecotoxicity strategies and methods not fit for purpose?

The review and interviews in the preceding sections suggest that there are a limited number of important areas in which current ecotoxicity testing strategies and methods are not wholly fit for purpose. These are:

1. The toxicity of macroscale forms of a substance may not reflect the toxicity of nanoscale forms.

2. The use of solvents or sonication to produce a homogeneous dispersion of nanoparticles at the beginning of ecotoxicity tests may not reflect the behaviour of nanoparticles in the natural environment in which it is possible that agglomerations predominate.

3. The standard reliance in most environmental risk assessment frameworks (including REACH) on acute toxicity data plus large Assessment Factors to predict the chronic effects of chemical substances is not yet supported by sufficient empirical data on acute-to-chronic ratios for nanoparticles. There is little empirical information on the ecotoxicity or bioaccumulation potential of nanoparticles and, depending on exposure pathways, these data need to be generated for aquatic and terrestrial microbes, plants and animals to reduce uncertainties about the hazards posed by these substances.

4. Reporting of ecotoxicity results for nanoparticles as mass concentration does not take account of their unique properties, which are better reflected by measurements of number concentration or surface activity. These parameters need to be measured in test systems - reliance on nominal concentrations is unreliable. These parameters also need to be accurately predicted for, or directly measured in, the environment so that Predicted Environmental Concentrations can be meaningfully compared to Predicted No Effect Concentrations from ecotoxicity tests.

5. There are some concerns about whether or not the partition coefficient test works for nanomaterials. This has implications for risk assessment strategies that use the partition coefficient as a trigger for requiring either sediment toxicity tests or bioaccumulation studies.

In other respects it is likely that existing strategies and methods are sufficiently fit for purpose.

5.3 How should current ecotoxicity strategies and methods be amended for testing nanoparticles?

1. The toxicity of nanoparticles must be measured in separate studies to those used to test the ecotoxicity of other physical forms. Measurement may be by use of appropriate rapid tests to demonstrate that the nanoparticle has similar hazard properties to other physical forms, *so long as the mode of toxic action of the nanoparticle is understood*. When there is uncertainty about the mode of action, a full suite of standard aquatic ecotoxicity tests with algae, invertebrates and fish should be performed with the nanoparticle.

2. Nanoparticles should be tested in ecotoxicity studies in the form in which they occur in the environment. If a reasonable worst case exposure scenario is that a nanoparticle will always be present in agglomerated form, then this should be the form in which it is tested, so that Predicted Environmental Concentrations of the agglomerated form can be compared with Predicted No Effect Concentrations for the same form. There is a lack of knowledge about the fate and behaviour of nanoparticles in the environment - this is the single most important area for research that is identified in this report. Without reliable information on realistic worst case exposure scenarios it is impossible to design appropriate exposure systems in laboratory ecotoxicity tests. As a result of this, many resources and test organisms will be wasted in performing irrelevant ecotoxicity tests that cannot be used in risk assessments.

3. Chronic ecotoxicity data on the effects of nanoparticles need to be generated to determine the potential for establishing reliable acute-to-chronic ratios and standard Assessment Factors. Preliminary information suggests that acute testing may not be a practical way of determining nanoparticle hazards, because of practical difficulties in exposing test organisms to high concentrations of nanoparticles and the slow uptake of these substances by organisms. It may therefore be the case that chronic ecotoxicity testing remains the only practical approach. If this is the case, then a limit test approach, as recommended by RIP 3.3 (EC 2007a) for fish testing under REACH, is likely to be the most cost-effective solution. The results from limit tests with algae (OECD 201), *Daphnia* (OECD 211) and fish early life stages (OECD 212), used in combination with information on the toxicity of other physical forms of a substance should be sufficient to perform an adequate environmental risk assessment for a nanoparticle.

4. Exposure in ecotoxicity tests with nanoparticles must be measured and expressed as number concentration or surface activity, as well as mass concentration. Reporting of nominal concentrations alone is inadequate.

6. RECOMMENDATIONS

We focus below on what we believe are the most important recommendations that emerge from the findings in this report.

1. Research on establishing appropriate ecotoxicity test strategies and methods for nanoparticles should currently focus primarily on defining realistic worst case exposure scenarios for nanoparticles in the environment and then testing the toxicity of nanoparticles under these scenarios. This research programme should consider the fate and behaviour of nanoparticles in the environment, with or without the presence of natural and anthropogenic substances and conditions that may influence aggregation state. The programme should include the following elements:
   - A desk study to characterise fully the environmental parameters that influence the fate and behaviour of the main classes of nanomaterials likely to be released to the environment.
   - Collation of data on these influential parameters for surface waters and terrestrial systems in the UK, and derivation of realistic worst case exposure scenarios for nanomaterials likely to be released to the environment.
   - Chronic laboratory ecotoxicity tests with microbes, algae and fish, which simulate these realistic worst case exposure scenarios. These studies should include sediment ecotoxicity studies designed to test the accuracy and precision for nanoparticles of estimates based on partitioning theory and partition coefficients.
   - Identification of appropriate QA/QC procedures and robust nanoparticle characterisation methods that can be used in long-term laboratory experiments and in field surveys of different media. This should include identification of any requirements for laboratory inter-calibration exercises.

   Ideally such a project would be a joint initiative between the UK Government and the nanotechnology industries, so that funding and the input of appropriate expertise are maximised.

2. A set of rapid, cost-effective tests should be agreed between regulators, industry and other stakeholders that are able to demonstrate that a nanoparticle has similar hazard properties to other physical forms of a substance. These should include, *inter alia*, tests to identify overall toxicity (e.g., cell viability assay or microbial population growth test (e.g., with *Vibrio fischeri*)), and also to check for specific modes of toxicity that may not be detected by a general toxicity screen, but are relevant for that type of nanomaterial. These might include genotoxicity (abbreviated Ames test or similar), immunotoxicity assays, and an oxidative stress assay.

3. When rapid tests are unable to demonstrate similar hazard properties to other physical forms, the *chronic* effects of nanoparticles should be measured in a limit test design.

7. REFERENCES

Aitken RJ, Chaudhry MQ, Boxall ABA, Hull M. 2006. Manufacture and use of nanomaterials: current status in the UK and global trends. Occupational Medicine 56: 300-306.

Aitken RJ, Tran CL, Donaldson K, Stone V, Cumpson P, Johnstone J, Chaudhry Q, Cash S. 2007. Reference materials for engineered nanoparticle toxicology and metrology. Draft Report May 2007 for UK Department for Environment, Food and Rural Affairs.

Asuri P, Karajanagi SS, Sellitto E. 2006. Water-soluble carbon nanotubes-enzyme conjugates as functional biocatalytic formulations. Biotechnology and Bioengineering 95: 804-811

Barnhill H.N, Reuther R. Ferguson PL, Dreher T, Wang Q. 2007. Turnip yellow mosaic virus as a chemoaddressable bionanoparticle. Bioconjugate Chemistry 18:852-859.

Barlow PG, Donaldson K, MacCallum J, Clouter A, Stone V. 2005. Serum exposed to nanoparticle carbon black displays increased potential to induce macrophage migration. Toxicology Letters 155:397-401.

BAuA (German Federal Institute for Occupational Health and Safety). 2007. Nanotechnology: Health and Environmental Risks of Nanoparticles - Research strategy.[http://www.baua.de/nn_47716/sid_B0AA05CB8FF8141A55EAC94DDDFCC809/nsc_true/de/Themen-von-A-Z/Gefahrstoffe/Nanotechnologie/pdf/draft-research-strategy.pdf]

BCC report. Nanotechnology: A Realistic Market Assessment. 2006. Report highlights - http://www.bccresearch.com/nan/ (31/01/07)

Bergeron S, Archambault E. 2005. Canadian stewardship practices for environmental nanotechnology. Environment Canada, Science-Metrix Nanotechnology Stewardship.[http://science-metrix.com/eng/projects_2005_Nanotechnology_Environment.htm].

Bermudez E, Mangum JB, Wong BA, Asgharian B, Hext PM, Warheit DB, Everitt JI. 2004. Pulmonary responses of mice, rats, and hamsters to subchronic inhalation of ultrafine titanium dioxide particles. Toxicological Sciences 77:347-357.

Biswas P, Wu C-Y. 2005. Nanoparticles and the environment. Journal of the Air and Waste Management Association 55:708-746.

Blanchard J, Grosell M. 2006. Copper toxicity across salinities from freshwater to seawater in the euryhaline fish *Fundulus heteroclitus*: Is copper an ionoregulatory toxicant in high salinities? Aquatic Toxicology 80:131-139

Braydich-Stolle L, Hussain S, Schlager JJ, Hofmann MC. 2005. In vitro cytotoxicity of nanoparticles in mammalian germline stem cells. Toxicological Sciences 88:412-419.

Chen KA, Elimelech M. 2007. Influence of humic acid on the aggregation kinetics of fullerenes (C_{60}) nanoparticles in monovalent and divalent electrolyte solutions. Journal of Colloid and Interface Science (in press).

Colvin VL. 2003. The potential environmental impact of engineered nanoparticles. Nature Biotechnology 21:1166-1170.

Cui DX, Tian FR, Ozkan CS, Wang M, Gao HJ. 2005. Effect of single wall carbon nanotubes on human HEK293 cells.Toxicology Letters 155:73-85.

Cui Z, Lockman PR, Atwood CS, Hsu C-H, Gupte A, Allen DD. 2005. Novel $_D$-penicillamine carrying nanoparticles for metal chelation therapy in Alzheimer's and other CNS diseases. European Journal of Pharmacology and Biopharmacology 59:263-72.

Dobson J. 2001. Nanoscale biogenic iron oxides and neurodegenerative disease. FEBA Letters 496:1-5.

Dreher KL. 2004. Health and environmental impact of nanotechnology. Toxicological Sciences 77:3-6.

EC. 2003. Technical Guidance Document in support of Commission Directive 93/67/EEC on risk assessment for new notified substances, Commission Regulation (EC) No. 1488/94 on Risk Assessment for Existing Substances and Directive 98/8/EC of the European Parliament and of the Council concerning the placing of biocidal products on the market. Ispra (IT): EC.

EC. 2007a. RIP 3.3.2 EWG08 Aquatic (Pelagic) Toxicity. Revised draft 26 October 2006.

EC. 2007b. RIP 3.3.2 EWG ToR Appendix 2. Draft EWG11 Terrestrial_version3.doc. Created 19 February 2007.

Eddy FB. 1982. Osmotic and ionic regulation in captive fish with particular reference to salmonids. Comparative Biochemistry and Physiology B-Biochemistry and Molecular Biology 73:125-141.

EMEA. 2006. Guideline on the environmental risk assessment of medicinal products for human use. European Medicines Evaluation Agency, London, UK.[http://www.emea.eu.int/pdfs/human/swp/444700en.pdf]

Environmental Defense – DuPont Nano Partnership. 2007. Nano Risk Framework. Draft 26 February 2007.[http://nanoriskframework.com/page.cfm?tagID=1081]

Federici G, Shaw BJ, Handy R D. 2007. Toxicity of titanium dioxide nanoparticles to rainbow trout, (*Oncorhynchus mykiss*): gill injury, oxidative stress, and other physiological effects. Aquatic Toxicology (in press).

Fortner JL, Lyon D, Sayer CM, Boyd A, Frehner JG, Hotze EM, Alemann LB, Hughes J. 2005. C^{60} in water: nanocrystal formation and microbial response. Environmental Science and Technology 39:4307-4316.

Frater L, Stokes E, Lee R, Oriola T. 2006. An overview of the framework of current regulation affecting the development and marketing of nanomaterials. A Report for the DTI. ESRC Centre for Business Relationships Accountability Sustainability and Society (BRASS). Cardiff University, December 2006. [http://www.dti.gov.uk/science/science-in-govt/st_policy_issues/nanotechnology/page20218.html]

Freitas RA. 2005. What is nanomedicine? Nanomedicine 1:2-9.

Galloway TS, Handy RD. 2003. Immunotoxicity of organophosphorus pesticides. Ecotoxicology 12:345-363.

Grubek-Jaworska H, Nejman P, Czuminska K, Przybylowski T, Huczko A, Lange H, Bystrzejewski M, Baranowski P, Chazan R. 2006. Preliminary results on the pathogenic effects of intratracheal exposure to one-dimensional nanocarbons, Carbon 44:1057-1063.

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

Ham HT, Choi YS, Chung IJ. 2005. An explanation of dispersion states of single-walled carbon nanotubes in solvents and aqueous surfactant solutions using solubility parameters. Journal of Colloid and Interface Science 286:216-223.

Handy RD, Eddy FB. 2004. Transport of solutes across biological membranes in eukaryotes: An environmental perspective. In Physicochemical Kinetics and Transport at Chemical-Biological Interphases (editors HP van Leeuwen and W Köster), IUPAC series, pp. 337-356, John Wiley, Chichester.

Handy RD, Shaw BJ. 2007a. Toxic effects of nanoparticles and nanomaterials: Implications for public health, risk assessment and the public perception of nanotechnology. Health, Risk and Society 9:125-144.

Handy RD, Shaw BJ. 2007b. Ecotoxicity of nanomaterials to fish: Challenges for ecotoxicity testing. Integrated Environmental Assessment and Management 3:458-460.

Herbert DW, Alabaster JS, Dart MC, Lloyd D.1961. The effect of china-clay wastes on trout streams. International Journal of Air and Water Pollution 5:56-74.

Hirano K, Nitta H, Sawada K. 2005. Effect of sonication on the photo-catalytic mineralization of some chlorinated organic compounds. Ultrasonics Sonochemistry 12:271-276.

HMG. 2005. Response to the Royal Society and Royal Academy of Engineering Report: Nanoscience and nanotechnologies: opportunities and uncertainties, February 2005.[http://www.dti.gov.uk/science/science-tech-and-dti/page27229.html]

HSE. 2004. Nanoparticles: An occupational hygiene review. Research Report 274 by Aitken et al. HSE books, Norwich.

Hund-Rinke K, Simon M. 2006. Ecotoxic effect of photocatalytic active nanoparticles TiO_2 on algae and daphnids. Environmental Science and Pollution Research 13:225-232.

Hussain SM, Hess KL, Gearhart JM, Geiss KT, Schlager JJ. 2005. In vitro toxicity of nanoparticles in BRL 3A rat liver cells. Toxicology in Vitro 19:975-983.

Hyung H, Fortner JD, Hughes JB, Kim J-H. 2007. Natural organic matter stabilizes carbon nanotubes in the aqueous phase. Environmental Science and Technology 41:179-184.

Johnson DW, Haley MV, Hart GS, Muse WT, Landis WG. 1986. Acute toxicity of brass particles to *Daphnia magna*. Journal of Applied Toxicology 6:225-228.

Jobling S, Casey D, Rodgers-Gray T, Oehlmann J, Schulte-Oehlmann U, Pawlowski S, Braunbeck T, Turner AP, Tyler CR. 2003. Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. Aquatic Toxicology 66:211.

Karajanagi SS, Yang HC, Asuri P. 2006. Protein-assisted solubilisation of single-walled carbon nanotubes. Langmuir 22:1392 – 1395

Kashiwada S. 2006. Distribution of nanoparticles in the see-through medaka (*Oryzias latipes*). Environmental Health Perspectives 114:1697-1702.

Konishi Y, Ohno K, Saitoh N, Nomura T, Nagamine S, Hishida H, Takahashi Y, Uruga T. 2007. Bioreductive deposition of platinum nanoparticles on the bacterium *Shewanella algae*. Journal of Biotechnology 128:648-653.

Koyama S, Endo M, Kim YA, Hayashi T, Yanagisawa T, Osaka K, Koyama H, Haniu H, Kuroiwa N. 2006. Role of systemic T-cells and histopathological aspects after subcutaneous implantation of various carbon nanotubes in mice, Carbon 44:1079-1092.

Kreuter J. 2005. Application of nanoparticles for the delivery of drugs to the brain. International Congress Series 1277, Drug Transport(ers) and the Diseased Brain: 85-94.

Kuzma J, VerHage P. 2006. Nanotechnology in Agriculture and Food Production: Anticipated Applications. Report published 4th September 2006, Washington DC: Woodrow Wilson International Centre for Scholars.

Lam CW, James JT, McCluskey R, Hunter RL. 2004. Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. Toxicological Sciences 77:126-134.

Lead JR Wilkinson KJ. 2006. Aquatic colloids and nanoparticles: Current knowledge and future trends. Environmental Chemistry 3:159-171.

Lin Y, Allard LF, Sun YP. 2003. Protein-affinity of single-walled carbon nanotubes in water. Journal of Physical Chemistry B 108:3760-3764

Lovern SB, Klaper RD. 2006. *Daphnia magna* mortality when exposed to titanium nanoparticles and fullerene (C60) nanoparticles. Environmental Toxicology and Chemistry 25:1132-1137.

Lyven B, Hasselov M, Turner DR, Haraldsson C, Anderson K. 2003. Competition between iron and carbon based colloidal carriers for trace metals in a freshwater assessed using Flow Field-Flow Fractionation coupled to ICPMS. Geochimica et Cosmochimi Acta 67:3791-3802.

Masciangioli T, Zhang W-X. 2003. Environmental technologies at the nanoscale. Environmental Science and Technology 102-108.

Monteiro-Riviere NA, Nemanich RJ, Inman AO, Wang YYY,, Riviere J E. 2005. Multi-walled carbon nanotube interactions with human epidermal keratinocytes. Toxicology Letters 155:377-384.

Moore MN. 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? Environment International 32:967-976.

Muller J, Huaux F, Moreau N, Misson P, Heilier JF, Delos M, Arras M, Fonseca A, Nagy JB, Lison D. 2005. Respiratory toxicity of multi-wall carbon nanotubes. Toxicology and Applied Pharmacology 207:221-231.

NRCG. 2005. Characterising the potential risks posed by engineered nanopartiles. A first UK Government research report. Defra, London UK.[http://www.defra.gov.uk/environment/nanotech/research/index.htm].

NRCG. 2006. Characterising the potential risks posed by engineered nanopartiles. UK Government research – a progress report. Defra, London UK.[http://www.defra.gov.uk/environment/nanotech/research/index.htm].

Oberdörster E. 2004. Manufactured nanomaterials (Fullerenes, C_{60}) induce oxidative stress in the brain of juvenile Largemouth Bass. Environmental Health Perspectives 112:1058-1062.

Oberdörster E, Ortiz-Acevedo A, Xie H, Pantano P, Baughman RH, Dieckmann GR. 2005. Exposure of fathead minnow to fullerence and single walled carbon nanotubes. Official Journal of the Society of Toxicology 84:325.

Oberdörster E, Zhu S, Blickley TM, McClellan-Green P, Haasch ML. 2006. Ecotoxicology of carbon-based engineered nanoparticles: effects of fullerene (C60) on aquatic organisms. Carbon 44:1112-1120.

Oberdörster G, Oberdörster E, Oberdörster J. 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environmental Health Perspectives 113:823-839.

Oberdörster G et al. 2005. Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Particle and Fibre Toxicology 2:8.

Oberdörster G, Oberdörster E, Oberdörster J. 2007. Concepts of nanoparticle dose metric and response metric. Environmental Health Perspectives 115:A290-A291.

Oberdörster G, Ferin J, Gelein R, Soderholm SC, Finkelstein J. 1992. Role of the alveolar macrophage in lung injury - studies with ultrafine particles. Environmental Health Perspectives 97:193-199.

Owen R, Depledge M. 2005. Nanotechnology and the environment: Risks and rewards. Marine Pollution Bulletin 50:609-612.

Pantarotto D, Partidos CD, Graff R. 2003. Synthesis, structural characterisation, and immunological properties of carbon nanotubes functionalised with peptides. Journal of the American Chemical Society 125:6160-6164.

Prevodnik A, Gardeström J, Lilja K, Elfwing T, McDonagh B, Petrovíc N, Tedengren M, Sheehan D, Bollner T. 2007. Oxidative stress in response to xenobiotics in the blue mussel *Mytilus edulis L*: Evidence for variation along a natural salinity gradient of the Baltic Sea Aquatic Toxicology (in press).

Rickerby DG, Morrison M. 2007. Nanotechnology and the environment: A European perspective. Science and Technology of Advanced Materials 8:19-24.

Roberts AP, Mount AS, Seda B, Souther J, Qiao R, Lin SJ, Ke PC, Rao AM, Klaine SJ. 2007. In vivo biomodification of lipid-coated carbon nanotubes by *Daphnia magna*, Environmental Science and Technology 41:3025-3029.

Roco MC. 2003. Nanotechnology: convergence with modern biology and medicine. Current Op Biotech 14:337-346.

Rothen-Rutishauser BM, Schurch S, Haenni B, Kapp N, Gehr P. 2006. Interaction of fine particles and nanoparticles with red blood cells visualized with advanced microscopic techniques. Environmental Science and Technology 40:4353-4359.

Royal Society and Royal Academy of Engineering. 2004. Nanoscience and nanotechnologies: opportunities and uncertainties. The Royal Society, London.[www.nanotec.org.uk/finalReport.htm].

Sayes CM, Wahi R, Kurian PA, Liu YP, West JL, Ausman KD, Warheit DB, Colvin VL. 2006. Correlating nanoscale titaniam structure with toxicity: A cytotoxicity and inflammatory response study with human dermal fibroblasts and human lung epithelial cells. Toxicological Sciences 92:174-185.

SCENIHR. 2005. Opinion on the appropriateness of existing methodologies to assess the potential risks associated with engineered and adventitious products of nanotechnologies. Scientific Committee on Emerging and Newly Identified Health Risks, European Commission SCENIHR/002/05.

SCENIHR. 2007. 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. Scientific Committee on Emerging and Newly Identified Health Risks, European Commission 29th March 2007.

Scarano G., Morelli E. 2003. Properties of phytochelatin-coated CdS nanocrystallites formed in a marine phytoplanktonic alga (*Phaeodactylum tricornutum* Bohlin) in response to Cd", Plant Science 165:803-810.

Serita F, Kyono, H, Seki Y. 1999. Pulmonary clearance and lesions in rats after a single inhalation of ultrafine metallic nickel at dose levels comparable to the threshold limit value, Industrial Health 37:353-363.

Shvedova AA, Castranova V, Kisin ER, Schwegler-Berry D, Murray AR, Gandelsman VZ, Maynard A, Baron P. 2003. Exposure to carbon nanotube material: Assessment of nanotube cytotoxicity using human keratinocyte cells. Journal of Toxicology and Environmental Health-Part A 66:1909-1926.

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

Stolpe B, Hassellöv M. 2007. Changes in size distribution of fresh water nanoscale colloidal matter and associated elements on mixing with seawater. Geochimica et Cosmochimica Acta 71:3292–3301.

Stone V, Fernández TF, Ford A, Christofi N. 2006. Suggested strategies for the ecotoxicological testing of new nanomaterials. Symposium Proceedings. Materials Research Society Symposium Proceedings 895.

Takenaka S, Karg E, Kreyling WG, Lentner B, Schulz H, Ziesenis A, Schramel P, Heyder J. 2004. Fate and toxic effects of inhaled ultrafine cadmium oxide particles in the rat lung. Inhalation Toxicology 16:83-92.

Taylor MG, Simkiss K. 2004. Transport of solutes across biological membranes in eukaryotes: An environmental perspective. In: Physicochemical Kinetics and Transport at Chemical-Biological Interphases (editors HP van Leeuwen and W Köster), IUPAC series, pp. 337-356, John Wiley, Chichester.

Tian F, Cui D, Schwarz H, Estrada GG, Kobayashi H. 2006. Cytotoxicity of singe-wall carbon nanotubes on human fibroblasts. Toxicology in vitro 20:1202-1212.

Tran CL, Donaldson K, Stone V, Fernandes TF, Ford A, Christofi N, Ayres JG, Steiner M, Hurley JF, Aitken RJ, Seaton A. 2005. A scoping study to identify hazard data needs for addressing the risks presented by nanoparticles and nanotubes. IOM Research Report, Defra, UK.

USEPA. 2007. Nanotechnology White Paper. Science Policy Council, US Environmental Protection Agency, Washingr=ton DC, EPA 100/B-07/001, February 2007.[http://www.epa.gov/OSA/nanotech.htm]

Warheit DB. 2006. What is currently known about the health risks related to carbon nanotube exposures? Carbon 44:1064-1069.

Warheit DB, Laurence BR, Reed KL, Roach DH, Reynolds GAM, Webb TR. 2004. Comparative pulmonary toxicity assessment of single-wall carbon nanotubes in rats, Toxicological Sciences 77:117-125.

Warheit DB, Brock WJ, Lee KP, Webb TR, Reed KL. 2005. Comparative pulmonary toxicity inhalation and instillation studies with different TiO_2 particle formulations: Impact of surface treatments on particle toxicity. Toxicological Sciences 88:514-524.

Watanabe T, Nakajima A, Wang R, Minabe M, Koizumi S, Fujishima A, Hashimoto K. 1999. Photocatalytic activity and photoinduced hydrophilicity of titanium dioxide coated glass. Thin Solid Films 351:260-263.

Weltens R, Goossens R, Van Puymbroeck S. 2000. Ecotoxicity of contaminated suspended solids for filter feeders (*Daphnia magna*). Archives of Environmental Contamination and Toxicology 39:315-323.

Wu YL, Lim CS, Fu S, Tok AIY, Lau HM, Boey FYC, Zeng XT. 2007. Surface modifications of ZnO quantum dots for bio-imaging. Nanotechnology 18.

Yu J, Grossior N, Koning CE, Loos J. 2007. Controlling the dispersion of multi-wall carbon nanotubes in aqueous surfactant solution. Carbon 45:618-623.

Zhu S, Oberdörster E, Haasch ML. 2006. Toxicity of an engineered nanoparticle (fullerene, C60) in two aquatic species, *Daphnia* and fathead minnow. Marine Environmental Research 62:S5-S9.

APPENDIX 1 TERMINOLOGY

Nomenclature and defining nanomaterials and nanoparticles

There needs to be a pragmatic solution to nomenclature that enables consideration of materials that are already available, as well as naming of materials for the purpose of toxicity testing. The suggestion that "nanoscale" or "nano-substances" should be defined as anything < 100 nm is arbitrary, and from the view point of toxicology we should be flexible and allow for nanomaterials that form much larger aggregates. For toxicity testing, the prime purpose of nomenclature is to uniquely identify the substance being tested. Given that toxicity may be a function of particle size, shape, and chemical structure, and its behaviour in solution, then at least some of these factors should be used in nomenclature. This should include:

(i) The full SI chemical name of the material, its chemical formulae, and molecular weight.

(ii) The common name and/or product name.

(iii) Exact dimensions of the fundamental unit of the material e.g. maximum internal and external diameters, length, and pore size of nanotubes. This should include mean and SEM of actual measurements in batches of the material, not just the manufacturer's desired target size/shape.

(iv) In the case of materials made of mixtures of crystal structures, then a schematic of the crystal structure and its current geochemical name included. The relative proportions of each crystal structure in a mixture needs to be reported (e.g. anastase versus rutile forms of TiO2), and the stoichiometry of the key component (e.g. exactly how much Ti metal in anastase and rutile forms).

(v) Chemical analysis of the main trace contaminants in the material is required. For example, background levels of toxic metals in formulations. These can be quite high (1-2%) in some carbon nanotubes for example, and mg/L solutions of the material might easily generate toxic <g/L concentrations of metals. Similar arguments would apply to residual solvents that may have been used in the manufacture. These should at least be identified (and be included in the test protocol if needed).

(vi) Aggregation. The question of aggregation will be a function of the solution the material are dissolved in, but the manufacturer should at least identify the particle size and distribution of aggregates in the stock solution of the material that is provided for testing. A more detailed analysis of these chemical properties perhaps should be added to existing chemical characterisation protocols: see the main text of this report for a discussion.

A report of the above factors would at least identify the material for the purposes of starting a toxicity test. These would be in addition to some of usual physico-chemical parameters (solid, liquid, powder, flash point etc) that would be part of the chemical properties evaluation of the new material. However, nanomaterials can be complex substances with sometimes long chemical formulae according to SI unit rules, and it would be impractical to use SI names all the time in risk assessment documents. Common names should be used, but this will involve construction of a suitable database with agreed common names matched up with the full chemical formulae (similar to the approach with dyes and stains for example).

There is also the issue of substances that are already available with an existing CAS number. The problem of nanomaterials with the same chemical formula as an existing macroscale compound could be resolved by adding a prefix to the CAS number that helps identify the structure. This might involve the use of abbreviations for categories such as carbon nanotube (CNT) and some information about structure (e.g., tube length). For example, single walled carbon nanotubes and ordinary graphite are both made of carbon, and therefore currently have the same CAS number (CAS No. 7440-44-0). Perhaps the carbon nanotube version could be renumbered (e.g. CAS No. 7440-44-0-CNT-4 to indicate carbon nanotubes of 4 nm length). Similar arguments would apply to the metal oxides. For example, titanium dioxide powder and titanium dioxide nanoparticles can currently have the same number (CAS No. 13463-67-7). The alternative is to come up with a completely new set of CAS numbers for nanomaterials, but this approach might lose the linkage in data bases with the nearest equivalent powder of normal form of the same/similar compound.

Classification of nanomaterials

This is an area of expanding activity with a range of nanomaterials, nanoparticles, "nano-products" being manufactured, as well as the realisation that there are also natural nanoparticles in the environment. There are several fundamental options for classification which include:

(i) Classification by type of product or overall material properties.
(ii) Classification by chemical structure.
(iii) Classification by chemical properties or biological reactivity.
(iv) Combinations of the above.

The way in which these materials are classified will depend on the nature of the problem and task. So for example, if we are considering the problem of how many products are in the market place, the location, and/or nature of the manufacturing processes, or methods for monitoring tonnes of production. Then it may be better to use a materials classification system that enables the tracking of materials by product type. Several authors have reviewed nanomaterials from perspective of product type or use (Aitken et al. 2006; Kuzma and VerHage, 2006). A product classification has been suggested (discussed in Handy and Shaw, 2007). Nanomaterials can be organised broadly into three major product types (S. F. Hansen, Technical University of Denmark, personal communication):

(i) materials where the macroscale is made of nanostructure
(ii) materials with nanostructures on the surface (e.g. products with coatings) and
(iii) materials containing nanoparticles

This approach may be useful regarding legislation involved in product life cycle assessment or aspects of waste disposal regulations, but is less logical from the view point of REACH or toxicity testing.

A classification based on chemical structure and physico-chemical properties is likely to be more useful for practical toxicity testing (Fig. 1). The first step is to decide whether or not the substance is a nanomaterial. This could be based on a particle size cut off value (e.g. <100 nm for the unit structure), and allowing for aggregate formation (< 1 <m?). However, the material may not be in particle form, so some consider of the nanoscale of the materials structure is needed. If it is not a nanomaterial, then existing chemical classifications can be used. If the material is a

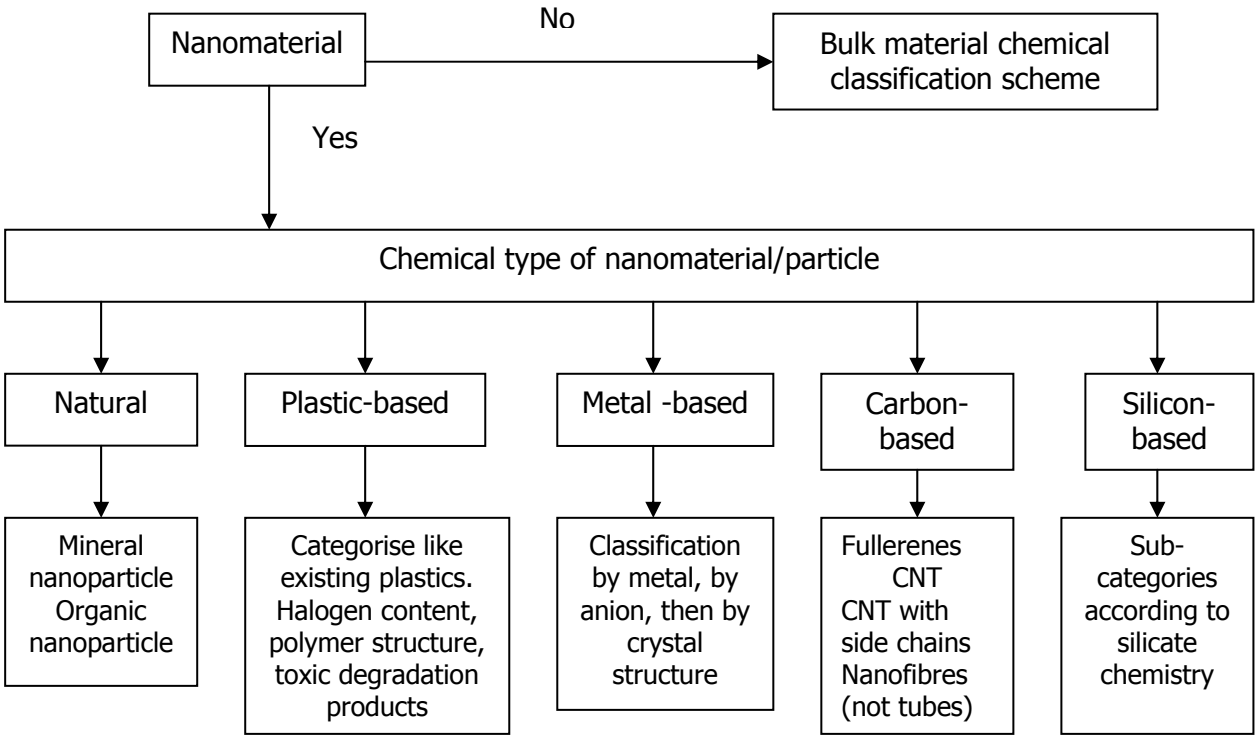
nanomaterial or nanoparticle (it may be better to use a catch-all phrase such as "nanosubstance") then it could be categorised by major chemical types. This would be based on structural chemistry not material properties at this stage in the scheme. So for example, materials that contained mostly carbon or where the basic unit of the structure was carbon, would be classified as "carbon-based nano-substances". Similar arguments could be used to derive other major categories of nano-substances. A "natural nano-substance" is also included as it may be that some manufactured nanoparticles are derived from simply grinding up rocks, or other naturally occurring material. In those cases perhaps the materials could be classified in the final tier according to their geochemical properties or organic C, N, or H content for organic matter (ie. using existing geochemical classifications). For plastic and plasticpolymers, the third tier could use existing classifications for plastics. However, it would be useful to consider the halogen content or major degradation products from the view point of toxicity assessments required.

For metal-based nano-substances an initial classification by metal content would useful (e.g. titanium-based materials etc). This could then be sub-divided by anion (metal sulphates, oxides, phosphates etc) and because these may involve several crystal structures, report these by crystal structure type as well (or the proportions of each crystal structure where a mixture of structures are present). Finally, any such chemical classification scheme will have materials that do not fit any one major category. Composites are a case in point, "Other" could include these or equally a separate classification for composites, or categorise them into one of the other groups based on the major ingredient. In any event, there may be completely novel materials emerging for which a category has not been invented and "Other" would be a useful interim measure.

The above classification scheme does not account for the physical shape or size of the material, but would lend itself to rational toxicity testing relative to existing chemicals. Another alternative is to abandon this chemical approach in favour of a material classification scheme based on particle size rather than chemical structure. The particle-size approach has some fundamental flaws: (i) you may need a sub-classification by chemical in any event, (ii) particle size may not be important if the reactivity is not on the surface of the material, (iii) many manufactured materials are not sold in particulate form, (iv) the scaling of biological reactivity with particle size is likely to vary with different chemical types (and shapes) of particles. It would therefore seem more logical to use a chemical classification, and identify particle-type as part of the "identity" of the new material at the start of the toxicity testing.

In summary, the balance of the argument is to identify the full chemical name of the material, but use a recognised list of common names for routine nomenclature. The nomenclature scheme should include variables that identify the physical size/shape of the nanomaterial. It may be possible to adapt the existing CAS numbers in the case of nano-substances that are very similar to existing macroscale chemicals. A chemical classification system has a number of merits from the view point of toxicity testing, and is preferred over a "product" or material "physical property" type scheme.

Figure 1 Chemical classification of nanomaterials and particles



APPENDIX 2: SUMMARY OF CHARACTERISATION TECHNIQUES FOR NANOPARTICLES

A detailed review of methods and technical aspects of particle characterisation is beyond the scope of this report, some discussion of the practical use of various techniques is given elsewhere (e.g. Taylor and Simkiss, 2004; Lead and Wilkinson, 2006). However, a few general points have emerged about the approaches to characterisation. Firstly, try to experimentally verify the particle size and impurities present in the material in your stock solutions, rather than just relying on reported data on the raw material from the manufacturer. Secondly, no one technique will resolve all the characterisation issues and it is expected that combinations of approaches will be needed such as electron micrographs to measure particle diameter and optical methods to verify if the entire solution is dispersed. There are advantages, and limitations of each approach which must be considered when reviewing the data collected.

The available approaches fall into 3 main categories for particle size determination:

(i) electron microscopy for particle size determination,

(ii) light scattering and ultracentrifuge methods that rely on hydrodynamic radius of the material,

(iii) laser reflection/refraction or UV-absorption techniques to indicate particle size in solutions.

In addition, there are techniques for fractioning or separating samples so that particles size ranges can be selected or determined. For impurities there are many approaches that will not be covered here, but inductively coupled plasma mass spectrometry (ICP-MS) can be used to determine metals, and a plethora of HPLC methods for organic chemicals. NMR may be particularly useful for carbon and other organic matter. Some of the common methods for particle separation or particle size determination are listed below:

Electron microscopy (EM); these techniques are well established and the use of scanning electron microscope or transmission electron microscopy have been used. Both approaches rely on drying the sample, and while individual particle size can be measured, there is no guarantee that aggregates present on the EM grid are true representations of aggregates in the solution since drying will tend to increase aggregation.

Atomic Force Microscopy (AFM); Has some advantages over conventional EM work in that samples do not necessarily need to be dried, but the fundamental operation of the instrument depends on reading forces on the surface of the material. It is not yet clear if novel materials introduce measurement artefacts.

Dynamic Light Scattering; This approach relies on the ability of particles to scatter light in a predictable way. When a beam of light passes through a dispersed particle

suspension (preferably monochromatic light or a laser), the particles will scatter light. The angle of the light scattering and time it takes to scatter can be used to estimate particle size distributions. When the particles are very small compared with the wavelength of the light, the intensity of the scattered light will be uniform in all directions (known as Rayleigh scattering). For larger particles (above about 250 nm in diameter), the intensity is angle dependent (known as Mie scattering). The main limitations of the technique is sensitivity, the more dilute the solution the less light scattering occurs. Also, if the instrument contains a photomultiplier to detect the light scattering, then there could be interferences or detector quenching from high concentrations of any photoactive engineered nanomaterials.

Zeta Potential Analysers; Surface reactivity or surface charge may be an important factor in particle toxicology. These analysers measure small voltage changes (electro-acoustics) sometimes in relation to titrating the surface of the particles in suspension. These instruments often have a capability for particle size determination based on sound attenuation through the sample as well. Of course, the application of solvents or capping agents to the nanomaterials may alter the surface coat and therefore the zeta potential. So some consideration of what the particles are made of is needed when interpreting the data.

BET Surface area; This approach relies on the rules governing the adsorption of gas molecules onto the particle surface. Stephen Brunauer, Paul Hugh Emmett, and Edward Teller published an article about this theory in 1938. "BET" comes from the first initials of their surnames (Brunauer et al. J. Am. Chem. Soc., 1938, 60, 309). Samples are usually heated while simultaneously flowing gas over the sample. The samples are then cooled with liquid nitrogen and analyzed by measuring the volume of gas adsorbed at specific pressures. This method relies on the assumption of gas adsorption to the surface of the material, so very porous materials that can trap gas inside the nanoparticle could, in theory, give some false positive results for "external surface area". This is a matter for data interpretation. However, techniques are available to measure porosity.

Field Flow Fractionation (FFF); Separation in FFF relies on particle size separation from the suspension during flow along a thin film of water (or solvent) in a flow channel. The channel has laminar flow because the film of water is thin. If a force is introduced perpendicular to the flow (e.g. hydrostatic, through a porous membrane) then particles will separate from the flow according to particle size. This approach is very effective but also has the disadvantage that the sample volume may be quiet large (tens of mls) and environmental samples may need pre-concentrating in order to achieve sensitivity for a good analysis of size distribution. FFF can be coupled with other techniques such as HPLC to analyse each fraction.

APPENDIX 3: SUMMARY OF STANDARD ECOTOXICITY TEST METHODS

Test Name	**OECD 201: Freshwater Alga and Cyanobacteria Growth Inhibition Test.**
Date adopted	23 March 2006.
Summary of test design	The purpose of the test is to determine the effects of a substance on the growth of freshwater microalgae and/or cyanobacteria. Exponentially growing test organisms are exposed to the test substance in batch cultures over a period of 72 hours. The test endpoint is inhibition of growth, expressed as the logarithmic increase in biomass (average specific growth rate) during the exposure period. The concentration bringing about a specified x% inhibition of growth rate (e.g. 50%) is determined and expressed as the ErCx (e.g. ErC50). In addition, the lowest observed effect concentration (LOEC) and the no-observed effect concentration (NOEC) may be statistically determined.
Test Species	*Pseudokirchneriella subcapitata* *Desmodesmus subspicatus* *Navicula pelliculosa* *Anabaena flos-aquae* *Synechococcus leopoliensis.*
Number of organisms per vessel	5×10^3 - 10^5 depending on species used
Level of replication	At least 3 replicates.
Concentration range	Five concentrations in a geometric series with a factor preferably not exceeding 3.2.
Test medium	OECD and AAP (US EPA) growth medium.
Dosing method	The test chemical should preferably be dosed directly into the test medium. Solvents, e.g. acetone, t-butyl alcohol and dimethyl formamide, may be used. The concentration of solvent should not exceed 100 µl/l and the same concentration of solvent should be added to all cultures (including controls) in the test series.
Measurement of test concentrations	Analysis recommended at varying degrees depending on the test system and properties of the test substance
Reference material	3,5-dichlorophenol and potassium dichromate.
Test Duration	72 hours.
Endpoints measured	ErC10/20/50, LOEC, NOEC.
Test validity criteria	The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72-hour test period. The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2 and 2-3, for 72-hour tests) in the control cultures must not exceed 35%. The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7% in tests with *Pseudokirchneriella subcapitata*

	and *Desmodesmus subspicatus*. For other species the value should not exceed 10%.

Test Name	**OECD 202: *Daphnia Sp.* acute immobilisation test.**
Date adopted	13 April 2004.
Summary of test design	Young daphnids, aged less than 24 hours at the start of the test are exposed to the test substance at a range of concentrations for a period of 48 hours. Immobilisation is recorded at 24 hours and 48 hours and compared with control values. The results are analysed in order to calculate the EC50 at 48 hours. Determination of the EC50 at 24 hours is optional.
Test Species	*Daphnia magna* Straus is the preferred test species although other suitable *Daphnia* species can be used (e.g. *Daphnia pulex*).
Number of organisms per vessel	20.
Level of replication	4 (4 x 5 animals).
Concentration range	Five concentrations in a geometric series with a factor preferably not exceeding 2.2.
Test medium	Natural water (surface or ground water), reconstituted water or dechlorinated tap water are acceptable as holding and dilution water if daphnids will survive in it for the duration of the culturing, acclimation and testing without showing signs of stress.
Dosing method	The test chemical should preferably be dosed directly into the test medium. As far as possible, the use of solvents, emulsifiers or dispersants should be avoided. Preferably the test substance solutions should not exceed the limit of solubility in the dilution water.
Measurement of test concentrations	The concentration of the test substance should be measured, at a minimum, at the highest and lowest test concentration, at the beginning and end of the test.
Reference material	Potassium dichromate.
Test Duration	48 hours.
Endpoints measured	EC50.
Test validity criteria	In the control, including the control containing the solubilising agent (if used), not more that 10% of the daphnids should have been immobilised or show other signs of disease or stress, for example, discoloration or unusual behaviour such as trapping at the surface of water. The dissolved oxygen concentration at the end of the test should be ≥3 mg/l in control and test vessels.

Test Name	**OECD 203: Fish, Acute Toxicity Test.**
Date adopted	17 July 1992.
Summary of test design	The fish are exposed to the test substance preferably for a period of 96 hours. Mortalities are recorded at 24, 48, 72 and 96 hours and the concentrations which kill 50 per cent of the fish (LC50) are determined where possible.
Test Species	*Brachydanio rerio* (Zebra fish) *Pimephales promelas* (Fathead minnow) *Cyprinus carpio* (Carp) *Oryzias latipes* (Rice fish) *Poecilia reticulata* (Guppy) *Lepomis macrochirus* (Bluegill sunfish) *Oncorhynchus mykiss* (Rainbow trout).
Number of organisms per vessel	7 (1.0 g fish/litre).
Level of replication	Not stated.
Concentration range	Five concentrations in a geometric series with a factor preferably not exceeding 2.2.
Test medium	Good quality natural water or reconstituted water is preferred, although drinking water (dechlorinated if necessary) may also be used. Waters with total hardness of between 10 and 250 mg $CaC0_3$ per litre, and with a pH 6.0 to 8.5 are preferable.
Dosing method	The test chemical should preferably be dosed directly into the test medium. If necessary, vehicles such as organic solvents, emulsifiers or dispersants of low toxicity to fish may be used. The concentration of organic solvents, emulsifiers or dispersants should not exceed 100 mg/l.
Measurement of test concentrations	There must be evidence that the concentration of the substance being tested has been maintained at preferably 80% of the nominal concentration throughout the test.
Reference material	Not stated.
Test Duration	96 hours.
Endpoints measured	LC50.
Test validity criteria	The mortality in the control(s) should not exceed 10% (or one fish if less than ten are used) at the end of the test. Constant conditions should be maintained as far as possible throughout the test. The dissolved oxygen concentration must have been at least 60% of the air saturation value throughout the test. There must be evidence that the concentration of the substance being tested has been maintained at preferably 80% of the nominal concentration throughout the test. If the deviation from the nominal concentration is greater than 20%, results should be based on the measured concentration.

Test Name	**OECD 204: Fish, Prolonged Toxicity Test: 14-day Study.**
Date adopted	4 April 1984.
Summary of test design	The test assesses the longer-term effects of chemicals on fish. Threshold levels of lethal and other observed effects and NOECs are determined at intervals during the test period, which is at least fourteen days long.
Test Species	*Brachydanio rerio* (Zebra fish) *Pimephales promelas* (Fathead minnow) *Cyprinus carpio* (Carp) *Oryzias latipes* (Rice fish) *Poecilia reticulata* (Guppy) *Lepomis macrochirus* (Bluegill sunfish) *Oncorhynchus mykiss* (Rainbow trout).

Test Species	*Brachydanio rerio* (Zebra fish) *Pimephales promelas* (Fathead minnow) *Cyprinus carpio* (Carp) *Oryzias latipes* (Rice fish) *Poecilia reticulata* (Guppy) *Lepomis macrochirus* (Bluegill sunfish) *Oncorhynchus mykiss* (Rainbow trout).
Number of organisms per vessel	10 (1.0 g fish/litre).
Level of replication	Not stated.
Concentration range	The test concentrations chosen must permit the determination both of the threshold levels for the lethal and other observable effects and of the NOEC value.
Test medium	Drinking water supply (dechlorinated if necessary), good quality natural water or reconstituted water. Waters with a total hardness of between 50 and 250 mg of CaCO3 per litre, and with a pH 6.0 to 8.5 are preferable.
Dosing method	The test chemical should preferably be dosed directly into the test medium. Stock solutions of test substances of low water solubility may be prepared by mechanical dispersion or, if necessary, by use of vehicles, such as organic solvents, emulsifiers or dispersants of low toxicity to fish. The concentration of organic solvents, emulsifiers or dispersants should not exceed 100 mg/l.
Measurement of test concentrations	In the flow-through test, the concentration of the substance in the test solution may be determined at the beginning of the test. In the semi-static test at the beginning, immediately prior to the first renewal of the test solution and at the termination of the test. Appropriate procedures other than analysis for giving evidence that adequate concentrations of the test substance have been maintained can also be used.
Reference material	Not stated.
Test Duration	14 days.
Endpoints measured	EC/LC50 and NOEC.

Test validity criteria	The mortality in the control(s) should not exceed 10% (or one fish if less than ten are used) at the end of the test. Constant conditions should be maintained as far as possible throughout the test. The dissolved oxygen concentration must have been at least 60% of the air saturation value throughout the test. There must be evidence that the concentration of the substance being tested has been maintained at preferably 80% of the nominal concentration throughout the test. If the deviation from the nominal concentration is greater than 20%, results should be based on the measured concentration.

Test Name	**OECD 205: Avian Dietary Toxicity Test.**
Date adopted	4 April 1984.
Summary of test design	Birds are fed a diet containing the test substance at a range of concentrations for a period of five days. Beginning on day 6, the birds are fed the basal diet, free of the test substance, for a minimum of three additional days. Mortalities and signs of toxicity are recorded daily.

Test Species	*Anas platyrhynchos* (mallard duck) *Colinus virginianus* (bobwhite quail) *Columba livia* (pigeon) *Coturnix coturnix japonica* (Japanese quail) *Phasianus colchicus* (ring-necked pheasant) *Alectoris rufa* (redlegged partridge).
Number of organisms per vessel	5-10 birds (Pigeons 1 bird).
Level of replication	Two control groups and one treatment group for each of the five (at least) dietary levels of the test substance should be used.
Concentration range	A minimum of five test diets, each containing different concentrations of the test substance is required for the test. Each level should be separated by a constant factor preferably not exceeding 2.0.
Test medium	Basal diet.
Dosing method	Diets containing the required amount of the test substance are prepared by uniformly mixing the appropriate amount of the test substance with the prescribed basal diet for young birds. Uniform distribution of the test substance in the food is the criterion for selecting the method of mixing. If necessary, a carrier of low toxicity to birds may be used to ensure uniform distribution. Carriers should not exceed 2 per cent by weight of the diet and when used should also be added to the diets of the birds in the control. Water, corn oil or other carriers for which there is well-documented evidence that they do not interfere with the toxicity of test substances are acceptable.
Measurement of test concentrations	Guideline only states that "there must be evidence that the concentration of the substance being tested has been satisfactorily maintained in the diet".
Reference material	Not stated .
Test Duration	8 days (5 days on the test diet followed by a minimum of 3 days on normal diet).
Endpoints measured	LC50.

Test validity criteria	The mortality in the controls should not exceed 10% at the end of the test. There must be evidence that the concentration of the substance being tested has been satisfactorily maintained in the diet (it should be at least 80% of the nominal concentration) throughout the first five days of the test period. The lowest treatment level should not result in compound-related mortality or other observable toxic effects.

Test Name	**OECD 206: Avian Reproduction Test.**
Date adopted	4 April 1984.

Summary of test design	Birds are fed a diet containing the test substance in various concentrations for a period of not less than 20 weeks. Birds are induced, by photoperiod manipulation, to lay eggs. Eggs are collected over a ten-week period, artificially incubated and hatched, and the young maintained for 14 days. Mortality of adults, egg production, cracked eggs, egg shell thickness, viability, hatchability and effects on young birds are compared with the corresponding parameters in the controls.
Test Species	*Anas platyrhynchos* (mallard duck) *Colinus virginianus* (bobwhite quail) *Coturnix coturnix japonica* (Japanese quail).
Number of organisms per vessel	Birds may be kept in pens as pairs or as groups of one male and two (bobwhite quail and Japanese quail) or three (mallard duck) females.
Level of replication	For tests with pairs, at least twelve pens of birds should be used for each test concentration and for the control. For tests with groups, at least 8 pens of mallard ducks and twelve pens of bobwhite quail or Japanese quail should be used for each test concentration and for the control.
Concentration range	A minimum of three dietary concentrations of the test substance is required for the test. The highest concentrations should approximate one half of the LC10 (based on data from OECD 205). Lower concentrations should be geometrically spaced at fractions of the highest dose (e.g. 1/6 and 1/36 of the highest dose). The maximum recommended test concentration is 1000 ppm.
Test medium	Basal diet.
Dosing method (e.g. water/solvent)	Diets containing the required amount of the test substance are prepared by uniformly mixing the appropriate amount of the test substance with the prescribed basal diet for young birds. Uniform distribution of the test substance in the food is the criterion for selecting the method of mixing. If necessary, a carrier of low toxicity to birds may be used to ensure uniform distribution. Carriers should not exceed 2 per cent by weight of the diet and when used should also be added to the diets of the birds in the control. Water, corn oil or other carriers for which there is well-documented evidence that they do not interfere with the toxicity of test substances are acceptable.
Measurement of test concentrations	During the first week of the test, diets containing the highest and lowest concentrations should be analysed immediately after the initial mixing and again within four hours of replacing with freshly mixed diet, unless the stability of the test substance in the diet can be adequately demonstrated. If all analyses are within 80 per cent of expected concentrations, no further analyses are required, and the test diet should be renewed frequently enough to ensure maintenance of the concentrations.
Reference material	Not stated.
Test Duration	20 weeks.
Endpoints measured	NOEC and any statistically significant effect levels.

Test validity criteria	The mortality in the controls should not exceed 10% at the end of the test. The average number of 14-day-old survivors per hen in the controls should be at least 14, 12 and 24 for mallard duck, bobwhite quail and Japanese quail, respectively. The average egg shell thickness for the control group should be at least 0.34, 0.19 and 0.19 mm for mallard duck, bobwhite quail and Japanese quail, respectively. There must be evidence that the concentration of the substance being tested has been satisfactorily maintained in the diet (it should be at least 80% of the nominal concentration) throughout the test period.

Test Name	OECD 207: Earthworm, Acute Toxicity Tests.
Date adopted	4 April 1984.
Summary of test design	A screening test (filter paper contact test) can be used which involves exposing earthworms to test substances on moist filter paper in order to identify potentially toxic chemicals to earthworms in soil. The artificial soil test involves keeping earthworms in samples of a precisely defined artificial soil to which a range of concentrations of the test substance has been applied. Mortality is assessed 7 and 14 days after application.
Test Species	*Eisenia. foetida foetida* *Eisenia foetida Andrei*.
Number of organisms per vessel	1 (filter paper test). 10 (soil test).
Level of replication	10 (filter paper test). 4 (soil test).
Concentration range	Five concentrations in a geometric series are used.
Test medium	Filter paper: 80 to 85 g/m2, approximately 0.2 mm thick, medium grade. Artificial soil (10% sphagnum peat, 20% kaolin clay and 70% industrial sand, pH 6 ±0.5).
Dosing method (e.g. water/solvent)	For the filter paper test the substance is dissolved in water (if soluble up to a concentration of 1000 mg/l) or in a suitable organic solvent (e.g. acetone, hexane or chloroform), as appropriate, to give a range of known concentrations. For the soil tests an emulsion or dispersion of the test substance in deionised water is mixed with the artificial soil or sprayed evenly over it with a fine chromatographic or similar spray. If insoluble in water, the test substance can be dissolved in as small a volume as possible of a suitable organic solvent (e.g. hexane, acetone or chloroform). The solvent should be allowed to evaporate. If the test substance is not soluble, dispersible or emulsifiable, 10 g of a mixture of fine ground quartz sand and quantity of test substance corresponding to 750 g wet weight of artificial soil are mixed with 740 g wet artificial soil for each test container.
Measurement of test concentrations	Not stated.
Reference material	Chloracetamide.
Test Duration	72 hours (filter paper test). 14 days (soil test).
Endpoints measured	LC50.
Test validity criteria	The mortality in the control(s) should not exceed 10% at the end of either of the test.

Test Name	**OECD 208: Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test**
Date adopted	19 July 2006
Summary of test design	The test assesses effects on seedling emergence and early growth of higher plants following exposure to the test substance in the soil (or other suitable soil matrix). Seeds are placed in contact with soil treated with the test substance and evaluated for effects following usually 14 to 21 days after 50 % emergence of the seedlings in the control group. Endpoints measured are visual assessment of seedling emergence, dry shoot weight (alternatively fresh shoot weight) and in certain cases shoot height, as well as an assessment of visible detrimental effects on different parts of the plant.
Test Species	There are up to 32 species (monocotyledonous and dicotyledonous) that can be used for the test (Annex 2 of OECD 208).
Number of organisms per vessel	3 - 10 seeds per 100 cm^2 depending to the size of the seeds.
Level of replication	At least 4.
Concentration range	At least five in a geometric series plus untreated control, and spaced by a factor not exceeding three.
Test medium	Commercial potting soil or synthetic soil mix that contains up to 1.5 percent organic carbon may be used. Clay soils should not be used if the test substance is known to have a high affinity for clays. In addition, acid washed quartz sand, mineral wool and glass beads (e.g. 0.35 to 0.85 mm in diameter) can be used. These have been found to be suitable inert materials that minimally absorb the test substance, ensuring that the substance will be maximally available to the seedling .
Dosing method (e.g. water/solvent)	Substances which are water soluble or suspended in water can be added to water, and then the solution is mixed with soil with an appropriate mixing device. Substances with low water solubility should be dissolved in a suitable volatile solvent (e.g. acetone, ethanol) and mixed with sand. The solvent can then be left to volatilise. The treated sand is mixed with the soil. For solid, insoluble test substances, dry soil and the chemical are mixed in a suitable mixing device. For crop protection products, spraying the soil surface with the test solution is often used for application of the test substance.
Measurement of test concentrations	For soluble substances, verification of all test concentrations can be confirmed by analysis of the highest concentration test solution used with documentation on subsequent dilution. For insoluble substances, verification of compound material must be provided with weights of the test substance added to the soil. If demonstration of homogeneity is required, analysis of the soil may be necessary.
Reference material	Reference material may be used, but specific chemicals not specified.
Test Duration	14 to 21 days after 50 % of the control plants.
Endpoints measured	ECx (e.g. EC50) NOEC and LOEC.
Test validity criteria	Seedling emergence in controls is at least 70%. The control seedlings do not exhibit visible phytotoxic effects (e.g. chlorosis, necrosis, wilting, leaf and stem deformations) and the plants exhibit only normal

	variation in growth and morphology for that particular species. The mean survival of emerged control seedlings is at least 90% for the duration of the study. Environmental conditions for a particular species are identical and growing media contain the same amount of soil matrix, support media, or substrate.

Test Name	**OECD 209: Activated Sludge, Respiration Inhibition Test.**
Date adopted	4 April 1984.
Summary of test design	The respiration rate of an activated sludge fed with a standard amount of synthetic sewage feed is measured after a contact time of 30 minutes or 3 hours, or both. The respiration rate of the same activated sludge in the presence of various concentrations of the test substance under otherwise identical conditions is also measured. The inhibitory effect of the test substance at a particular concentration is expressed as a percentage of the mean respiration rates of two controls. An EC50 value is calculated from determinations at different concentrations.
Test Species	Various, activated sludge from a sewage treatment plant is normally used as the microbial innoculum for the test.
Number of organisms per vessel	200 ml of microbial innoculum (suspended solids level of 4 g/l (± 10 per cent)).
Level of replication	Not stated.
Concentration range	At least five concentrations, spaced by a constant factor preferably not exceeding 3.2.
Test medium	Activated sludge from a sewage treatment plant is normally used as the microbial innoculum for the test. Where possible, activated sludge should be obtained from a sewage work treating predominantly domestic sewage. The tests are run using synthetic sewage feed and microbial innoculum from the sewage sludge.
Dosing method (e.g. water/solvent)	The test chemical should preferably be dosed directly into the test medium Solutions of the test substance are freshly prepared at the start of the study using a stock solution. A stock solution concentration of O.5 g/l is appropriate. There is no mention of the use of solvents.
Measurement of test concentrations	Not stated.
Reference material	3,5-dichlorophenol.
Test Duration	30 minutes and 3 hours.
Endpoints measured	LC20/50/80.
Test validity criteria	The two control respiration rates are within 15% of each other. The EC50 (3 hours) of the reference substance 3,5-dichlorophenol is in the accepted range (5 to 30 mg/l).

Test validity criteria	The dissolved oxygen concentration must be between 60 and 100% of air saturation value at the test temperature.
Test Name	**OECD 210 Fish, Early life Stage Toxicity Test.**
Date adopted	**17th July 1992.**
	The water temperature must not differ by more than ±1.5°C between test chambers or between successive days at any time during the test, and should be within the temperature ranges specified for the test species.
Summary of test design	The early life stages of fish are exposed to a range of concentrations of the test substance dissolved in water, preferably under flow-through conditions. The test is begun by placing fertilised eggs in the test chambers and is continued at least until all the control fish are free-feeding. Lethal and sub-lethal effects are assessed and compared with control values to determine the lowest observed effect concentration and the no observed effect concentration. Evidence must be available to demonstrate that the concentrations of the test substance in solution have been satisfactorily maintained within ±20% of the mean measured values.
Test Species	*Brachydanio rerio* (Zebra fish) *Pimephales promelas* (fathead minnow) *Oryzias latipes* (Rice fish) *Oncorhynchus mykiss* (Rainbow trout) *Cyprinodon variegates* (Sheepshead minnow).
Number of organisms per vessel	At least 60 eggs.
Level of replication	2 replicates.
Concentration range	Normally five concentrations of the test substance spaced by a constant factor not exceeding 3.2.
Test medium	Any water in which the test species shows good control survival (described in the guideline) is suitable as a test water
Dosing method	The test chemical should preferably be dosed directly into the test medium For flow-through tests, a system which continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) is required. The flow rates of stock solutions and dilution water should be checked at intervals during the test and should not vary by more than 10%. A flow rate equivalent to at least five test chamber volumes per 24 hours is recommended. The use of solvents or dispersants (solubilising agents) may be required in some cases in order to produce a suitably concentrated stock solution (solvents should not be greater than 0.1 ml/l in the test solutions).
Measurement of test concentrations	A minimum of five determinations is necessary. In studies lasting more than one month determinations should be made at least once a week.
Reference material	Not stated.
Test Duration	The test should continue at least until all the control fish have been free-feeding. Test duration will depend upon the species used.
Endpoints measured	NOEC and LOEC.
	Overall survival of fertilised eggs in the controls and, where relevant, in the solvent controls must be greater than or equal to the limits defined in the guideline. When a solubilising agent is used it must have no significant effect on survival nor produce any other adverse effects on the early-life stages as revealed by a solvent-only control.

Test Name	**OECD 211: *Daphnia magna* Reproduction Test.**
Date adopted	21st September 1998.
Summary of test design	The primary objective of the test is to assess the effect of chemicals on the reproductive output of *Daphnia magna*. Young female *Daphnia* (the parent animals), aged less than 24 hours at the start of the test, are exposed to the test substance added to water at a range of concentrations. The test duration is 21 days. At the end of the test, the total number of living offspring produced per parent animal alive at the end of the test is assessed. The reproductive output of the animals exposed to the test substance is compared to that of the control(s) in order to determine the lowest observed effect concentration (LOEC) and the no-observed effect concentration (NOEC). In addition, the data are analysed using a regression model in order to estimate the concentration that would cause a x% reduction in reproductive output, i.e. ECx
Test Species	*Daphnia magna* Straus (preferably Clone A) (24 hours old and must not be first brood progeny).
Number of organisms per vessel	1 (10 x1 in static tests). 10 (4 x 10 in flowthrough tests).
Level of replication	10 (10 x 1 in static tests). 4 (4 x 10 animals).
Concentration range	At least five test concentrations arranged in a geometric series with a separation factor preferably not exceeding 3.2.
Test medium	Defined medium such as Elendt M4 and M7 media. However, other media are acceptable provided the performance of the *Daphnia* culture is shown to meet the validity criteria for the test.
Dosing method	Stock solutions should preferably be prepared by dissolving the substance in test medium. Organic solvents (e.g. acetone, ethanol, methanol, dimethylformamide and triethylene glycol) or dispersants (e.g. cremophor RH40, methylcellulose 0.01% and HCO-40) may be used, but every effort should be made to avoid the use of such materials. Solvents and dispersants should be ≤ 0.1 ml/l of the test solution.
Measurement of test concentrations	In semi-static tests where the concentration of the test substance is expected to remain within ± 20% of the nominal, the highest and lowest test concentrations should be analysed when freshly prepared and at the time of renewal on one occasion during the first week of the test. These determinations should be repeated at least at weekly intervals thereafter. For tests where the concentration of the test substance is not expected to remain within ± 20% of nominal, it is necessary to analyse all test concentrations, when freshly prepared and at renewal. If a flow-through test is used, a similar sampling regime to that described for semi-static tests is appropriate. However, it may be advisable to increase the number of sampling occasions during the first week to ensure that the test concentrations are remaining stable.
Reference material	Not stated.
Test Duration	21 days.
Endpoints measured	ECx NOEC and LOEC.
Test validity criteria	The control mortality of the parent animals (female *Daphnia*) does not exceed 20% at the end of the test.

	The mean number of live offspring produced per control parent animal surviving at the end of the test is ≥ 60.

Test Name	**OECD 212: Fish, Short-term Toxicity Test on Embryo and Sac-fry Stages.**
Date adopted	21st September 1998.
Endpoints measured	EC/LC, NOEC and LOEC
Summary of test design	The embryo and sac-fry stages of fish are exposed to a range of concentrations of the test substance dissolved in water. The test is begun by placing fertilised eggs in the test chambers and is terminated just before the yolk-sac of any larvae in any of the test chambers has been completely absorbed or before mortalities by starvation start in controls. Lethal and sub-lethal effects are assessed and compared with control values to determine the lowest observed effect concentration and hence the no-observed effect concentration. Alternatively, they may be analysed using a regression model in order to estimate the concentration that would cause a given percentage effect (ECx).
Test Validity criteria	The dissolved oxygen concentration must be between 60 and 100% of the air saturation value throughout the test. The water temperature must not differ by more than ± 1.5°C between test chambers or between successive days at any time during the test and should be within the temperature ranges specified for the test species. Overall survival of fertilised eggs in the controls and, where relevant, in the solvent controls must be greater than or equal to the limits defined in the guideline.
Test Species	*Brachydanio rerio* (Zebra fish) — *Carassius auratus* (Goldfish) *Pimephales promelas* (fathead minnow) — *Gadus morhua* (Cod) *Oryzias latipes* (Rice fish) — *Clupea harengus* (Herring) *Cyprinus carpio* (Common carp) — *Menidia peninsulae* (Silverside) *Oncorhynchus mykiss* (Rainbow trout) — *Cyprinodon variegates* (Sheepshead minnow) *Lepomis macrochirus* (Bluegill)
Number of organisms per vessel	At least 30 eggs.
Level of replication	3 replicates.
Concentration range	Five concentrations spaced by a constant factor not exceeding 3.2.
Test medium	Any water in which the test species shows good control survival (described in the guideline) is suitable as a test water.
Dosing method	The test chemical should preferably be dosed directly into the test medium. For flow-through tests, a system which continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) is required. The flow rates of stock solutions and dilution water should be checked at intervals during the test and should not vary by more than 10%. A flow rate equivalent to at least five test chamber volumes per 24 hours is recommended. For semi-static tests, two different renewal procedures may be followed; either new test solutions are prepared and surviving eggs and larvae gently transferred into the new vessels in a small volume of old solution or the test organisms are retained in the test vessels whilst a proportion (at least three-quarters) of the test water is changed. The use of solvents or dispersants (solubilising agents) may be required in some cases in order to produce a suitably concentrated stock solution (solvents should not be greater than 0.1 ml/l in the test solutions).
Measurement of test concentrations	In semi-static tests where the concentration is expected to remain within ± 20% of nominal, the highest and lowest test concentrations should be analysed when freshly prepared and immediately prior to renewal on at least three occasions spaced evenly over the test . For tests where the concentration of the test substance is not expected to remain within ± 20% of the nominal, it is necessary to analyse all test concentrations, when freshly prepared and at renewal. If a flow-through test is used, a similar sampling regime to that described for semi-static tests is appropriate. However, it may be advisable to increase the number of sampling occasions during the first week to ensure that the test concentrations are remaining stable.
Reference material	Not stated.

Test Name	**OECD 213: Honeybees, Acute Oral Toxicity Test.**
Date adopted	21st September 1998.
Summary of test design	Adult worker honeybees are exposed to a range of doses of the test substance dispersed in sucrose solution. The bees are then fed the same diet, free of the test substance. Mortality is recorded daily during at least 48 hours and compared with control values. If the mortality rate is increasing between 24 and 48h whilst control mortality remains at an accepted level, i.e. ≤10%, it is appropriate to extend the duration of the test to a maximum of 96 hour. The results are analysed in order to calculate the LD_{50} at 24 hours and 48 hours and, in case the study is prolonged, at 72 hours and 96 hours.
Test Species	*Apis mellifera.*
Number of organisms per vessel	10.
Level of replication	At least 3.
Concentration range	Five doses in a geometric series, with a factor not exceeding 2.2.
Test medium	Each test group of bees should be provided with 100-200 ml of 50% sucrose solution in water, containing the test substance at the appropriate concentration.
Dosing method	Where the test substance is a water miscible compound this may be dispersed directly in 50% sucrose solution. For technical products and substances of low water solubility, vehicles such as organic solvent, emulsifiers or dispersants of low toxicity to bees may be used (e.g. acetone, dimethylformamide, dimethylsulfoxide). The concentration of the vehicle depends on the solubility of the test substance and it should be the same for all concentrations tested. However, a concentration of the vehicle of 1% is generally appropriate and should not be exceeded.
Measurement of test concentrations	Not stated.
Reference material	Dimethoate.
Test Duration	48 – 96 hours.
Endpoints measured	LD50.
Test validity criteria	The average mortality for the total number of controls must not exceed 10% at the end of the test. The LD50 of the toxic standard meets the specified range.

Test Name	**OECD 214: Honeybees, Acute Contact Toxicity Test.**
Date adopted	21st September 1998.
Summary of test design	Adult worker honeybees are exposed to a range of doses of the test substance dissolved in appropriate carrier, by direct application to the thorax (droplets). The test duration is 48 hours. If the mortality rate is increasing between 24 and 48 hours whilst control mortality remains at an accepted level, i.e. ≤10%, it is appropriate to extend the duration of the test to a maximum of 96 hours. Mortality is recorded daily and compared with control values. The results are analysed in order to calculate the LD_{50} at 24 and 48 hours and in case the study is prolonged at 72 hours and 96 hours.

Summary of test design	Adult worker honeybees are exposed to a range of doses of the test substance dissolved in appropriate carrier, by direct application to the thorax (droplets). The test duration is 48 hours. If the mortality rate is increasing between 24 and 48 hours whilst control mortality remains at an accepted level, i.e. ≤10%, it is appropriate to extend the duration of the test to a maximum of 96 hours. Mortality is recorded daily and compared with control values. The results are analysed in order to calculate the LD_{50} at 24 and 48 hours and in case the study is prolonged at 72 hours and 96 hours.
Test Species	*Apis mellifera.*
Number of organisms per vessel	10.
Level of replication	At least 3.
Concentration range	Five doses in a geometric series, with a factor not exceeding 2.2.
Test medium	The test substance is to be applied as solution in a carrier, i.e. an organic solvent or a water solution with a wetting agent. As an organic solvent, acetone is preferred but other organic solvents of low toxicity to bees may be used (e.g. dimethylformamide, dimethylsulfoxide). For water dispersed formulated products and highly polar organic substances not soluble in organic carrier solvents, solutions may be easier to apply if prepared in a weak solution of a commercial wetting agent (e.g. Agral, Citowett, Lubrol, Triton, Tween).
Dosing method	The collected bees may be anaesthetized with carbon dioxide or nitrogen for application of the test substance. A volume of 1 ml of solution containing the test substance at the suitable concentration should be applied with a micro-applicator to the dorsal side of the thorax of each bee.
Measurement of test concentrations	Not stated.
Reference material	Dimethoate.
Test Duration	48 – 96 hours.
Endpoints measured	LD50.
Test validity criteria	The average mortality for the total number of controls must not exceed 10% at the end of the test. The LD50. of the toxic standard meets the specified range.

Test Name	**OECD 215: Fish, Juvenile Growth Test.**
Date adopted	21st January 2000.
Summary of test design	Juvenile fish in exponential growth phase are placed, after being weighed, in test chambers and are exposed to a range of sub-lethal concentrations of the test substance dissolved in water preferably under flow-through or semi static conditions for 28 days. Fish are fed daily. At the end of the test, the fish are weighed again. Effects on growth rates are analysed using a regression model in order to estimate the concentration that would cause a x% variation in growth rate, i.e. ECx . Alternatively, the lowest observed effect concentration (LOEC) and hence the no-observed effect concentration (NOEC) may be calculated.
Test Species	*Oncorhynchus mykiss* (Rainbow trout) *Oryzias latipes* (Rice fish) *Danio Rerio* (Zebra fish).
Number of organisms per vessel	16 trout of 3-5g in a 40-litre volume.
Level of replication	Replication not required for ECx calculation. 2 Replicates suggested for NOEC calculation.
Concentration range	Normally five concentrations of the test substance spaced by a constant factor not exceeding 3.2.
Test medium	Any water in which the test species shows good control survival (described in the guideline) is suitable as a test water.
Dosing method	The stock solution should preferably be prepared by simply mixing or agitating the test substance in the dilution water by using mechanical means (e.g. stirring or ultrasonication). Organic solvents or dispersants may be used and should be should be ≤ 0.1 ml/l of the test solution. For semi-static (renewal) tests, the frequency of medium renewal will depend on the stability of the test substance, but a daily water renewal is recommended. For flow-through tests, a system which continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) is required. The flow rates of stock solutions and dilution water should be checked preferably daily, during the test and should not vary by more than 10% throughout the test. A frequency of water removal during the test of 6 litres/g of fish/day is acceptable.
Measurement of test concentrations	In flow-through tests, the flow rates of diluent and toxicant stock solution should be checked at intervals, preferably daily, and should not vary by more than 10% throughout the test. In semi-static tests where the concentration of the test substance is expected to remain within ± 20% of the nominal values the highest and lowest test concentrations should be analysed when freshly prepared and immediately prior to renewal at the start of the study and weekly thereafter. For tests where the concentration of the test substance is not expected to remain within ± 20% of nominal, all test concentrations must be analysed following the same regime as for more stable substances.
Reference material	Not stated.
Test Duration	28 days.
Endpoints measured	ECx NOEC and LOEC.
Test validity criteria	The mortality in the control(s) must not exceed 10% at the end of the test. The mean weight of fish in the control(s) must have increased enough to permit the detection of the minimum variation of growth rate considered as significant (i.e. 50% of their mean initial weight over 28 days). The dissolved oxygen concentration must have been at least 60% of the air saturation value (ASV) throughout the test. The water temperature must not differ by more than ± 1 °C between test chambers at any one time during the test and should be maintained within a range of 2 °C within the temperature ranges specified for the test species.

Test Name	OECD 216: Soil Microorganisms: Nitrogen Transformation Test.
Date adopted	21st January 2000.

Summary of test design	Sieved soil is amended with powdered plant meal and either treated with the test substance or left untreated (control). After 0, 7, 14 days and 28 days of incubation, samples of treated and control soils are extracted with an appropriate solvent, and the quantities of nitrate in the extracts are determined. The rate of nitrate formation in treated samples is compared with the rate in the controls, and the percent deviation of the treated from the control is calculated. All tests run for at least 28 days. If, on the 28th day, differences between treated and untreated soils are equal to or greater than 25%, measurements are continued to a maximum of 100 days. If non agrochemicals are tested, a series of concentrations of the test substance are added to samples of the soil, and the quantities of nitrate formed in treated and control samples are measured after 28 days of incubation. Results from tests with multiple concentrations are analysed using a regression model, and the ECx values are calculated.
Test Species	Natural micro-organism community.
Number of organisms per vessel	Not applicable.
Level of replication	3 replicates.
Concentration range	If agrochemicals are tested, at least two concentrations should be used. The lower concentration should reflect at least the maximum amount expected to reach the soil under practical conditions whereas the higher concentration should be a multiple (e.g. x5) of the lower concentration. If non-agrochemicals are tested, a geometric series of at least five concentrations is used.
Test medium	One single soil is used. The use of soils freshly collected from the field is preferred. The recommended soil characteristics are as follows: • sand content: not less than 50% and not greater than 75% • pH: 5.5 - 7.5 • organic carbon content: 0.5 - 1.5 % • the microbial biomass should be measured and its carbon content should be at least 1% of the total soil organic carbon.
Dosing method (e.g. water/solvent)	The test substance is normally applied using a carrier. The carrier can be water (for water soluble substances) or an inert solid such as fine quartz sand (particle size: 0.1-0.5 mm). Liquid carriers other than water (e.g. organic solvents such as acetone, chloroform) should be avoided since they can damage the microflora. If sand is used as a carrier, it can be coated with the test substance dissolved or suspended in an appropriate solvent. In such cases, the solvent should be removed by evaporation before mixing with the soil. For an optimum distribution of the test substance in soil, a ratio of 10 g of sand per kilogram of soil (dry weight) is recommended.
Measurement of test concentrations	Not stated.
Reference material	Nitrapyrin.
Test Duration	28 days up to 100 days.
Endpoints measured	ECx (e.g. EC50).

Test validity criteria	Evaluations of test results with agrochemicals are based on relatively small differences (i.e. average value ± 25%) between nitrate concentrations in control and treated soil samples, so large variations in the controls can lead to false results. Therefore, the variation between replicate control samples should be less than ± 15%.

Test Name	OECD 217: Soil Microorganisms: Carbon Transformation Test.

Date adopted	21st January 2000.
Summary of test design	Sieved soil is either treated with the test substance or left untreated (control). After 0, 7, 14 and 28 days incubation, samples of treated and control soils are mixed with glucose, and glucose-induced respiration rates are measured for 12 consecutive hours. Respiration rates are expressed as carbon dioxide released (mg carbon dioxide/kg dry soil/h) or oxygen consumed (mg oxygen/kg soil/h). The mean respiration rate in the treated soil samples is compared with that in control and the percent deviation of the treated from the control is calculated. All tests run for at least 28 days. If, on the 28th day, differences between treated and untreated soils are equal to or greater than 25 % measurements are continued in 14 day intervals for a maximum of 100 days. If chemicals other than agrochemicals are tested, a series of concentrations of the test substance are added to samples of the soil, and glucose induced respiration rates (i.e. the mean of the quantities of carbon dioxide formed or oxygen consumed) are measured after 28 days. Results from tests with a series of concentrations are analysed using a regression model, and the ECx values are calculated.
Test Species	Natural micro-organism community.
Number of organisms per vessel	Not applicable.
Level of replication	3 replicates.
Concentration range	If agrochemicals are tested, at least two concentrations should be used. The lower concentration should reflect at least the maximum amount expected to reach the soil under practical conditions whereas the higher concentration should be a multiple (e.g. x5) of the lower concentration. If non-agrochemicals are tested, a geometric series of at least five concentrations is used.
Test medium	One single soil is used. The use of soils freshly collected from the field is preferred. The recommended soil characteristics are as follows: • sand content: not less than 50% and not greater than 75% • pH: 5.5 - 7.5 • organic carbon content: 0.5 - 1.5 % • the microbial biomass should be measured and its carbon content should be at least 1% of the total soil organic carbon.
Dosing method (e.g. water/solvent)	The test substance is normally applied using a carrier. The carrier can be water (for water soluble substances) or an inert solid such as fine quartz sand (particle size: 0.1-0.5 mm). Liquid carriers other than water (e.g. organic solvents such as acetone, chloroform) should be avoided since they can damage the microflora. If sand is used as a carrier, it can be coated with the test substance dissolved or suspended in an appropriate solvent. In such cases, the solvent should be removed by evaporation before mixing with the soil. For an optimum distribution of the test substance in soil, a ratio of 10 g of sand per kilogram of soil (dry weight) is recommended.
Measurement of test concentrations	Not stated.
Reference material	Not stated.
Test Duration	28 days up to 100 days.
Endpoints measured	ECx (e.g. EC50).

Test validity criteria	Evaluations of test results with agrochemicals are based on relatively small differences (i.e. average value ± 25%) between the carbon dioxide released or the oxygen consumed in (or by) control and treated soil samples, so large variations in the controls can lead to false results. Therefore, the variation between replicate control samples should be less than ± 15%.

Test Name	**OECD 218: Sediment-water chironomid toxicity test using spiked sediment.**
Date adopted	13th April 2004.
Summary of test design	First instar chironomid larvae are exposed to a concentration range of the test chemical in sediment - water systems. The test substance is spiked into the sediment and first instar larvae are subsequently introduced into test beakers in which the sediment and water concentrations have been stabilised. Chironomid emergence and development rate is measured at the end of the test. Larval survival and weight may also be measured after 10 days if required. These data are analysed either by using a regression model in order to estimate the concentration that would cause x% reduction in emergence, larval survival or growth, or by using statistical hypothesis testing to determine a NOEC/LOEC.
Test Species	*Chironomus riparias* *Chironomus yoshimatsui* *Chironomus tentans.*
Number of organisms per vessel	20 first instar larvae.
Level of replication	3-4 replicates.
Concentration range	Five concentrations of the test substance spaced by a constant factor not exceeding 2.
Test medium	Formulated sediment (4-5 % (d wt) finely ground peat; 20 % (d wt) kaolin clay and 75-76 % (d wt) quartz sand) or a well characterised natural sediment with any water which conforms to the chemical characteristics of acceptable dilution water as stated in the guideline. A sediment-water volume ratio of 1:4 is recommended.
Dosing method (e.g. water/solvent)	Static systems are used. Semi-static or flow-through systems with intermittent or continuous renewal of overlying water might be used in exceptional cases. Spiked sediments are usually prepared by addition of a solution of the test substance directly to the sediment. The stock solution of the test substance mixed with the formulated sediment by rolling mill, feed mixer or hand mixing. If poorly soluble in water, the test substance can be dissolved in a suitable organic solvent (e.g. hexane, acetone or chloroform). This solution is then mixed with 10 g of fine quartz sand for one test vessel. The solvent is allowed to evaporate and the sand is then mixed with the suitable amount of sediment per test beaker.
Measurement of test concentrations	At a minimum, samples of the overlying water, the pore water and the sediment should be analysed at the start and at the end of the test, at the highest concentration and a lower one.
Reference material	Lindane, trifluralin, pentachlorophenol, cadmium chloride or potassium chloride.
Test duration	28 days for *C. riparias* and *C. yoshimatsui* and 65 days for *C. tentans.*
Endpoints measured	ECx NOEC and LOEC.
Test validity criteria	The emergence in the controls must be at least 70% at the end of the test. *C. riparias* and *C. yoshimatsui* emergence to adults from control vessels should occur between 12 and 23 days after their insertion into the vessels; for *C. tentans* a period of 20 to 65 days is necessary.

	At the end of the test the oxygen concentration should be at least% of the air saturation value (ASV) at the temperature used and the pH of overlying water should be in the 6-9 range in all test vessels. The water temperature should not differ by more than ±1.0 °C. The water temperature could be controlled by isothermal room and in that case the room temperature should be confirmed at appropriate time intervals.

Test Name	**OECD 219: Sediment-water chironomid toxicity test using spiked water**.
Date adopted	13th April 2004.
Summary of test design	First instar chironomid larvae are exposed to a concentration range of the test chemical in sediment-water systems. The test starts by placing first instar larvae into the test beakers containing the sediment-water system and subsequently spiking the test substance into the water. Chironomid emergence and development rate is measured at the end of the test. Larval survival and weight may also be measured after 10 days if required. These data are analysed either by a regression model in order to estimate the concentration that would cause x% reduction in emergence, larvae survival or growth or by using statistical hypothesis testing to determine a NOEC/LOEC.
Test Species	*Chironomus riparias* *Chironomus yoshimatsui* *Chironomus tentans.*
Number of organisms per vessel	20 first instar larvae.
Level of replication	3-4 replicates.
Concentration range	Five concentrations of the test substance spaced by a constant factor not exceeding 2.
Test medium	Formulated sediment (4-5 % (d wt) finely ground peat; 20 % (d wt) kaolin clay and 75-76 % (d wt) quartz sand) or a well characterised natural sediment with any water which conforms to the chemical characteristics of acceptable dilution water as stated in the guideline. A sediment-water volume ratio of 1:4 is recommended.
Dosing method (e.g. water/solvent)	Static systems are used. Semi-static or flow-through systems with intermittent or continuous renewal of overlying water might be used in exceptional cases. Test concentrations are calculated on the basis of water column concentrations. Test solutions are usually prepared by dilution of a stock solution. Stock solutions should preferably be prepared by dissolving the test substance in test medium. The use of solvents or dispersants may be required in some cases in order to produce a suitably concentrated stock solution. Examples of suitable solvents are acetone, ethanol, methanol, ethylene glycol monoethyl ether, ethylene glycol dimethyl ether, dimethylformamide and triethylene glycol. Dispersants which may be used are Cremophor RH40, Tween 80, methylcellulose 0.01% and HCO-40. The solubilising agent concentration in the final test medium should be minimal (i.e. ≤ 0.1 ml/l) and should be the same in all treatments.
Measurement of test concentrations	At a minimum, samples of the overlying water, the pore water and the sediment should be analysed at the start and at the end of the test, at the highest concentration and a lower one.
Reference material	Lindane, trifluralin, pentachlorophenol, cadmium chloride or potassium chloride.
Test duration	28 days for *C. riparias* and *C. yoshimatsui* and 65 days for *C. tentans.*
Endpoints measured	ECx NOEC and LOEC.
Test validity criteria	The emergence in the controls must be at least 70% at the end of the test. *C. riparias* and *C. yoshimatsui* emergence to adults from control vessels

	should occur between 12 and 23 days after their insertion into the vessels; for *C. tentans* a period of 20 to 65 days is necessary. At the end of the test the oxygen concentration should be at least 60% of the air saturation value (ASV) at the temperature used and the pH of overlying water should be in the 6-9 range in all test vessels. The water temperature should not differ by more than ±1.0 °C. The water temperature could be controlled by isothermal room and in that case the room temperature should be confirmed at appropriate time intervals.

Test Name	OECD 220: Enchytraeid reproduction test.

Date adopted	13th April 2004.
Summary of test design	Adult enchytraeid worms are exposed to a range of concentrations of the test substance mixed into an artificial soil. The test assesses the total number of juveniles produced by parent animals and the survival of parent animals. The duration of the definitive test is six weeks. After the first three weeks, the adult worms are removed and morphological changes are recorded. After an additional three weeks, the number of offspring, hatched from the cocoons produced by the adults, is counted. The reproductive output of the animals exposed to the test substance is compared to that of the control(s) in order to determine the no-observed effect concentration (NOEC) and/or the ECx by using a regression model to estimate the concentration that would cause a x% reduction in reproductive output.
Test Species	*Enchytraeus albidus* *Enchytraeus buchholzi* *Enchytraeus crypticus.*
Number of organisms per vessel	10.
Level of replication	4.
Concentration range	Five concentrations in a geometric series spaced by a factor not exceeding 1.8.
Test medium	Artificial soil (10% sphagnum peat, 20% kaolin clay and 70% air dried quartz sand, pH 6±0.5).
Dosing method (e.g. water/solvent)	A solution of the test substance is prepared in deionised water in a quantity sufficient for all replicates of one test concentration. For chemicals insoluble in water but soluble in organic solvents, the test substance can be dissolved in the smallest possible volume of a suitable vehicle (e.g. acetone). The vehicle is sprayed on or mixed with a small amount, for example 2.5 g, of fine quartz sand. The vehicle is eliminated by evaporation and the mixture of quartz sand and test substance added to the pre-moistened soil and thoroughly mixed after adding an appropriate amount of deionised water to obtain the moisture required. For substances that are poorly soluble in water and organic solvents, the equivalent of 2.5 g of finely ground quartz sand per test vessel is mixed with the quantity of test substance to obtain the desired test concentration. This mixture of quartz sand and test substance is added to the pre-moistened soil and thoroughly mixed after adding an appropriate amount of de-ionised water to obtain the required moisture content.
Measurement of test concentrations	Not stated.
Reference material	Carbendazim.
Test Duration	42 days.
Endpoints measured	EC/LCx NOEC and LOEC.

Test validity criteria	Control adult mortality should not exceed 20% at the end of the range-finding test and after the first three weeks of the reproduction test. Assuming that 10 adults per vessel were used in setting up the test, an average of at least 25 juveniles per control vessel should have been produced at the end of the test. The coefficient of variation around the mean number of control juveniles should not be higher than 50% at the end of the reproduction test.

Endpoints measured	ECx, NOEC and LOEC.
Test Name	**OECD 221: *Lemna* sp. Growth Inhibition Test.**
Test validity criteria	For the test to be valid, the doubling time of frond number in the control must be less than 2.5 days (60 hours), corresponding to approximately a seven-fold increase in seven days and an average specific growth rate of 0.275 d^{-1}.
Date adopted	23rd March 2006.
Summary of test design	Exponentially growing plant cultures of the genus *Lemna* are allowed to grow as monocultures in different concentrations of the test substance over a period of seven days. The objective of the test is to quantify substance-related effects on vegetative growth over this period based on assessments of selected measurement variables. Frond number is the primary measurement variable. At least one other measurement variable (total frond area, dry weight or fresh weight) is also measured. To quantify substance-related effects, growth in the test solutions is compared with that of the controls and the concentration bringing about a specified x% inhibition of growth (e.g. 50 %) is determined and expressed as the ECx. In addition, the lowest observed effect concentration (LOEC) and the no-observed effect concentration (NOEC) may be statistically determined.
Test Species	*Lemna gibba* *Lemna minor*.
Number of organisms per vessel	Each test vessel should contain a total of 9 to 12 fronds.
Level of replication	3 replicates.
Concentration range	Five concentrations in a geometric series with a factor preferably not exceeding 3.2.
Test medium	A modification of the Swedish standard (SIS) *Lemna* growth medium is recommended for culturing and testing with *L. minor.* The growth medium, 20X – AAP is recommended for culturing and testing with *L. gibba*.
Dosing method	Test solutions are usually prepared by dilution of a stock solution. Stock solutions of the test substance are normally prepared by dissolving the substance in growth medium. For test substances of low water solubility it may be necessary to prepare a concentrated stock solution or dispersion of the substance using an organic solvent or dispersant in order to facilitate the addition of accurate quantities of the test substance to the test medium. However, every effort should be made to avoid the use of such materials. If a solvent or dispersant is used, its final concentration should be reported and kept to a minimum (≤ 100 µL/L). If a preliminary stability test shows that the test substance concentration cannot be maintained over the 7 day test duration, a semi-static test regime is recommended. In this case, the colonies should be exposed to freshly prepared test solutions on at least two occasions during the test.
Measurement of test concentrations	In static tests, the minimum requirement is to determine the concentrations at the beginning and at the end of the test. In semi-static tests where the concentration of the test substance is not expected to remain within 20 % of the nominal concentration, it is necessary to analyse all freshly prepared test solutions and the same solutions at each renewal.
Reference material	3,5-dichlorophenol.
Test Duration	7 days.

Test Name	**OECD 222: Earthworm reproduction test (*Eisenia fetida/Eisenia andrei*).**
Date adopted	13th April 2004.
Summary of test design	Adult worms are exposed to a range of concentrations of the test substance either mixed into the soil or, in case of pesticides, applied into or onto the soil using procedures consistent with the use pattern of the substance. Mortality and growth effects on the adult worms are determined after 4 weeks of exposure. The adults are then removed from the soil and effects on reproduction assessed after a further 4 weeks by counting the number of offspring present in the soil. The reproductive output of the worms exposed to the test substance is compared to that of the control(s) in order to determine the no-observed effect concentration (NOEC) and/or ECx using a regression model to estimate the concentration that would cause an x% reduction in reproductive output.
Test Species	*Eisenia foetida*, *Eisenia Andrei.*
Number of organisms per vessel	10 earthworms in 500 - 600 g dry mass of artificial soil.
Level of replication	At least 2.
Concentration range	Five concentrations in a geometric with a factor preferably not exceeding 2.
Test medium	Artificial soil (10% sphagnum peat, 20% kaolin clay and 70% air dried quartz sand, pH 6 ±0.5).
Dosing method (e.g. water/solvent)	A solution of the test substance in de-ionised water is prepared immediately before starting the test in a quantity sufficient for all replicates of one concentration. For water insoluble substances the substance is dissolved in a small volume of a suitable organic solvent (e.g. acetone) and then mixed into, a small quantity of fine quartz sand. The solvent is then removed by evaporation. The treated sand is then mixed thoroughly with the pre-moistened artificial soil. For insoluble substances a mixture comprised of 10 g of finely ground industrial quartz sand with a quantity of the test substance necessary to achieve the test concentration in the soil is prepared. The mixture is then mixed thoroughly with the pre-moistened artificial soil. For surface applications the soil is treated after the worms are added. The test substance should be applied to the surface of the soil as evenly as possible using a suitable laboratory-scale spraying device to simulate spray application in the field.
Measurement of test concentrations	Chemical analysis of the test substance at the start and the end of the test is recommended.
Reference material	Carbendazim or benomyl.
Test Duration	56 days.
Endpoints measured	EC/LCx NOEC and LOEC.
Test validity criteria	Each control replicate (containing 10 adults) to have produced ≥30 juveniles by the end of the test. The coefficient of variation of control reproduction to be ≤30%.

	Control adult mortality over the initial 4 weeks of the test to be ≤10%.

Test Name	OECD 224: Determination of the inhibition of the activity of anaerobic bacteria – reduction of gas production from anaerobically digesting (sewage) sludge.

Date adopted	8th January 2007.
Summary of test design	Aliquots of a mixture of anaerobically digesting sludge (20 g/L to 40 g/L total solids) and a degradable substrate solution are incubated alone and simultaneously with a range of concentrations of the test substance in sealed vessels for up to 3 days. The amount of gas (methane plus carbon dioxide) produced is measured by the increase in pressure (Pa) in the bottles. The percentage inhibition of gas production brought about by the various concentrations of the test substance is calculated from the amounts produced in the respective test and control bottles. The EC50 and other effective concentrations are calculated from plots of percentage inhibition against the concentration of the test chemicals or, more usually, its logarithm.
Test Species	Various, activated sludge from a sewage treatment plant is normally used as the microbial inoculum for the test.
Number of organisms per vessel	100 ml of microbial inoculum in a 160 ml bottle.
Level of replication	The number of necessary replicates depends on the degree of precision required for the inhibition indices. 3 are suggested..
Concentration range	At least five concentrations.
Test medium	Actively digesting sludge from a digester at a wastewater treatment plant, or alternatively, from a laboratory digester, treating sludge from predominantly domestic sewage. The solids concentration is usually between 2% and 4% (w/v). The test substrate consists of 10 g nutrient broth (e.g. Oxoid), 10 g of yeast extract and 10 g of D-glucose in deionised water and dilute to 100 ml.
Dosing method (e.g. water/solvent)	The test chemical should preferably be dosed directly into the test medium. Water soluble substances are dissolved in water to produce the stock solution. For water insoluble substances a solution of an appropriate concentration is made up in a suitable volatile solvent, for example, acetone, di-ethylether. The required volumes of solvent solution are added to the empty test bottles and the solvent evaporated before the addition of sludge.
Measurement of test concentrations	Not stated.
Reference material	3, 5-dichlorophenol.
Test Duration	3 days.
Endpoints measured	EC50.
Test validity criteria	The test is considered to be valid when an inhibition of greater than 20% is obtained in the reference control containing 150 mg/L of 3,5-dichlorophenol. More than 50 ml of gas per g of dry matter is produced in the blank

	control. The pH value is within the range of 6.2 to 7.5 at the end of the test.

Test Name	OECD 227: Terrestrial Plant Test: Vegetative Vigour Test.

Date adopted	19 July 2006.
Summary of test design	The test assesses the potential effects on plants following deposition of the test substance on the leaves and above-ground portions of plants. Plants are grown from seed usually to the 2- to 4- true leaf stage. Test substance is then sprayed on the plant and leaf surfaces at appropriate rate(s). After the application, the plants are evaluated against untreated control plants for effects on vigour and growth at various time intervals through 21 - 28 days from treatment. Endpoints measured are dry shoot weight (alternatively fresh shootweight), and in certain cases shoot height, as well as an assessment of visible detrimental effects on different parts of the plants. Appropriate statistical analysis are used to obtain an effective concentration ECx or an effective application rate ERx for the most sensitive parameter(s) of interest. Also, the no-observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) can be calculated in this test.
Test Species	There are up to 32 species (monocotyledonous and dicotyledonous) that can be used for the test (Annex 2 of OECD 227).
Number of organisms per vessel	3 - 10 seeds per 15 cm pot depending to the size of the seeds.
Level of replication	At least 4.
Concentration range	At least five in a geometric series plus untreated control, and spaced by a factor not exceeding 3.
Test medium	Commercial potting soil or synthetic soil mix that contains up to 1.5 percent organic carbon may be used. Clay soils should not be used if the test substance is known to have a high affinity for clays. Field soil should be sieved to 2 mm particle size in order to homogenize it and remove coarse particles. In addition, acid washed quartz sand, mineral wool and glass beads (e.g. 0.35 to 0.85 mm in diameter) can be used.
Dosing method (e.g. water/solvent)	The substance should be applied in an appropriate carrier (e.g. water, acetone, ethanol, polyethylene glycol and gum Arabic). Formulated products and formulations containing active ingredients and various adjuvants can also be tested. The test substance is sprayed onto the plant surface simulating typical spray tank applications. Generally, spray volumes should be in the range of normal agricultural practice, and not to exceed plant runoff. If solvents or carriers are applied, a second group of control plants should be established receiving only the solvent/carrier.
Measurement of test concentrations	The concentrations/rates of application must be confirmed by appropriate analytical verification. For soluble materials, verification of all test concentrations/rates can be confirmed by analysis of the highest concentration used for the test with documentation on subsequent dilution and use of calibrated application equipment. For insoluble materials verification of compound material must be provided with weights of test material added to the soil.
Reference material	Reference material may be used, but specific chemicals not specified.
Test Duration	21 - 28 days from treatment.
Endpoints measured	ECx (e.g. EC50) NOEC and LOEC.
Test validity criteria	Control seedling emergence is at least 70%. Control plants do not exhibit visible phytotoxic effects. Plants exhibit only normal variation in growth and morphology for that particular species.

	The mean plant survival is at least 90% for the duration of the study. Environmental conditions for a particular species are identical and growing media contain the same amount of soil matrix, support media, or substrate from the same source.

Test Name	OECD 301 A: DOC Die away test.

Date adopted	17th July 1992.
Summary of test design	A measured volume of inoculated mineral medium, containing a known concentration of the test substance (10-40 mg DOC/l) as the nominal sole source of organic carbon, is aerated in the dark or diffuse light at 22 ± 2°C. Degradation is followed by DOC analysis at frequent intervals over a 28-day period. The degree of biodegradation is calculated by expressing the concentration of DOC removed (corrected for that in the blank inoculum control) as a percentage of the concentration initially present. Primary biodegradation may also be calculated from supplemental chemical analysis for parent compound made at the beginning and end of incubation.
Test Species	Various depending on source of inoculum.
Number of organisms per vessel	10^7 - 10^8 cells per litre.
Level of replication	2 replicates.
Concentration range	1 test concentration.
Test medium	Deionised or distilled water, free from inhibitory concentrations of toxic substances (e.g. Cu^{2+} ions) is used. It must contain no more than 10% of the organic carbon content introduced by the test material. Mineral media are prepared from stock solutions of appropriate concentrations of mineral components, namely, potassium and sodium phosphates plus ammonium chloride, calcium chloride, magnesium sulphate and iron (III) chloride. The inoculum may be derived from a variety of sources: activated sludge; sewage effluents (unchlorinated); surface waters and soils; or from a mixture of these.
Dosing method (e.g. water/solvent)	Introduce 800 ml portions of mineral medium into 2-litre conical flasks and add sufficient volumes of stock solutions of the test and reference substances to separate flasks to give a concentration of chemical equivalent to 10-40 mg DOC/l. Inoculate the flasks with activated sludge or other source of inoculum to give a final concentration not greater than 30 mg suspended solids/l. The test is not suitable for low solubility or volatile substances.
Measurement of test concentrations	Chemical analysis for parent compound can be made at the beginning and end of incubation.
Reference material	Aniline (freshly distilled), sodium acetate and sodium benzoate.
Test Duration	28 days.
Endpoints measured	D_t.
Test validity criteria	A test is considered valid if the difference of extremes of replicate values of the removal of the test chemical at the plateau, at the end of the test or at the end of the 10-d window, as appropriate, is less than 20% and if the percentage degradation of the reference compound has reached the pass levels by day 14. If in a toxicity test, containing both the test substance and a

	reference compound, less than 35% degradation (based on total DOC) or less than 25% (based on total ThOD or $ThCO_2$) occurs within 14 days, the test substance can be assumed to be inhibitory.

Test Name	**OECD 301 B: CO_2 evolution test.**
Date adopted	17th July 1992.

Summary of test design	A measured volume of inoculated mineral medium, containing a known concentration of the test substance (10-20 mg DOC or TOC/l) as the nominal sole source of organic carbon is aerated by the passage of carbon dioxide-free air at a controlled rate in the dark or in diffuse light. Degradation is followed over 28 days by determining the carbon dioxide produced. The CO_2 is trapped in barium or sodium hydroxide and is measured by titration of the residual hydroxide or as inorganic carbon. The amount of carbon dioxide produced from the test substance (corrected for that derived from the blank inoculum) is expressed as a percentage of $ThCO_2$. The degree of biodegradation may also be calculated from supplemental DOC analysis made at the beginning and end of incubation.
Test Species	Various depending on source of inoculum.
Number of organisms per vessel	10^7 - 10^8 cells per litre.
Level of replication	2 replicates.
Concentration range	1 test concentration.
Test medium	Deionised or distilled water, free from inhibitory concentrations of toxic substances (e.g. Cu^{2+} ions) is used. It must contain no more than 10% of the organic carbon content introduced by the test material. Mineral media are prepared from stock solutions of appropriate concentrations of mineral components, namely, potassium and sodium phosphates plus ammonium chloride, calcium chloride, magnesium sulphate and iron (III) chloride. The inoculum may be derived from a variety of sources: activated sludge; sewage effluents (unchlorinated); surface waters and soils; or from a mixture of these.
Dosing method (e.g. water/solvent)	To each 5-litre flask add 2,400 ml mineral medium. Add an appropriate volume of the prepared activated sludge to give a concentration of suspended solids of not more than 30 mg/l in the final 3 litres of inoculated mixture. Add the test material and reference compound, separately, as known volumes of stock solutions, to replicate flasks to yield concentrations, contributed by the added chemicals, of 10 to 20 mg DOC or TOC/l. Add poorly soluble test substances directly to the flasks on a weight or volume basis. An emulsifier or solvent can be used, but it should not be toxic to bacteria and must not be biodegraded or cause foaming.
Measurement of test concentrations	Chemical analysis for parent compound can be made at the beginning and end of incubation.
Reference material	Aniline (freshly distilled), sodium acetate and sodium benzoate.
Test Duration	28 days.
Endpoints measured	$\%ThCO_2$
Test validity criteria	A test is considered valid if the difference of extremes of replicate values of the removal of the test chemical at the plateau, at the end of the test or at the end of the 10-d window, as appropriate, is less than 20% and if the percentage degradation of the reference compound has reached the pass levels by day 14. If in a toxicity test, containing both the test substance and a reference compound, less than 35% degradation (based on total DOC) or less than 25% (based on total ThOD or $ThCO_2$) occurred within 14 days, the test substance can

	be assumed to be inhibitory. The inorganic carbon content of the test substance suspension in the mineral medium at the beginning of the test must be less than 5% of the total carbon, and the total $C0_2$ evolution in the inoculum blank at the end of the test should not normally exceed 40 mg/l medium. If values greater than 70 mg CO_2/l are obtained, the data and experimental technique should be examined critically.

Test Name	OECD 301 C: Modified MITI test (I).

Date adopted	17th July 1992.
Summary of test design	The oxygen uptake by a stirred solution, or suspension, of the test substance in a mineral medium, inoculated with specially grown, unadapted micro-organisms, is measured automatically over a period of 28 days in a darkened, enclosed respirometer at 25 ± 1°C. Evolved carbon dioxide is absorbed by soda lime. Biodegradation is expressed as the percentage oxygen uptake (corrected for blank uptake) of the theoretical uptake (ThOD). The percentage primary biodegradation is also calculated from supplemental specific chemical analysis made at the beginning and end of incubation and optionally ultimate biodegradation by DOC analysis.
Test Species	Various depending on source of inoculum.
Number of organisms per vessel	10^7 - 10^8 cells per litre.
Level of replication	2 replicates.
Concentration range	1 test concentration.
Test medium	Deionised or distilled water, free from inhibitory concentrations of toxic substances (e.g. Cu^{2+} ions) is used. It must contain no more than 10% of the organic carbon content introduced by the test material. Mineral media are prepared from stock solutions of appropriate concentrations of mineral components, namely, potassium and sodium phosphates plus ammonium chloride, calcium chloride, magnesium sulphate and iron (III) chloride. The inoculum may be derived from fresh samples from no fewer than ten sites, mainly in areas where a variety of chemicals are used and discharged. From sites such as sewage treatment works, industrial waste-water treatment works, rivers, lakes, seas.
Dosing method (e.g. water/solvent)	When the solubility exceeds 1 g/l dissolve 1-10 g, as appropriate, of test or reference substance in water and make up to 1 litre. Otherwise prepare stock solutions in the mineral medium or add the chemical directly to the mineral medium. Poorly soluble test substances should be added directly on a weight or volume basis . Solvents or emulsifying agents should not be used.
Measurement of test concentrations	Chemical analysis for parent compound can be made at the beginning and end of incubation.
Reference material	Aniline (freshly distilled), sodium acetate and sodium benzoate.
Test Duration	28 days.
Endpoints measured	%ThOD.
Test validity criteria	A test is considered valid if: The oxygen uptake of the inoculum blank is normally 20-30 mg 0_2/l and should not be greater than 60 mg 0_2/l in 28 days. If the pH value should be within the range 6-8.5 and the oxygen consumption by the test substance more than 60%. The difference of extremes of replicate values of the removal of the test

	substance at the plateau or at the end of the test, as appropriate is less than 20% and if the percentage degradation of aniline calculated from the oxygen consumption exceeds 40% after 7 days and 65% after 14 days.

Test Name	OECD 301 D: Closed bottle test.

Date adopted	17th July 1992.
Summary of test design	The solution of the test substance in mineral medium, usually at 2-5 mg/l, is inoculated with a relatively small number of micro-organisms from a mixed population and kept in completely full closed bottles in the dark at constant temperature. Degradation is followed by analysis of dissolved oxygen over a 28-d period. The amount of oxygen taken up by the microbial population during biodegradation of the test substance, corrected for uptake by the blank inoculum run in parallel, is expressed as a percentage of ThOD or COD.
Test Species	Various depending on source of inoculum.
Number of organisms per vessel	$10^4 - 10^6$ cells per litre.
Level of replication	2 replicates.
Concentration range	2 test concentrations.
Test medium	Deionised or distilled water, free from inhibitory concentrations of toxic substances (e.g. Cu^{2+} ions) is used. It must contain no more than 10% of the organic carbon content introduced by the test material. Mineral media are prepared from stock solutions of appropriate concentrations of mineral components, namely, potassium and sodium phosphates plus ammonium chloride, calcium chloride, magnesium sulphate and iron (III) chloride. The inoculum is normally derived from the secondary effluent of a treatment plant or laboratory-scale unit receiving predominantly domestic sewage. An alternative source for the inoculum is surface water.
Dosing method (e.g. water/solvent)	If the solubility exceeds 1 g/l, dissolve 1-10 g, as appropriate, of test or reference substance in water and make up to 1 litre. Otherwise, prepare stock solutions in mineral medium or add the chemical directly to the mineral medium making sure that the chemical dissolves. An emulsifier or solvent can be used, but it should not be toxic to bacteria and must not be biodegraded or cause foaming.
Measurement of test concentrations	Chemical analysis for parent compound can be made at the beginning and end of incubation.
Reference material	Not stated.
Test Duration	28 days.
Endpoints measured	% ThOD or COD.
Test validity criteria	A test is considered valid if the difference of extremes of replicate values of the removal of the test chemical at the plateau, at the end of the test or at the end of the 10-d window, as appropriate, is less than 20% and if the percentage degradation of the reference compound has reached the pass levels by day 14. If in a toxicity test, containing both the test substance and a reference compound, less than 35% degradation (based on total

	DOC) or less than 25% (based on total ThOD or $ThCO_2$) occurred within 14 days, the test substance can be assumed to be inhibitory. Oxygen depletion in the inoculum blank should not exceed 1.5 mg dissolved oxygen/l after 28 days.

Test Name	**OECD 301 E: Modified OECD screening test**.

Date adopted	17th July 1992.
Summary of test design	A measured volume of mineral medium containing a known concentration of the test substance (10-40 mg DOC/l) as the nominal sole source of organic carbon is inoculated with 0.5 ml effluent per litre of medium. The mixture is aerated in the dark or diffused light at 22 + 2°C. Degradation is followed by DOC analysis at frequent intervals over a 28 d period. The degree of biodegradation is calculated by expressing the concentration of DOC removed (corrected for that in the blank inoculum control) as a percentage of the concentration initially present. Primary biodegradation may also be calculated from supplemental chemical analysis for the parent compound made at the beginning and end of incubation.
Test Species	Various depending on source of inoculum.
Number of organisms per vessel	10^5 cells per litre.
Level of replication	2 replicates.
Concentration range	1 test concentration.
Test medium	Deionised or distilled water, free from inhibitory concentrations of toxic substances (e.g. Cu^{2+} ions) is used. It must contain no more than 10% of the organic carbon content introduced by the test material. Mineral media are prepared from stock solutions of appropriate concentrations of mineral components, namely, potassium and sodium phosphates plus ammonium chloride, calcium chloride, magnesium sulphate and iron (III) chloride. The inoculum is derived from the secondary effluent of a treatment plant or laboratory-scale unit receiving predominantly domestic sewage
Dosing method (e.g. water/solvent)	Introduce 800 ml portions of mineral medium into 2-litre conical flasks and add sufficient volumes of stock solutions of the test and reference substances to separate flasks to give a concentration of chemical equivalent to 10-40 mg DOC/l. Inoculate the flasks with activated sludge or other source of inoculum to give a final concentration not greater than 30 mg suspended solids/l.
Measurement of test concentrations	Chemical analysis for parent compound can be made at the beginning and end of incubation.
Reference material	Aniline (freshly distilled), sodium acetate and sodium benzoate.
Test Duration	28 days.
Endpoints measured	D_t.
Test validity criteria	A test is considered valid if the difference of extremes of replicate values of the removal of the test chemical at the plateau, at the end of the test or at the end of the 10-d window, as appropriate, is less than 20% and if the percentage degradation of the reference compound has reached the pass levels by day 14. If in a toxicity test, containing both the test substance and a reference compound, less than 35% degradation (based on total

	DOC) or less than 25% (based on total ThOD or $ThCO_2$) occurred within 14 days, the test substance can be assumed to be inhibitory.

Test Name	OECD 301 F: Manometric respirometry test.
Date adopted	17th July 1992.

Summary of test design	A measured volume of inoculated mineral medium, containing a known concentration of test substance (100 mg test substance/l giving at least 50-100 mg ThOD/l) as the nominal sole source of organic carbon, is stirred in a closed flask at a constant temperature (± 1°C or closer) for up to 28 days. The consumption of oxygen is determined either by measuring the quantity of oxygen (produced electrolytically) required to maintain constant gas volume in the respirometer flask, or from the change in volume or pressure (or a combination of the two) in the apparatus. Evolved carbon dioxide is absorbed in a solution of potassium hydroxide or another suitable absorbent. The amount of oxygen taken up by the microbial population during biodegradation of the test substance (corrected for uptake by blank inoculum, run in parallel) is expressed as a percentage of ThOD or COD. Optionally, primary biodegradation may also be calculated from supplemental specific chemical analysis made at the beginning and end of incubation, and ultimate biodegradation by DOC analysis.
Test Species	Various depending on source of inoculum.
Number of organisms per vessel	10^7 - 10^8 cells per litre.
Level of replication	2 replicates.
Concentration range	1 test concentration.
Test medium	Deionised or distilled water, free from inhibitory concentrations of toxic substances (e.g. Cu^{2+} ions) is used. It must contain no more than 10% of the organic carbon content introduced by the test material. Mineral media are prepared from stock solutions of appropriate concentrations of mineral components, namely, potassium and sodium phosphates plus ammonium chloride, calcium chloride, magnesium sulphate and iron (III) chloride. The inoculum may be derived from a variety of sources: activated sludge; sewage effluents (unchlorinated); surface waters and soils; or from a mixture of these.
Dosing method (e.g. water/solvent)	Prepare solutions of the test and reference substances, in separate batches, in mineral medium equivalent to a concentration, normally, of 100 mg chemical/l (giving 50-100 mg ThOD/l), using stock solutions. An emulsifier or solvent can be used, but it should not be toxic to bacteria and must not be biodegraded or cause foaming.
Measurement of test concentrations	Chemical analysis for parent compound can be made at the beginning and end of incubation.
Reference material	Aniline (freshly distilled), sodium acetate and sodium benzoate.
Test Duration	28 days.
Endpoints measured	% ThOD or COD.
Test validity criteria	A test is considered valid if the difference of extremes of replicate values of the removal of the test chemical at the plateau, at the end of the test or at the end of the 10-d window, as appropriate, is less than 20% and if the percentage degradation of the reference compound has reached the pass levels by day 14. If in a toxicity test, containing both the test substance and a reference compound, less than 35% degradation (based on total DOC) or less than 25% (based on total ThOD or $ThCO_2$) occurred within 14 days, the test substance can be assumed to be inhibitory.

	The oxygen uptake of the inoculum blank is normally 20-30 mg 02/l and should not be greater than 60 mg/l in 28 days. If the pH value is outside the range 6-8.5 and the oxygen consumption by the test substance is less than 60%, the test should be repeated with a lower concentration of test substance.

Test Name	**OECD 302 A: Inherent Biodegradability: Modified SCAS Test.**
Date adopted	12 May 1981.
Summary of test design	Activated sludge from a sewage treatment plant is placed in an aeration (SCAS) unit. The test compound and settled domestic sewage are added, and the mixture is aerated for 23 hours. The aeration is then stopped, the sludge allowed to settle and the supernatant liquor is removed. The sludge remaining in the aeration chamber is then mixed with a further aliquot of test compound and sewage and the cycle is repeated. Biodegradation is established by determination of the dissolved organic carbon content of the supernatant liquor. This value is compared with that found for the liquor obtained from a control tube dosed with settled sewage only.

Test Species	Various depending on source of inoculum.
Number of organisms per vessel	Not stated.
Level of replication	Not stated.
Concentration range	1 test concentration.
Test medium	A sample of mixed liquor from an activated sludge plant treating predominantly domestic sewage is obtained. Approximately 150 ml of the mixed liquor are required for each aeration unit.
Dosing method (e.g. water/solvent)	The method is applicable to any non-volatile, water soluble, organic compound. The test chemical should preferably be dosed directly into the test medium. Stock solutions of the test compounds are prepared as 400 mg/litre organic carbon which gives a test compound concentration of 20 mg/litre carbon at the start of each aeration cycle if no biodegradation is occurring. 100 ml of settled sewage are added to the control units and 95 ml plus 5 ml of the appropriate test compound stock solution (400 mg/l) to the test units.
Measurement of test concentrations	Not stated.
Reference material	4-acetyl aminobenzene sulphonate, Tetra propylene benzene sulphonate, 4-nitrophenol, Diethylene glycol, Aniline.
Test Duration	Months.
Endpoints measured	% biodegradation.
Test validity criteria	At the end of the aeration period about 10 mg/litre of dissolved organic carbon remain in the supernatant liquor of the control experiment. Assuming that the dissolved organic carbon determination is within ± 5 per cent and a level of 20 mg/litre of carbon as test material is added at the start of the aeration period, then the assessment of the extent of biodegradation should be within ± 6% for the range 80-100% biodegradation.

Test Name	**OECD 302 B: Zahn-Wellens/EMPATest.**
Date adopted	17th July 1992.
Summary of test design	A mixture containing the test substance, mineral nutrients and a relatively large amount of activated sludge in aqueous medium is agitated and aerated at 20-25°C in the dark or in diffuse light for up to 28 days. The biodegradation process is monitored by determination of DOC (or COD) in filtered samples taken at daily or other time intervals. The ratio of eliminated DOC (or COD), corrected for the blank, after each time interval, to the initial DOC value is expressed as the percentage biodegradation at the sampling time. The percentage biodegradation is plotted against time to give the biodegradation curve.

Test Species	Various depending on source of inoculum.
Number of organisms per vessel	Not stated.
Level of replication	2 replicates.
Concentration range	1 test concentration.
Test medium	Deionised or distilled water, free from inhibitory concentrations of toxic substances (e.g. Cu^{2+} ions) is used. It must contain no more than 10% of the organic carbon content introduced by the test material. Mineral media are prepared from stock solutions of appropriate concentrations of mineral components, namely, potassium and sodium phosphates plus ammonium chloride, calcium chloride, magnesium sulphate and iron (III) chloride. The inoculum is derived from fresh effluent from a sewage treatment works (BOD_5 of effluent should be < 25 mg/l).
Dosing method (e.g. water/solvent)	Chemicals which are non-volatile and are soluble in water to at least 50 mg DOC/l may be assessed by this method, provided also that they do not significantly adsorb, are not lost by foaming and do not inhibit bacteria at the concentration tested. 500 ml mineral medium and the appropriate amounts of test substance and inoculum are mixed to reach respectively between 50 and 400 mg DOC/l (between 100 and 1000 mg COD/l) and 0.2-1.0 g dry matter/l in the final volume.
Measurement of test concentrations	Chemical analysis for parent compound can be made at the beginning and end of incubation.
Reference material	Ethylene glycol, diethylene glycol, lauryl sulfonate and aniline.
Test Duration	28 days.
Endpoints measured	$D_{t.}$
Test validity criteria	The test is considered valid if the procedural control shows the removal of the reference compound by at least 70% within 14d and if the removal of DOC (or COD) in the test suspension took place relatively gradually over days or weeks.

Test Name	OECD 302 C: Inherent biodegradability: modified MITI test (II).

Date adopted	12th May 1981.
Summary of test design	This test method is based on the following conditions: • test chemicals as sole organic carbon sources • no adaptation of micro-organisms to test chemicals An automated closed-system oxygen consumption measuring apparatus (BOD-meter) is used. Chemicals to be tested are inoculated in the testing vessels with micro-organisms. During the test period, the biochemical oxygen demand is measured continuously by means of a BOD meter. Biodegradability is calculated on the basis of BOD and supplemental chemical analysis, such as measurement of the dissolved organic carbon concentration, concentration of residual chemicals, etc.
Test Species	Various depending on source of inoculum.
Number of organisms per vessel	Not stated.
Level of replication	3 replicates.
Concentration range	1 test concentration.
Test medium	Deionised or distilled water, free from inhibitory concentrations of toxic substances (e.g. Cu^{2+} ions) is used. It must contain no more than 10% of the organic carbon content introduced by the test material. Mineral media are prepared from stock solutions of appropriate concentrations of mineral components, namely, potassium and sodium phosphates plus ammonium chloride, calcium chloride, magnesium sulphate and iron (III) chloride. The inoculum may be derived from fresh samples from no fewer than ten sites, mainly in areas where a variety of chemicals are used and discharged. From sites such as sewage treatment works, industrial waste-water treatment works, rivers, lakes, seas..
Dosing method (e.g. water/solvent)	The test chemical should preferably be dosed directly into the test medium. In case the test compound is not soluble in water at the desired test concentration, the test compound pulverised as finely as possible is employed. In case the test compound is volatile, test chemicals should be well-cooled to prevent evaporation.
Measurement of test concentrations	Chemical analysis for parent compound can be made at the beginning and end of incubation.
Reference material	Aniline, sodium acetate or sodium benzoate.
Test Duration	28 days.
Endpoints measured	% biodegradation.
Test validity criteria	If the percentage degradation of aniline calculated from the oxygen consumption does not exceed 40% after 7 days and 65%t after 14 days, the test is regarded as invalid. If the recovery rate of the test compound is found to be in the order of 10% or less, the test is also regarded as invalid. If the percentage degradation of aniline calculated from the oxygen consumption does not exceed 40% after 7 days, and 65% after 14 days, the test is regarded as invalid.

Test Name	OECD 304 A: Inherent Biodegradability in Soil.
Date adopted	12th May 1981.

Summary of test design	In the basic test a small sample of soil is treated with the ^{14}C-labelled test chemical in a biometer flask apparatus. Release of $^{14}CO_2$ from the test chemical is measured by means of alkali absorption and liquid scintillation counting. In the evaporation test, when testing chemicals of a vapour pressure higher than 0.0133 Pa, a polyurethane foam plug is placed into the biometer flask apparatus to absorb the labelled volatile part of the parent compound and volatile metabolites for liquid scintillation counting. In the residue test, at the point of 50% mineralisation, the test soil may be extracted. The extractable portion of the compound, and its metabolites remaining in the soil, may be determined by liquid scintillation counting. Furthermore, data on the bound residue part may be obtained by measuring the $^{14}CO_2$ released after combustion of the soil.
Test Species	Various depending on source of inoculum.
Number of organisms per vessel	Not stated.
Level of replication	Not stated.
Concentration range	1 test concentration.
Test medium	Alfisol: pH between 5.5 and 6.5; organic C content between 1 and 1.5%; clay content (i.e. particles < 0.002 mm in diameter) between 10 and 20% and cation exchange capacity between 10 and 15 mval. Spodosol: pH between 4.0 and 5.0; organic C content between 1.5 and 3.5%; clay content £ 10 % and cation exchange capacity < 10 mval. Entisol: pH between 6.6 and 8.0; organic C content between 1 and 4%; clay content between 11 and 25% and cation exchange capacity > 10 mval.
Dosing method (e.g. water/solvent)	^{14}C-labelled compounds are dissolved in water or acetone to give radioactivity of 37-185 KBq (1-5 µCi)/100 µl. Using unlabelled material, this solution is made up to the required concentration (e.g. 0.5 mg/100 µl 10 mg/kg soil, or depending on the toxicity of the substance). 100 µl of the radioactive test solution are added in 50 drops over the whole soil surface (I) of each flask. Then, the soil is carefully mixed with a Pasteur pipette and left in the flask.
Measurement of test concentrations	Measured as ^{14}C at various time points.
Reference material	Not stated.
Test Duration	up to 64 days.
Endpoints measured	50% mineralization/volatilisation.
Test validity criteria	Not stated.

Test Name	OECD 305: Bioconcentration: Flow-through Fish Test.

Date adopted	14th June 1996.	
Summary of test design	The test consists of two phases: the exposure (uptake) and post-exposure (depuration) phases. During the uptake phase, separate groups of fish of one species are exposed to at least two concentrations of the test substance. They are then transferred to a medium free of the test substance for the depuration phase. The concentration of the test substance in/on the fish (or specified tissue thereof) is followed through both phases of the test. The uptake phase is run for 28 days unless it is demonstrated that equilibrium has been reached earlier. The depuration period is then begun by transferring the fish to the same medium but without the test substance in another clean vessel. Where possible the bioconcentration factor is calculated preferably both as the ratio (BCF_{ss}) of concentration in the fish (C_f) and in the water (C_w) at apparent steady-state and as a kinetic bioconcentration factor, BCF_k as the ratio of the rate constants of uptake (k_1) and depuration (k_2) assuming first-order kinetics.	
Test Species	*Danio rerio* (Zebra-fish) *Pimephales promelas* (Fathead Minnow) *Cyprinus carpio* (Common carp) *Oryzias latipes* (Ricefish)	*Poecilia reticulata* (Guppy) *Lepomis macrochirus* (Bluegill) *Oncorhynchus mykiss* (Rainbow trout) *Gasterosteus aculeatus* (Threespined Stickleback)
Number of organisms per vessel	At least 4 (0.1-1.0 g of fish (wet weight) per litre of water per day).	
Level of replication	Not specified.	
Concentration range	At least 2 test concentration.	
Test medium	Natural water is generally used in the test and should be obtained from uncontaminated and uniform quality source. The dilution water must be of a quality that will allow the survival of the chosen fish species for the duration of the acclimation and test periods without them showing any abnormal appearance or behaviour.	
Dosing method (e.g. water/solvent)	The stock solution should preferably be prepared by simply mixing or agitating the test substance in the dilution water. The use of solvents or dispersants (solubilising agents) is not recommended. However, in some cases they may be used but must not be present at concentrations greater than 0.1 ml/l. For flow-through tests, a system which continuously dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) is required to deliver the test concentrations to the test chambers. Preferably allowing at least five volume replacements through each test chamber per day.	
Measurement of test concentrations	Water and fish are sampled on at least five occasions during the uptake phase and at least on four occasions during the depuration phase.	
Reference material	Not stated.	
Test Duration	up to 90 days.	
Endpoints measured	BCF.	
Test validity criteria	The temperature variation is less than ± 2°C. The concentration of dissolved oxygen does not fall below 60% saturation. The concentration of the test substance in the chambers is maintained within ± 20% of the mean of the measured values during the uptake phase. The mortality or other adverse effects/disease in both control and treated fish is less than 10% at the end of the test; where the test is extended over several weeks or	

	months, death or other adverse effects in both sets of fish should be less than 5% per month and not exceed 30% in all.

Test Name	**OECD 306: Biodegradability in Seawater (Shake flask).**
Date adopted	**17th July 1992.**
Summary of test design	A pre-determined amount of the test substance is dissolved in the test medium to yield a concentration of 5-40 mg dissolved organic carbon (DOC)/l. The solution of the test substance in the test medium is incubated under agitation in the dark or in diffuse light under aerobic conditions at a fixed temperature (controlled to ± 2°C) which will normally be within the range 15-20°C. In cases where the objective of the study is to simulate environmental situations, tests may be carried out beyond this normal temperature range. The recommended maximum test duration is about 60 days. Degradation is followed by DOC measurements (ultimate degradation) and, in some cases, by specific analysis (primary degradation).
Test Species	Various depending on source of inoculum.
Number of organisms per vessel	Not stated.

Number of organisms per vessel	Not stated.
Level of replication	2 replicates.
Concentration range	1 test concentration.
Test medium	Field collected seawater with resident microbial population as inoculum. Mineral media are prepared from stock solutions of appropriate concentrations of mineral components, namely, potassium and sodium phosphates plus ammonium chloride, calcium chloride, magnesium sulphate and iron (III) chloride.
Dosing method (e.g. water/solvent)	Dissolve the test substances in the test medium - they may be conveniently added via a concentrated stock solution - to give the desired starting concentrations of normally 5-40 mg DOC/l.
Measurement of test concentrations	Not stated.
Reference material	Sodium benzoate, sodium acetate and aniline.
Test Duration	60 days.
Endpoints measured	$D_{t.}$
Test validity criteria	The results obtained with the reference compounds e.g. sodium benzoate, sodium acetate or aniline, should be comparable to results obtained in the ring test (see guideline).

Test Name	OECD 306: Biodegradability in Seawater (Closed bottle test).
Date adopted	**17th July 1992.**
Summary of test design	A pre-determined amount of the test substance is dissolved in the test medium in a concentration of usually 2-10 mg of test substance per litre (one or more concentrations may be used). The solution is kept in a filled closed bottle in the dark in a constant temperature bath or enclosure controlled to ± 1°C within a range of 15-20°C. The degradation is followed by oxygen analyses over a 28-day period.
Test Species	Various depending on source of inoculum.
Number of organisms per vessel	Not stated.
Level of replication	2 replicates.
Concentration range	1 or more test concentration.

Test medium	Field collected seawater with resident microbial population as inoculum. Mineral media are prepared from stock solutions of appropriate concentrations of mineral components, namely, potassium and sodium phosphates plus ammonium chloride, calcium chloride, magnesium sulphate and iron (III) chloride.
Dosing method (e.g. water/solvent)	The solubility of the substance should be at least 2 mg/l, though in principle less soluble compounds could be tested (e.g. using ultra sonication) as could volatile compounds. The test and reference substances are added either directly or by using a concentrated stock solution to the test bottles.
Measurement of test concentrations	Not stated.
Reference material	Sodium benzoate, sodium acetate and aniline.
Test Duration	60 days.
Endpoints measured	D_t.
Test validity criteria	The blank respiration should not exceed 30% of the oxygen in the test bottle. The results obtained with the reference compounds e.g. sodium benzoate, sodium acetate or aniline, should be comparable to results obtained in the ring test (see guideline).

Test Name	**OECD 308: Aerobic and Anaerobic Transformation in Aquatic Sediment Systems.**
Date adopted	24th April 2002.

Summary of test design	The method employs an aerobic and anaerobic aquatic sediment system which allows: • The measurement of the transformation rate of the test substance in a water-sediment system, • The measurement of the transformation rate of the test substance in the sediment, • The measurement of the mineralisation rate of the test substance and/or its transformation products (when 14C-labelled test substance is used), • The identification and quantification of transformation products in water and sediment phases including mass balance (when labelled test substance is used), • The measurement of the distribution of the test substance and its transformation products between the two phases during a period of incubation in the dark (to avoid, for example, algal blooms) at constant temperature. Half-lives, DT50, DT75 and DT90 values are determined where the data warrant, but should not be extrapolated far past the experimental period.
Test Species	Various depending on source of inoculum.
Number of organisms per vessel	Not stated.
Level of replication	2 replicates.
Concentration range	1 test concentration.
Test medium	Two sediments are normally used for the aerobic studies. One sediment should have a high organic carbon content (2.5-7.5%) and a fine texture, the other sediment should have a low organic carbon content (0.5-2.5%) and a coarse texture. For the strictly anaerobic study, two sediments (including their associated waters) should be sampled from the anaerobic zones of surface water bodies.
Dosing method (e.g. water/solvent)	Ideally the test substance should be applied as an aqueous solution into the water phase of the test system. If unavoidable, the use of low amounts of water miscible solvents (such as acetone, ethanol) is permitted for application and distribution of the test substance, but this should not exceed 1% v/v.
Measurement of test concentrations	Concentration of the test substance and the transformation products at every sampling time (at least 6 times) in water and sediment should be measured
Reference material	Not specified.
Test Duration	<100 days.
Endpoints measured	DT50, DT75 and DT90.
Test validity criteria	Chemical recoveries should range from 90% to 110% for labelled chemicals and from 70% to 110% for non-labelled chemicals.

Test Name	**OPPTS 850.2100: Avian acute oral toxicity test.**
Date adopted	April 1996.
Summary of test design	After birds have been obtained, they are acclimated for at least 14 days. The dosage levels for the definitive test are established, possibly requiring a range-finding test to be conducted first. Test birds are randomly assigned to the various dosage levels and controls. Birds are weighed and the test substance is administered as a single oral dose either by gavage or capsule. Birds are closely monitored for 60 to 120 min after doses are given and then observed regularly for mortality or any signs of intoxication throughout the observation period. Birds are weighed and feed consumption is estimated at least weekly. The mortality pattern is examined and subjected to the appropriate statistical analysis to derive the LD50, confidence limits, and slope of the dose-response line.

Summary of test design	After birds have been obtained, they are acclimated for at least 14 days. The dosage levels for the definitive test are established, possibly requiring a range-finding test to be conducted first. Test birds are randomly assigned to the various dosage levels and controls. Birds are weighed and the test substance is administered as a single oral dose either by gavage or capsule. Birds are closely monitored for 60 to 120 min after doses are given and then observed regularly for mortality or any signs of intoxication throughout the observation period. Birds are weighed and feed consumption is estimated at least weekly. The mortality pattern is examined and subjected to the appropriate statistical analysis to derive the LD50, confidence limits, and slope of the dose-response line.
Test Species	*Colinus virginianus* (Northern bobwhite) *Anas platyrhynchos* (Mallard).
Number of organisms per vessel	A minimum of 10 birds.
Level of replication	Not stated.
Concentration range	A minimum of five test diets.
Test medium	Basal diet.
Dosing method	Dosing by gavage is preferred. Where gavage is not feasible, doses may be administered by gelatin capsule. Doses are to be based on the individual body weight (bw) of each bird. If a carrier is used to administer the test substance, the preferred carrier is distilled or deionized water unless the test substance is known to hydrolyze readily. Other acceptable carriers include corn oil, propylene glycol, 1% carboxymethylcellulose, and gum acacia.
Measurement of test concentrations	Not stated.
Reference material	Not stated.
Test Duration	14-21 days.
Endpoints measured	LD50.
Test validity criteria	A test is unacceptable if more than 10 percent of the control birds die during the test.

Test Name	**EU TGD: Mesocosm tests.**
Date adopted	2003.
Summary of test design	Not stated.
Test Species	Several taxonomic groups, in more or less natural ecosystems.
Number of organisms per vessel	Not stated.
Level of replication	At least one replicate.
Concentration range	One control and at least two test concentrations.
Test medium	Not stated.
Dosing method	Not stated.
Measurement of test concentrations	The concentration of the test compound should be measured several times during the experiment.
Reference material	Not stated.
Test Duration	Not stated.
Endpoints measured	A reliable MS NOEC should be derived.
Test validity criteria	Not stated.