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Nanomaterials for environmental studies: Classification, reference material issues, and strategies for physico-chemical characterisation

Vicki Stone [a,*], Bernd Nowack [b], Anders Baun [c], Nico van den Brink [d], Frank von der Kammer [e], Maria Dusinska [f], Richard Handy [g], Steven Hankin [h], Martin Hassellöv [i], Erik Joner [j], Teresa F. Fernandes [a]

[a] School of Life Sciences, Edinburgh Napier University, 10 Colinton Road, Edinburgh EH10 5DT, UK
[b] Materials, Products and the Environment Group, Empa — Swiss Federal Laboratories for Materials Testing and Research, Lerchenfeldstrasse 5 CH – 9014 St. Gallen, Switzerland
[c] Department of Environmental Engineering, Technical University of Denmark, NanoDTU, Building 113, 2800 Kgs. Lyngby, Denmark
[d] Alterra, P.O. Box 47, 6700 AA Wageningen, The Netherlands
[e] Department of Environmental Geosciences, Vienna University, Althanstrasse 14, Wien 1090, Austria
[f] Health Effects Laboratory, Centre for Ecological Economics, Norwegian Institute for Air Research (NILU), Instituttveien, 18, 2027 Kjeller, Norway
[g] University of Plymouth, Davy Building, Drake Circus, Plymouth PL4 8AA, UK
[h] Institute of Occupational Medicine, Research Avenue North, Riccarton, Edinburgh EH14 4AP, UK
[i] Department of Chemistry, Environmental Nanoparticle Research Group, Göteborg University, SE-412 96 Göteborg, Sweden
[j] Bioforsk Soil and Environment, Fredrik A Dahls vei 20, N-1432 Aas, Norway

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ABSTRACT

NanoImpactNet is a European Commission Framework Programme 7 (FP7) funded project that provides a forum for the discussion of current opinions on nanomaterials in relation to human and environmental issues. In September 2008, in Zurich, a NanoImpactNet environmental workshop focused on three key questions:

1. What properties should be characterised for nanomaterials used in environmental and ecotoxicology studies?
2. What reference materials should be developed for use in environmental and ecotoxicological studies?
3. Is it possible to group different nanomaterials into categories for consideration in environmental studies?

Such questions have been, at least partially, addressed by other projects/workshops especially in relation to human health effects. Such projects provide a useful basis on which this workshop was based, but in this particular case these questions were reformulated in order to focus specifically on environmental studies. The workshop participants, through a series of discussion and reflection sessions, generated the conclusions listed below.

The physicochemical characterisation information identified as important for environmental studies included measures of aggregation/agglomeration/dispersability, size, dissolution (solubility), surface area, surface charge, surface chemistry/composition, with the assumption that chemical composition would already be known.

There is a need to have test materials for ecotoxicology, and several substances are potentially useful, including TiO_2 nanoparticles, polystyrene beads labelled with fluorescent dyes, and silver nanoparticles. Some of these test materials could then be developed into certified reference materials over time.

No clear consensus was reached regarding the classification of nanomaterials into categories to aid environmental studies, except that a chemistry-based classification system was a reasonable starting point, with some modifications. It was suggested, that additional work may be required to derive criteria that can be used to generate such categories, that would also include aspects of the material structure and physical behaviour.

1. Introduction

Nanotechnology, involves the production of a diverse array of nanomaterials (NM), which include nano-objects and nanoparticles (NP). Nanomaterials have one dimension less than 100 nm, whereas nano-objects have two dimensions less than 100 nm (e.g., carbon nanotubes) and nanoparticles are defined as particles with three

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* Corresponding author. Tel.: +44 0131 4552671; fax: +44 0131 4552291.
E-mail address: v.stone@napier.ac.uk (V. Stone).

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dimensions of less than 100 nm (British Standards, BSI, 2007a; SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks, 2007a). Due to the rapid expansion of nanotechnology and the increasing range of nanomaterials under production and development, it is essential that the potential impacts on human and environmental health are addressed. Due to their small size, NP exhibit relative surface areas that are greater than the corresponding conventional forms; in addition, the small size often results in higher reactivity and altered surface properties that can be exploited in a variety of consumer products such as paints, cosmetics, medicines, food and suntan lotions, as well as applications which directly release NPs into the environment, such as remediation of polluted environments (Aitken et al., 2006). Any potential deleterious effects therefore need to be assessed in order to understand environmental impacts and potential effects on human health. Such work will require the linking of physicochemical characteristics of NP to their biological behaviour.

It is widely accepted that much work is still needed to advance knowledge in the area of physicochemical characterisation of nanomaterials, and how characteristics and properties of these nanomaterials influence their fate and behaviour in the environment and their potential to induce toxicity in different environmental receptors (The Royal Society and The Royal Academy of Engineers, 2004; Colvin, 2003; Klaine et al., 2008; SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks, 2009). Human nanotoxicology, compared to environmental studies of nanomaterials, has the advantage of decades of particle respiratory toxicology for a wide range of particles, including asbestos, coal mine dust, silica and air pollution. Results from this work have highlighted key mechanistic pathways observed on exposure to air-borne particulate material and can be used as a basis to understand how to design, conduct and interpret nanomaterial toxicology experiments in alternative models. While the information from the human toxicology can provide a useful knowledge base on which to address effects in other species, coupled with the recent increase in available data in this area (e.g., see reviews by Handy et al., 2008; Klaine et al., 2008, SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks, 2009), knowledge is still lacking regarding the uptake, biological fate, effects and modes of action of nanomaterials in species other than rodent and mammalian models, especially in relation to uptake routes other than via the air (e.g., waterborne exposure, sediment exposures). Many standardised protocols are available to assess the hazards of substances released into the environment, but while these have been developed for standard chemicals they are not always appropriate for nanomaterials, potentially leading to misleading effects. Modifications of such protocols are required for nanomaterials, which then brings into question the relevance and reproducibility of the protocols. In addition, the behaviour and fate of engineered nanomaterials in the environment are largely unknown, and difficult to assess due to many complicating factors such as in such heterogeneous systems where it is difficult to detect nanomaterials over the background of naturally occurring particles. Although there is a wealth of knowledge on colloids and other natural nanoscale materials (e.g., Lead and Wilkinson, 2006; SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks, 2009), more work is needed to provide relevant input to risk assessment and management approaches for engineered nanomaterials. While there is the potential to transfer knowledge between the human and environmental areas, especially in the field of hazard identification, much work needs to be done in order to establish the basis for the development and use of relevant experimental approaches, so that hazards can be adequately assessed (e.g., Crane et al., 2008, Klaine et al., 2008; SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks, 2009).

In order to elucidate the modes of action of toxicity of nanomaterials, to underpin the processes of their environmental fate and behaviour, and to be able to extrapolate results between nanomaterials, it is essential to characterise the materials used in the different studies as far as possible and necessary. Furthermore, in light of the vast amount of work to be done, it is essential to prioritise the order in which nanomaterials should be approached, and to relate this to the identification of which materials should be developed for further testing and for use as benchmarks to validate and compare test results. To bring the discussions on these matters a step forward, in this paper we present the outcomes of a workshop regarding the selection of properties for characterisation of nanomaterials, prioritisation of materials to be tested and the development of reference materials.

1.1. Characterisation of nanomaterials

Discussions regarding the characterisation of nanomaterials and the requirements for hazard assessment have taken place in the scientific literature (e.g., Bucher et al., 2004; Oberdorster et al., 2005; Powers et al., 2006; Thomas et al., 2006; Balbus et al., 2007; Park et al., 2007; SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks, 2007b, Scientific Committee on Emerging and Newly Identified Health Risks, 2007a; Handy et al., 2008; Klaine et al., 2008; Warheit, 2008; SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks, 2009), and others, although much of the recent literature does not add substantial novel information. There is, however, still debate regarding the key 'ideal' or 'essential' characterisation information required for hazard identification purposes, and if there should be any differences in these requirements between human and environmental studies. Warheit (2008) suggests that lists of properties have been proposed so often that he calls them a 'laundry list of physicochemical characteristics' which 'does not have adequate prioritisation'. Even though there seems to be a limited number of fundamental properties that researchers in the field generally agree must be addressed, these have only recently been discussed in the context of ecotoxicological studies of nanomaterials (SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks, 2007a, Scientific Committee on Emerging and Newly Identified Health Risks, 2007b, Klaine et al., 2008; Handy et al., 2008, SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks, 2009, http://www.epa.gov/oppt/nano/stewardship.htm accessed 02/09/09). There is also no current consensus on the "minimum information" on characterisation that should be provided in an ecotoxicology study, although base-sets of data have been suggested as a starting point for regulatory tests (Klaine et al., 2008; Crane et al., 2008; SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks, 2009). A recent review of the published literature, which included 482 toxicity studies, revealed that while information on the chemical composition was given for all of the 965 nanomaterials included in the papers, only 66% of the studies included information on the particle size, while only 16% provided the surface area measurements (Hansen et al., 2007).

One of the first initiatives in this field was a Workshop run in Florida in November 2004 (Bucher et al., 2004). There it was suggested that characterisation should be carried out on the materials themselves, as well as on materials within experimental media. The authors suggest the first should essentially focus on specific physical chemical properties, such as size, shape, surface area, surface porosity, roughness, morphology, crystallinity, solubility, chemical composition, surface chemistry, reactivity, and the second should include images, dispersibility and dosage. A comprehensive list of such characteristics/properties was compiled at the workshop and it was suggested that a minimum set should include the elemental composition of the particles (*sic*); as well as, surface morphology, degree of crystallinity, and imaging by TEM (transmission electron microscopy; Bucher et al., 2004).

Several other reviews indicate a range of physicochemical properties that should be characterised in research when assessing potential human and environmental hazards of nanomaterials (e.g., Thomas et al., 2006, Hansen et al., 2007, British Standards, BSI, 2007b, SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks, 2007a, Scientific Committee on Emerging and Newly Identified Health Risks, 2007b, SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks, 2009). The list of ideal properties that may be addressed is extensive, and it is recognised that characterisation of nanomaterials is time consuming, expensive, and may require specialist technical expertise, and thus its extent should depend on the objectives of the study. In fact, generation of a prescribed list of requirements is potentially dangerous at this stage, as it could lead to exclusion of potentially important characteristics. Whatever strategy is adopted, it should recognise the limitations of resources and capabilities, but it should also be mindful of achieving scientific robustness in the context of the objectives of a particular study. We suggest that an internationally coordinated strategic approach with a tiered physicochemical characterization testing strategy on test or reference materials can provide a much higher level of physicochemical characterization in a rather cost efficient manner. The Organisation for Economic Co-operation and Development (OECD) is currently spearheading a coordinated strategy focussing on an initial selection of nanomaterials and characterization properties (OECD, 2008).

According to the studies highlighted in Table 1 it is clear that although there is some variability amongst authors, the overall lists are similar, with the priority set including surface area, surface chemistry, shape and morphology, as well as and material composition/purity. SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks, 2007a, Scientific Committee on Emerging and Newly Identified Health Risks, 2007b) stressed that in any study of nanomaterials, it is important that the sample being characterised is representative of the substance, and that both particle size and shape characteristics should be measured in the most relevant dispersed state (SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks, 2007a, Scientific Committee on Emerging and Newly Identified Health Risks, 2007b). Furthermore, it is stated that the most appropriate metrics and methods for their evaluation should be used for both the material characterisation and the hazard assessment, which may include parameters such as number concentration and surface area (SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks (2007a), Scientific Committee on Emerging and Newly Identified Health Risks, (2007b)). There have also been discussions on whether such measurements should be conducted for the nanomaterial before addition into an experimental medium, on dispersion in the relevant medium (liquid or solid), at various time points throughout the study, and then again at the end. It is important, however, that such points are addressed rationally and an assessment of the usefulness of such thorough measurements is carefully assessed. SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks, 2007a, Scientific Committee on Emerging and Newly Identified Health Risks, 2007b) also indicate that characteristics should be measured under conditions that mimic those of the potential human and environmental exposure. Klaine et al. (2008) provide a comprehensive discussion on proposed characterisation approaches and suggest possible practical procedures to conduct such measurements and quantification. Powers et al. (2007) indicate the difficulties associated with the assessment of some of the listed characteristics, mainly in relation to methodological approaches as well as practicalities of sampling. Hassellov et al. (2008) and Tiede et al. (2009) provide information on possible methods to be used in characterising properties of nanomaterials and their shortcomings. Handy et al. (2008) suggest prioritising characterisation measurements on the basis of their known or suspected influence on ecotoxicity. This would also include measuring key abiotic factors in the aqueous media, as appropriate, that may influence characterisation such as media pH, Ca^{2+} concentration, the presence of natural organic matter and ionic strength.

Table 1
Properties to characterise nanomaterials in media (stock solution) proposed by a range of authors.

Property	Oberdorster et al. (2005)	Powers et al. (2006, 2007)	Thomas et al. (2006)	Warheit (2008)	Klaine et al. (2008)
Size distribution	*	**	**	**	**
Agglomeration state/dispersion	*	**	*	**	*
Crystal structure	*	*	*	**	
Chemical composition	*	*	*		**
Surface area and Porosity	*	**	*	**	**
Surface chemistry		**	*	**	*
Surface charge		*	*	**	*
Shape and morphology		**	**		*
Dissolution/ Solubility		*	*		**
Physical/chemical properties (purity)		**		**	
Methods of synthesis				**	

*: Of importance; **: Priority.

1.2. Prioritisation and 'reference' materials

It would be useful for the scientific community to have access to well characterised materials that may be appropriate in the short term as test materials for hazard assessment, and in the longer term, could be used for bench marking toxic effects. There are concerns that some of the materials which are used for bench marking in mammalian respiratory toxicology may not be appropriate to ecotoxicology (Crane et al., 2008), but nonetheless, developing a list of potential test, benchmark or reference materials for environmental studies would be important. In addition, with so many materials available, with so many potentially important properties to characterise, some prioritisation of the nanomaterials to test first is essential. Several criteria may be applicable for prioritisation. OECD has produced a list that takes into account those materials which are already in production (or close to commercial use), as well as considerations of production volume, the likely availability of materials for testing and the existing information (OECD, 2008). The OECD list comprises fullerenes (e.g., C_{60}), single- and multi-walled carbon nanotubes (SWCNTs and MWCNTs, respectively), carbon black, polystyrene, dendrimers, nanoclays, and nanoparticles of Ag, Fe, TiO_2, Al_2O_3, CeO_2, ZnO, and SiO_2 (OECD, 2008).

To validate and evaluate potential tests there are in fact several kinds of standardized materials available: certified reference materials, reference materials and test materials, as described by ISO (International Organisation for Standardization). A number of meetings have discussed such materials such as the REFNANO project (Aitken et al. 2008), the ERDC-NIST workshop on nano-silver (http://nanobiology.ncifcrf.gov/groups/silver/), and a number of initiatives are under way to develop such materials, for example at NIST (National Institute of Standards and Technology, USA). and at the JRC-IRMM (Joint Research Centre — Institute for Reference Materials and Measurements, European Commission). From the different categories of standardized materials, the simplest is the test material, which is often used in basic research and can be employed for the development of protocols or instrumentation. According to ISO, a reference material is a material (pure or mixed) which is "used for calibration, method validation, the establishment of metrological traceability, method development, and other various quality control purposes"[1]. Additionally, a certified reference material is a material which is "accompanied by a certificate, one or more of whose property values are certified by a procedure which establishes traceability to an accurate realisation of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty at a stated level of confidence" (ISO/IEC Guide 99:2007). The highest level measurement benchmarks are provided by certified reference materials[2]. As identified in the REFNANO report, materials in each of these three categories can be of some use in this field, and the ultimate goal of achieving certified reference material status may not be feasible for all desired candidate 'test' nanomaterials.

The aim of this paper is to provide direction regarding the needs of characterisation, classification, prioritisation and derivation of potential test, benchmark or reference materials in the hazard assessment of nanomaterials in the environmental area, in contrast with the requirements for human studies. Therefore the issue of prioritising which properties researchers in the field generally agree must be addressed. The aim of the paper is not to suggest new lists of properties to be characterized but rather to build on established lists and setting the properties mentioned into an environmental perspective. The authors, however, recognise that characterisation of nanomaterials is time consuming, expensive, and complex, and thus its extent should depend on the objectives of the study.

3. Methods

NanoImpactNet, funded under European Commission FP7 (CSA-CA 218539), is a multidisciplinary network of experts in the area of nanosciences. This network spans 24 different European research groups that are actively involved in studies of the potential health and environmental impacts of nanomaterials. One of the objectives of NanoImpactNet is to devise strategies for the investigation of nanomaterial exposure, hazard and hence risk in the environment. To address this objective, a group of 42 researchers consisting mainly of ecotoxicologists, environmental chemists and materials scientists, but also including toxicologists, risk assessors and industrial stakeholders, met over a period of two days to address the following three key questions:

1. What properties should be characterised for nanomaterials used in environmental and ecotoxicological studies?
2. What 'reference' materials should be used for environmental and ecotoxicology studies?
3. Is it possible to group different nanomaterials into categories/groups for consideration in environmental studies?

In order to address these questions all of the participants were randomly assigned to one of three working groups and all three groups conducted activities that would generate answers to the three questions listed above over a period of two days. In order to address question 1, the researchers were provided with a list of physicochemical properties that had been proposed in the literature (Table 1). Each working group was then asked to choose just three physicochemical characteristics that they deemed to be most important in ecotoxicological studies, plus two others that would be desirable.

For question 2, the three groups were provided with the priority list of materials identified in the REFNANO project (carbon black, TiO_2, ZnO, SWCNT/MWCNT, fluorescent polystyrene, Ag, other metals/oxides, combustion derived nanoparticles) (Aitken et al., 2008). The groups were asked to identify whether they agreed or disagreed that each REFNANO material would be suitable as a test or reference material for environmental and ecotoxicological studies, and to justify their answer. They were also invited to add up to two materials to their list. For question 3, the groups were asked to generate classifications of nanomaterials that would be useful in allowing them to design environmental and ecotoxicology studies.

The discussion sessions were designed to be interactive and reflective. After addressing a specific question, each group divided, sending representatives to discuss their results with one of the other groups. At the same time, a number of representatives remained at their original location to receive visitors from the other two groups and contrast their viewpoints. The original groups then reconvened to share information, to discuss their original conclusions and to make any amendments based upon the concepts of the other two groups. The outputs generated by each question and each group were compiled into an individual report and presented to all of the workshop participants in a summary discussion.

The NanoImpactNet group was keenly aware that many of these issues have been addressed mostly in relation to human health elsewhere by other groups of scientists, and in some instances the outcomes reported, e.g., Aitken et al (2008); SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks (2007a), Scientific Committee on Emerging and Newly Identified Health Risks (2007b); OECD (2008); Oberdorster et al. (2005). The NanoImpactNet group attempted to use such reports and papers as a starting point for discussions, building on existing work and expanding this to provide an environmental science perspective, therefore providing added value to the ongoing discussion, rather than repetition.

4. Results

Question 1: What properties should be characterised for nanomaterials investigated in environmental and ecotoxicology studies?

In the summary session of the discussion of question 1, it became apparent, that although the three groups approached the question from a different angle, they all agreed on six properties as the main priorities required for environmental and ecotoxicology studies (Table 2).

The suggestions shown in Table 2 were made under the assumption that the chemical composition (including impurities) is known, with all groups acknowledging the importance of composition information when assessing both fate in the environment and potential toxic effects. The shape of nanomaterials was mentioned by some of the participants as a seventh property to be characterised for environmental hazard identification.

It was also emphasized that the measurement of specific properties, via certain techniques, will sometimes generate an important set of data, rather than an individual value, within the same analysis. For example, analysis of agglomeration would provide data such as primary particle size, average particle size and size distribution. Similarly, elemental analysis could provide information on both chemical composition and the presence of impurities, while BET (Brunauer–Emmett–Teller; Brunauer et al., 1938) could provide both specific surface area and

Table 2
The six main properties to be characterised for nanomaterials investigated in environmental and ecotoxicology studies (in alphabetical order).

Aggregation/agglomeration/dispersability
Size
Dissolution[a]
Surface area
Surface charge
Surface composition/surface chemistry

[a] The term 'dissolution' is used here rather than 'solubility' since dissolution is the process by which a solid or liquid forms a homogeneous mixture with a solvent (solution) whereas the maximum equilibrium amount of solute that can be dissolved per amount of solvent is the solubility of that solute in that solvent under the specified conditions.

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[1] http://irmm.jrc.ec.europa.eu/html/reference_materials_catalogue/user_support/general_questions.htm.

[2] http://www.measurement.gov.au.

Table 3
The suitability of the nanomaterial panel identified by REFNANO for environmental studies.

Material	Suitable as test/reference material	Available as reference material today
Carbon black	No[a]	Yes
TiO_2	Yes	No
ZnO	No[a]	No
SWCNT/MWCNT	Not yet[a]	No
Polystyrene fluorescent	Yes	Yes
Ag	Yes	No
Other metals/ oxides	Yes (Cu/CuO Fe)	No
		No
Combustion derived	Yes/No[b]	Yes
Additions	Yes (Au)	Yes
	Yes (C60)	No
	Yes (SiO_2)	Yes

[a] No complete consensus was reached with regards to this conclusion. However a majority of the participants supported the indicated conclusion — though for different reasons (see comments in the text).

[b] The participants were divided on this issue (see comments in the text).

additional information on porosity. Techniques such as BET are conducted on dry powders, which could of course differ in porosity and accessible surface area, due to agglomeration in an aqueous state. It is important to note, however, that measuring the same property via different techniques can generate different results, often influenced by the technique itself and the exact parameter measured. A clear understanding of the technique employed can help to overcome this issue. It was also mentioned in the discussions that different properties may be important at different stages of research. For instance, different weight may be given to specific properties in aquatic rather than on sediment tests, or when comparing fate and hazard assessments, and so on. Therefore, it is not possible, or desirable, to list a complete or appropriate set of properties that needs to be characterised for each study, without a definition of the aim of the research. It was concluded that at this stage it is not yet possible to specify or 'enforce' the list from Table 2, but instead it provides current direction which will require updating in the future. In addition, it was agreed that properties should be characterised in the test system and not in "the bottle" in which they were supplied. This will increase the value of the information considerably, although this is not always easy due to the complex nature of the experimental media used in some studies, as well as the limits of detection for some techniques with low particle concentrations. Finally, due to the dynamic character of nanoparticulate systems, certain properties should not be listed as "states", but instead as "rates". For example, it is more appropriate to determine dissolution rates and agglomeration rates instead of dissolution and agglomeration state, although rates may not be as straightforward to measure. There may be a steady state (e.g., agglomeration counteracted by deagglomeration), but that is rarely the case as nanomaterials in water or air behave as colloidal systems that are slowly or rapidly changing.

The prioritisation of properties to be determined also implies the need to attach available and suitable methods to measure these properties. Unfortunately this is not always possible, for example, there is no method available to directly measure the specific surface area in an aqueous dispersion of particles. Another relevant example is that there is a high risk of producing biased results with the different sizing techniques available. There is still a deficit of information about how far the limitations of the different methods may influence the correct interpretation of test results, which means that the methods of characterisation and the data interpretation are sometimes a matter of debate. Bias from one technique could be reduced by the use of multiple techniques, although this may not be possible due to cost and time constrains. At the very least it is expected that the method employed to measure a specific parameter should be identified in any publication.

It should be noted that the groups were asked to focus on which characteristics should be assessed, but they were not asked to consider when in an experiment they should be measured. This therefore remains open for debate.

Question 2. What 'reference' materials should be developed for use in the area of environmental and ecotoxicology studies?

The groups discussed whether test- or reference materials would be required, and for which purposes. The development and use of test materials were deemed appropriate, and the groups suggested that in the future a subset of these test materials might be developed into certified reference materials. From the eight nanomaterials suggested by REFNANO, TiO_2, polystyrene beads labelled with fluorescent dyes, and Ag were all identified by the three groups as materials that would be useful to obtain as test materials (see Table 3).

Silver nanoparticles were deemed by some groups as a potential positive control, in that these particles appear to be associated with relatively high toxicity in a number of studies. The groups discussed the potential that this toxicity might be driven by release of soluble material, with a number of researchers agreeing that the particle form might alter exposure, bioavailability, potential for uptake and fate within organisms, therefore influencing toxicity (Luoma, 2008). Silver nanoparticles were also chosen because of their prevalence in consumer products such as health remedies, wound dressings, clothing, food processing surfaces and computer keyboards, therefore increasing the potential for exposure to humans and the environment[3].

Single walled and multiwalled carbon nanotubes were identified by all groups to be desirable as test materials, but concerns were raised about the reproducibility between batches (including possible impurities), and identification of a sample that might be suitably representative of the wide range available. While reproducibility between batches can also be a problem for other types of nanomaterials, the groups perceived this to be more of an issue for nanotubes.

Carbon black and zinc oxide received mixed support. Carbon black was deemed too difficult to work with due to its propensity to adsorb substances and to interfere in assays, therefore making the results difficult to interpret. Other researchers argued that carbon black is relevant due to the high production volumes and therefore the potential for exposure of both humans and the environment. ZnO was considered problematic due to its relatively high solubility, making it difficult to investigate in aquatic environments and in organisms, but some participants proposed it as suitable for hypothesis testing of dissolving nanoparticles. In addition, the prevalence of Zn within the environment or biological specimens makes identification of ZnO nanoparticles very difficult to achieve over and above this significant background.

For the other metal/metal oxide group, CuO was identified by two groups as being useful due to its relatively low dissolution rate but its potentially high toxicity towards organisms (e.g., Villem et al., 2009), though this remains to be shown in experimental studies. Zero-valent iron nanoparticles were mentioned as candidates for inclusion in the list due to the field-scale application of this product for groundwater remediation. It is important to note, however, that the redox chemistry of certain substances such as those containing iron, titanium or copper, may act as a complicating factor in the various media used for ecotoxicological testing.

There was no clear consensus regarding combustion derived particles, with all three groups giving different answers. In support of including such particles, the use of existing samples (e.g., NIST diesel exhaust particles) was discussed, especially since they would allow

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[3] http://www.nanotechproject.org/inventories/silver/.

Table 4
Nanomaterial group classifications for the purpose of environmental studies (nano clays excluded due to lack of consensus).

Categories	Alternatives	Particles
Carbon	(a) Functionalised (b) Non functionalised or (a) Low aspect ratio (b) High aspect ratio	Carbon black, nanotubes and fullerenes
Mineral based	(a) Redox active (b) Non-redox active	Metals, metal oxides,
Organic		Polymers, dendrimers, surfactant coatings
Composites/ hybrids	a) Mineral–mineral	Multicomponent nanomaterial, e.g. quantum dots
	b) Organic–mineral or	
	a) Binary compound	Doped metal/metal oxides (e.g. Pd-ZVI)
	b) Multiple elements	

direct comparisons between ecotoxicology models and human toxicology studies which have already been published. However, it was commented that such samples consist of a mixture of components and are therefore not sufficiently well characterised and defined to be suitable as a reference material for ecotoxicological studies, where the focus is not on inhalation studies. On the other hand it was also acknowledged that nanomaterials can act as carriers of impurities or already existing environmental contaminants, and therefore materials with well defined impurities could be interesting.

Regarding the optional additions to the test material list, consensus was reached on the inclusion of gold nanoparticles. This was due to their current availability as reference materials, their apparently slow dissolution, availability in a variety of sizes, surfaces that can be functionalised, use in labelling of nanomaterials, as well as ease of detection within biological specimens and environmental matrices.

It was suggested that for ecotoxicological studies different test materials might be required for different purposes depending upon the type of experiment conducted (e.g., uptake versus toxicity), the environment represented by the experiment (e.g., air, water, soil), and the target organism investigated. The form in which test materials should be supplied was also included in the discussion, i.e., whether test materials would be best provided as powders or dispersed in liquids. Powders were considered to result in problems of reproducibility of dispersion in some cases. Suspensions or dispersions were considered to be relatively more stable in general, but it was acknowledged that powder forms would be most appropriate, so that preparations can be prepared fresh just before exposures. This is particularly important in the case of nanomaterials that may dissolve, and thus may be unsuitable for storage as stock solutions. The additional disadvantage of dispersions/suspensions was identified as the limited concentration range that such a suspension would impose on subsequent experimentation, and the fact that additional substances may need to be added to stabilize the dispersions.

Question 3. Is it possible to group different nanomaterials into categories for consideration in environmental studies?

The ability to structure a broad set of substances into well defined categories reflects the state of knowledge that is available for these materials in terms of their behaviour, effects and finally relevance to environmental routes of exposure. During recent years nanomaterials have been identified as being of natural, anthropogenic, or industrial origin and they can be free or bound in matrices. In addition they may be organic, inorganic or mixed organic/inorganic (inorganic includes CNT and fullerenes, while organic refers to e.g., dendrimers, while mixed includes functionalized quantum dots). In order to decide upon classifications it is necessary to assess which properties are known and understood to play a role in the particle behaviour in the environment and in organisms. A well founded categorization could help to focus characterization protocols on to the most essential or priority measurements, and offer some rationale for grouping materials that share some common features into ecotoxicity testing strategies.

The three groups found that at present it is not possible to categorise nanomaterials on the basis of parameters such as their mechanism of toxicity. This is because insufficient information is currently available in relation to such parameters to allow such an assessment and categorisation to be derived. The groups did, however, come up with broadly similar classifications based around the notion of evolving from a chemical classification system (Table 4). However, it was agreed that current chemical classification systems used for normal substances were inadequate because they do not account for particle shape, size or other (non-chemical) physical properties. So for example, for nanotubes the aspect ratio is also important. In the future, it may be possible to reclassify nanomaterials as more information emerges (e.g., in terms of toxicity mechanism, environmental behaviour, surface reactivity or other physical property). It was agreed that this question would be revisited with regular intervals to include the advances in ecotoxicological knowledge.

There was a lengthy discussion on how to categorize nano clays. They were suggested to belong to the group of metal/metal oxide group, since clays mainly consist of silicon and aluminium oxides. They also have traces of iron oxides and even titanium oxides that substitute SiO_2 plus other elements deliberately added to the material or as remainders of the manufacturing process (e.g., lithium in *Laponite RD*). They are thus also considered composites made from multiple elements. In addition, in some cases they have high aspect ratios. With nano clays it was suggested that the surface reactivity is not as important in relation to applications as with metal/metal oxides. Therefore, no consensus was reached on this discussion.

It was suggested that composites should be considered on a case-by-case basis, using information from the other classes of particles to decide upon a strategy for testing that relates to the most prominent components, or the surface composition. Concerns were also raised that all nanomaterials have the potential to fall into the composite classification once mixed into a medium or with a dispersant that results in coating. Therefore, it was suggested that the classifications listed in Table 4 are only valid prior to introduction into a test system. In addition, in relation to the discussion of composites, it was identified that the role and importance of the core material should be balanced with the reactivity (or attenuation of reactivity) by the shell/coating material.

As an alternative way of handling nanomaterials according to a categorization scheme in relation to environmental studies, it was proposed that a flow diagram with decision points might be applicable (Fig. 1). Such an approach would require detailed prior knowledge of the physicochemical characteristics of the material but would generate a powerful and systematic approach to material handling and testing. Such approaches, such as TOXTREE[4] already exist for conventional chemicals, and could be developed or adapted for nanomaterials.

5. Discussion

The NanoImpactNet workshop addressed a number of key questions relating to environmental studies, that addressed nanomaterial characterisation, the use of ‘reference’ materials and the potential ability to categorise nanomaterials. The following main conclusions were made:

1. The physicochemical properties which were identified for environmental studies as being in need of characterisation included, aggregation/agglomeration/dispersability, size, dissolution (solubility),

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[4] http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c=TOXTREE.

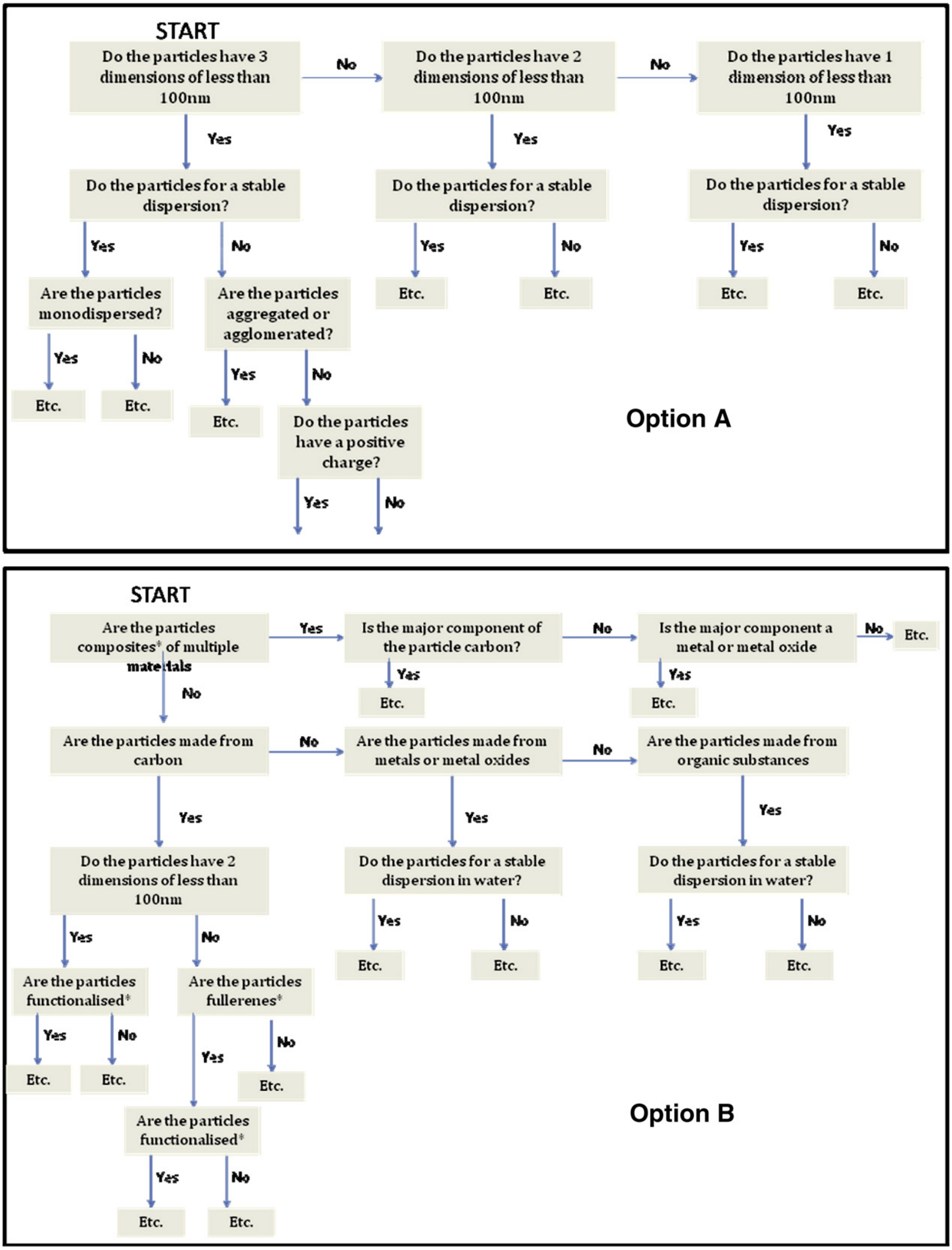

Fig. 1. Some suggested initiation points for decision trees that could be used for the classification of nanomaterials, for strategies to assess their toxicity, and/or strategies to inform safe handling of nanomaterials. Option A uses dimensions and shape as a starting point, while Option B uses composition as a starting point. Neither scheme is complete, but instead provides an indication of the types of formats and content a scheme might include. Clear definitions for each term would be required.

surface area, surface charge, surface chemistry/composition, with the assumption that total chemical composition would already be known;

2. The development of test materials was identified as a priority, and those identified as being useful for ecotoxicology studies included TiO_2, polystyrene beads labelled with fluorescent dyes, and Ag;

3. No clear consensus was reached regarding division of nanomaterials into categories to aid environmental studies; it was suggested, that additional work is required in order to generate the criteria needed to define such categories, and so this question will be revisited in three years time towards the end of the NanoImpactNet project funding.

The NanoImpactNet workshop therefore allowed identification of priorities for physicochemical characterisation and for the use/development of reference materials, with the added value that these outcomes were specific to environmental research rather than human toxicology, as has been published previously.

5.2. Particle characterisation

One of the main aims of this work was to attempt a prioritisation of the key physicochemical properties suggested in the literature relating to human toxicity studies, and to adapt this information in relation to environmental studies. The group of scientists representing the fields of environmental science and ecotoxicology generated a clear consensus of six priority physicochemical characterisation requirements of nanomaterials (Table 2). Reducing this list to two or three characteristics as minimum requirements was, however, problematic with the present state of knowledge. By suggesting a list of priority characteristics prematurely, we run the risk of overlooking one or more of the characteristics that later on turn out to be of vital importance for predicting the hazards of nanomaterials. On the other hand, a continuation of the call for more and more characterisation of nanomaterials in environmental studies may lead to a “paralysis by analysis” scenario (Hansen et al., 2008) in which relevant information is overlooked or, even worse, studies are not carried out/published due to lack of complete/appropriate characterisation of test materials before, during and after testing. Thus, it has to be realised that the scientific area of nanoecotoxicology is still in its infancy and there is a need thus to expand the database on fate, exposure and effects with studies of high quality, which may not always have a complete set of measured characteristics available.

Most publications on environmental fate and ecotoxicological effects of nanomaterials provide characteristics on pristine materials that are limited to some indicator of average size and composition (often manufacturer's data). Less frequently, other properties such as BET surface area and/or surface composition may also be given, although the latter is often limited to manufacturer's data on functionalization, coatings or oxide surface layers. Aspects that are not so often reported are the hydrodynamic diameter of the nanomaterials used, e.g., as obtained by dynamic light scattering (DLS), and surface charge, as measured by isoelectric focusing, or the zeta-potential often determined by laser-doppler anemometry. With respect to isoelectric focusing, nanomaterial suspensions alter their charge with changing pH and are destabilized at a pH corresponding to the point of zero net charge (PZC), resulting in precipitation, agglomeration and other related processes. The three listed parameters are measured in aqueous systems, and are thus highly relevant to environmental and biological systems, as environmental interactions of nanomaterials always include passage through aqueous phases. Environmental matrices such as surface water and soil differ widely in pH and ionic composition, thus agglomeration/aggregation and adsorption, and in turn mobility in the environment, may be predicted if pH dependent stability is described. Similarly, studies on nanomaterial translocation within organisms can also benefit from pH dependent characteristics, as it is well known that pH within an organism varies between organs, tissues and cellular compartments. Since nanomaterial suspensions are destabilized outside a narrow pH range defined by their point of zero charge, they are likely to precipitate in response to pH changes (Brant et al., 2007). This knowledge may allow predictions of pH dependant sequestration within an organism. In this respect it should be noted that in certain matrices it may be impossible to assess specific characteristics *in situ*. For instance within a dispersion it is not possible to assess the specific surface area, and in soils it is not feasible to assess hydrodynamic diameter by DLS or aggregation due to interference with the soil particles.

5.3. Reference or test materials

The working groups agreed with the requirement for test materials, some of which could be developed further to certified reference materials, once priorities and relevance become clearer. Starting with a pre-existing candidate panel identified by the REFNANO project, there was clear parity between the groups for the candidates of TiO_2, polystyrene beads and silver, but each candidate material was identified for different reasons including relevance in the environment (TiO_2 and silver), low solubility (TiO_2 and polystyrene beads, silver), ease of detection (polystyrene beads labelled with fluorescence) and potential high toxicity (silver). Some researchers demonstrated a strong interest in using materials which had been well studied in human toxicology models to provide a comparison between species. Others argued strongly against this point given that some materials may have a potentially lower relevance in the environment (e.g., less likely in certain environmental matrices), or that the material is already known to be not toxic to some aquatic species. It was also highlighted that such materials may provide issues relating to the ease of detection against large backgrounds of natural particles, organic matter, or trace metals in many environmental samples. Dissolution was clearly a factor that drove decision making, with easily dissolving materials being less attractive due to the difficulty in attributing toxicity to the particle or the chemical components. However, this concern was disregarded for silver due the knowledge that it is known to be toxic, and due to its extensive use and high relevance in the environment.

Single and multiwalled nanotubes were considered to be useful test materials in the future. All groups, in general, indicated that it would be difficult to obtain reproducible samples due to small differences in production conditions affecting the quality of the nanotubes and additional differences being introduced during purification. Until large volume methods are available that yield high quality, homogeneous batches of nanotubes over time, it would be unwise to include nanotubes among the test materials to be prioritized.

Since it is well-documented that the aspect ratio is of high relevance for toxicity prediction in rodent models (Poland et al., 2008), it was suggested that similar metrics should be identified for species relevant to ecological risk assessment. However, the potential toxicity of high aspect ratio particles in non-human, non-rodent models has not been well covered in the literature and it is therefore currently unclear if correlation does exist between aspect ratio and ecotoxicity.

5.4. Categorization of nanomaterials for environmental studies

Throughout the tasks employed to address the physiochemical characteristic requirements and the question of reference materials, all groups agreed with the classification of nanomaterials into broader categories. The advantages of generating classifications of materials are many. There was clear agreement that current classifications had no alternative than to be based upon material composition, due to the lack of information regarding other relevant properties such as surface reactivity or mechanism of toxicity. This of course could change with time, and NanoImpactNet was deemed a useful forum in which to revisit this issue in three years time. While all groups agreed on the use of material composition as broad umbrella headings for each material type, one group took this approach a significant step forward and proposed the use of hierarchical classification charts to allow a systematic approach to the handling, analysis and toxicity testing of such materials (Fig. 1). The advantage of such an approach is that it provides clear guidelines, for non-experts, industry and regulators as well as scientists with respect to how to handle a new nanomaterial for characterisation, hazard and exposure assessment purposes. The difficulty in generating a practical approach of this nature is the current lack of knowledge regarding which physico-chemical characteristics are most key and what decisions should be derived on the basis of such knowledge. This therefore provides a clear framework for directed research to establish such a hierarchical classification model.

It is important to realise, however, that current work on the development of test or reference materials focuses on their use for calibration, method validation, method development, and other various quality control purposes and not specifically for studying the effects of nanomaterials and/or examining the physical properties leading to toxicological impacts. For future use in ecotoxicologial studies it is essential to develop positive and negative controls for specific modes of action of toxicity and to address questions relating to Absorption, Distribution, Metabolism and Excretion (ADME) aspects in organisms.

5.5. Relevance to studies of environmental fate and ecotoxicity

For the purpose of environmental hazard identification of nanomaterials, the PBT-profile (persistence, bioaccumulation, toxicity) is of major importance as defined by REACH[5]. Thus, besides the present focus on ecotoxicity, degradability should receive increased attention in environmental studies of nanomaterials, as well as those characteristics that affect mobility and bioavailability in the environment and ultimately the processes leading to bioconcentration and/or biomagnification (equivalent to the ADME concept of in organisms) and (eco)toxicity. For this purpose, particle size can certainly be identified as a characteristic that will affect mobility and uptake in organisms, though it is not necessarily the smallest particles that are most mobile (Brant et al., 2007). On the other hand, coating with organic polymers seems to have a major impact on environmental mobility (Hyung et al., 2007) potentially for a number of reasons such as altered charge, and thus characteristics related to coatings and their persistence are of particular interest.

Persistence is a key issue for organic environmental contaminants like polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), as this will not only affect the toxic profile of a chemical (i.e., the risk of long-term exposure is low for ready biodegradable compounds) but a low degradability is a prerequisite for biomagnification to occur. It is important to stress that both abiotic and biotic degradation should be included in these considerations. Even though the persistence of metals is never discussed (as metals cannot be degraded) the dissolution and speciation of metals may be affected by abiotic and biological interactions yielding changes in metal bioavailability. Interestingly, the current focus on persistence of nanomaterials deviates from that of other chemicals, as carbon-based nanomaterials like fullerenes and carbon nanotubes, are, like carbon black, generally regarded as so persistent that they accumulate in soil (Kuzyakov et al., 2009). On the other hand metals and metal oxide nanomaterials that in their bulk form traditionally may be regarded as more or less stable against dissolution, can release ions into solutions and thus exhibit persistence that is similar to conventional contaminants. In this way by dissolving nanomaterials, which are accumulated by organisms as nanomaterials, the ADME of the compound may be governed by characteristics related to the particles form, while the effects may be related to the dissolved element. For instance a nanomaterial like ZnO with fast dissolution rates in aqueous media may be regarded less of a nano-specific hazard and therefore could be considered a less interesting candidate test material in an environmental context. Furthermore, the dissolution of nanomaterials like Ag seems to complicate mechanistic studies in terms of apportioning any effects to specific fractions. So that nanomaterials can be properly used in studies of environmental fate and uptake/excretion, as well as effects, it is desirable that they are well characterized with respect to persistence, and our choice of test and standard materials should reflect this.

To study bioaccumulation, it is essential to be able to trace and quantify nanomaterials within organisms, tissues and cells. Unless special labelling techniques are used, it is an advantage to work with nanomaterials that may easily be detected through unique chemical signatures or inherent properties that permit detection. Furthermore, it is important to consider the background levels in the studied organisms in order to be able to discriminate compounds taken up from those naturally occurring in the media or in the organisms. For this purpose nanomaterials like Au, Ag, TiO_2 and polystyrene are suitable as test materials.

As for bioaccumulation studies, the study of mobility of nanomaterials in the environment is feasible only if the materials studied have unique chemical signatures or carry some label (chemical, radioactive, fluorescent or immunologic). In environmental matrices like soils, sediments, sludges and water, the background levels of many elements are far higher than in organisms, rendering e.g., studies of TiO_2 and other more or less abundant elements problematic. This is one of the reasons that make rare earth elements like Au good for studying environmental fate and behaviour of nanomaterials.

The investigation of nanomaterial fate, behaviour and toxicity in the environment is a rapidly expanding area of research. This workshop aimed to provide some consensus on key issues, which at this time, will help to improve study design and provide the basis of considering the potential environmental impacts of nanotechnology.

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[5] http://ec.europa.eu/environment/chemicals/reach/reach_intro.htm.

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