Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

18-Dec-2012

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

GUIDANCE ON SAMPLE PREPARATION AND DOSIMETRY FOR THE SAFETY TESTING OF MANUFACTURED NANOMATERIALS

Series on the Safety of Manufactured Nanomaterials
No. 36

JT03332780

ENV/JM/MONO(2012)40
Unclassified

English - Or. English

OECD Environment, Health and Safety Publications

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Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials

IOMC
INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
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Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris, 2012

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EXECUTIVE SUMMARY

The unique properties of manufactured nanomaterials have raised the question as to whether the current OECD Test Guidelines are adequate to appropriately address their characterisation and the assessment of their toxicological properties. Since it was recognised that there is a need to develop a guidance document on sample preparation and dosimetry, to which special attention should be paid in using test guidelines when considering the chemical and physical characteristics of nanomaterials, the first version of the guidance document, Preliminary Guidance Notes on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials [ENV/JM/MONO(2010)25] was published in May 2010.

As this guidance document has been recognised as a living document, it is subject to amendment and refinement as researchers gain greater understanding of how to handle nanomaterials in test situations. It was proposed, at the 8th meeting of the WPMN held in March 2011, to set up a specific task group to review the first version of the Guidance Notes on Sample Preparation and Dosimetry (GNSPD) with experience gained through both the sponsorship programme analyses of data from the sponsored nanomaterials and other efforts.

This document is the revision which was initially prepared by the task group and was reviewed by Steering Group four of OECD Working Party on Manufactured Nanomaterials (WPMN) taking into account the latest experience on testing of manufactured nanomaterials as well as the scientific knowledge from relevant experts. Also, it is worth noting that the results/recommendations from the OECD-WPMN horizontal expert meeting with regard to inhalation toxicity testing for nanomaterials [ENV/JM/MONO(2012)14], held in October 2011, has contributed to this update. Section V-A (Physical-Chemical Properties) was updated taking into consideration the progress achieved by ISO. Several endpoints (such as dustiness, crystallite size) have been newly introduced.

This revised document “Guidance on Sample Preparation and Dosimetry” has also been circulated to the experts of Working Group of National Coordinators of the Test Guidelines Programme (WNT), for their comments.

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SECTION I: GENERAL INTRODUCTION

1. In its review of the OECD harmonised test guidelines, Steering Group 4 (SG4) of the Working Party on Manufactured Nanomaterials (WPMN) recommended the development of guidance on sample preparation and dosimetry for tests using manufactured nanomaterials. Such guidance would be a separate document from the OECD's existing guidance on difficult substances *Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures* [ENV/JM/MONO(2000)6] and would be written primarily to inform the WPMN's exploratory testing programme (OECD Sponsorship Programme for the Testing of Manufactured Nanomaterials) as well as other users involved in the testing of manufactured nanomaterials. A drafting group consisting of volunteer experts from the WPMN and the OECD member countries' delegations developed the guidance during 2008-2009. The first version of the guidance, *Preliminary Guidance Notes on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials* [ENV/JM/MONO(2010)25] was published on 31 May 2010.

2. From the beginning, it has been recognised that this guidance should be a living document, subject to amendments and refinements as researchers gain greater understanding of how to handle nanomaterials in test situations. Actually, as best methods for sample preparation, dosimetry, and safety testing do not yet have full consensus within the field, detailed methods cannot be prescribed. Thus, regular updates of this guidance can be anticipated. A significant outcome of the WPMN exploratory program is the knowledge gained in preparing test samples and administering doses across a wide range of testing scenarios and material types. Accordingly it was proposed, at the 8th meeting of the WPMN held in March 2011, to set up a task group to update/revise the Guidance Notes on Sample Preparation and Dosimetry. And in due course, the first version has been reviewed and revised during 2011-2012.

3. This document is the 2nd version which was developed between the 8th and the 10th WPMN (March 2011 to June 2012). It is worth noting that the results/recommendation from some of the WPMN horizontal expert meetings with regard to testing of nanomaterials have contributed this update.

4. The original purpose of this guidance was primarily to assist sponsors as they conduct testing in support of the WPMN exploratory program. It therefore focuses on the kinds of tests that address the endpoints and the types of nanomaterials being tested under the sponsorship programme. Nevertheless, the WPMN hopes that this guidance will also be of use to the scientific community at large, in particular to those investigators conducting tests to advance understanding of the environmental, health, and safety implications of manufactured nanomaterials. It is recognised, however, that due to the wide variety of nanomaterials, it is difficult to develop advice applicable to all nanomaterials; accordingly, the performer of a study will have to exert some judgment on a case-by-case basis on the applicability of the recommendations given in this guidance to their particular material.

5. Last but not least, this guidance refers and applies to water insoluble manufactured nanomaterials as the WPMN considered that soluble nanomaterials are unlikely to need different sample preparation techniques than other chemicals, apart from precautions dictated by the specific reactivity of each material. However their size will still affect where they are being deposited e.g. in the lung. It should be noted that nanomaterials that can release soluble species, e.g. silver, are also considered in this guidance. Because

few, if any, standard testing approaches have been developed for nanomaterials, this guidance is not a "book of recipes" for preparing samples and administering doses, but rather outlines – often in a general or descriptive manner – considerations based on early results with nanomaterials or other experience with chemicals and particulates. It is a guide in the most basic sense, designed to point researchers in directions that at present seem most promising for yielding meaningful and reproducible test results.

SECTION II: TERMINOLOGY

Dispersion versus solubility

6. Most dosing techniques require the test material to be in a liquid phase (generally aqueous) for delivery and (eco) toxicologists sometimes use the terms "in solution" or "solubility" to infer this. However, in particle chemistry these terms are inappropriate. The introduction of an insoluble or very sparingly soluble nanomaterial to a liquid or other aqueous medium with the intention of making a stock "solution" will involve dispersion. A stable dispersion of a nanomaterial in a liquid is referred to as a colloidal dispersion. The term "colloid" applies to particles or other dispersed material in the 1 nm - 1μm size range (IUPAC 1997). Colloids are *dispersed* rather than *dissolved* in a medium, and the term "dispersion" rather than "solution" is used in this document to mean the addition of solid nanomaterial to a liquid phase, where the solid and liquid phases co-exist. Some metal nanoparticles may release ions from the surface into the surrounding water (corrosion/degradation) and it is therefore possible that these nanomaterials will eventually degrade completely (Liu and Hurt 2010). Because of the particle size of many nanomaterials, it can be difficult to distinguish between when a nanomaterial is dispersed and when it is dissolved. The term "dissolved" is used in other OECD documents and historically in toxicology to mean the component of a liquid sample that has passed through a 0.45 μ m (or similar) filter. However, as (colloidal) dispersions of nanoparticles might also pass through such filters, it is recommended that where both liquid and particulates are present the term "dispersed" should be used. The terminology used in this document comes directly from colloid science and may need revision for more complex (second generation and beyond) nanomaterials.

7. In addition, nanoparticles may interact with the liquid phase components, partially or totally yielding degradation or transformation products that may influence the overall toxicity and fate processes. This possibility needs to be taken into account when selecting the media and procedures as well as in the assessment of the results of any experiment.

8. The formation of other colloidal systems, such as emulsions (dispersed liquid drops in another immiscible liquid) have not yet been considered in this guidance, although they will become more relevant as manufactured nanomaterials are further modified and functionalised.

Consideration of stability in sample preparation

9. Many nanoparticles are prepared in the form of aqueous dispersion (some may exist in the form of organic or oil-based dispersions). The particles may be maintained in dispersion by stabilizers (for example surfactants and polymers), surface modifications/coatings, or by charge repulsion. Generally, three different forces are encountered in normal dispersions of particles: electrostatic and steric hindrance, and Van der Waals forces, and, for magnetic particles, an additional magnetic attraction force (the same forces would apply to a dispersion of nanoparticles in an aerosol for inhalation tests). Nanoparticles in such

dispersions can exist as primary particles, agglomerates, or aggregates. The stability of the dispersion depends on the net effect of all these forces, which is determined mainly by the properties of the particle and the dispersing medium and particle surface properties, i.e. surface chemistry. For example, particles in an aqueous dispersion sterically stabilised by non-ionic surfactants would be less sensitive to pH and electrolyte conditions than those that are only electrostatically stabilised. For particles stabilised by (extra) surfactants, dilution during sample preparation may lead to desorption of the surfactant from the particle surface and hence agglomeration of particles may occur. For particles stabilised by surface charges, pH and ionic strength in the medium used for sample preparation may cause agglomeration of the particles. The test results for agglomerated particles may differ from what would be the case in the stable dispersion. In such cases, consideration of suspension chemistry is important to create kinetically stable suspensions. It is also important to consider the fundamental relevance of surface treatment or modifications of nanomaterials. In absence of these, many nanomaterials will tend to form large agglomerates and the primary particles will not disperse in water. Sonication or stirring can break up agglomerates into smaller sizes and can result in their temporary suspension in the medium. However, once sonication or stirring is stopped, in the absence of stabilizers the smaller agglomerates will tend to re-agglomerate into larger ones and precipitate.

10. The zeta potential (at a specified pH and ionic strength) and/or the isoelectric point of the particles (in case the particles are stabilised by surface charges) should be determined and provided so it can be used for the fate assessment of particles in a dispersion. For sterically stabilised particles, the zeta-potential may not be a suitable parameter to estimate the fate of the particles *a priori*.

Agglomerate (Working definition: from ISO TS27687 2008)[1]

11. Agglomerate: collection of weakly bound **particles, aggregates** or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components

- NOTE 1. The forces holding an agglomerate together are weak forces, for example van der Waals forces, or simple physical entanglement.

- NOTE 2. Agglomerates are also termed secondary **particles** and the original source particles are termed primary particles.

Aggregate (Working definition from ISO TS28687 2008)[1]

12. Aggregate: particle comprising strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components

- NOTE 1. The forces holding an **aggregate** together are strong forces, for example covalent bonds, or those resulting from sintering or complex physical entanglement.

- NOTE 2. **Aggregates** are also termed secondary **particles** and the original source **particles** are termed primary particles.

[1] These definitions differ from the ones described in the British Standards Institution Standard (BS 2955: 1993) and Nichols, Gary, *et al*. A Review of the Terms Agglomerate and Aggregate with a Recommendation for Nomenclature Used in Powder and Particle Characterization, Journal of Pharmaceutical Science, Vol. 91 2103-2109, 2002. The more up-to-date and currently more widely accepted definitions from ISO are preferred in this document.

SECTION III: CONSIDERATIONS ON APPROPRIATE DOSE-METRICS

13. Dosimetry refers to estimating or measuring the amount (in terms of mass, number, surface area, volume, etc.) of a particle at a specific biological target site at a particular point in time[2]. Mass is currently the most commonly used dose-metric in ecotoxicity and mammalian toxicity studies. Accordingly, it is usually expected that results of such studies should always report mass concentration (e.g. mg/l). This dose-metric is the basis of the current risk assessment process and the linkage to past work in both exposure and (eco) toxicology.

14. However, the mass metric appears not always to be the most appropriate or relevant one. Indeed for some nanomaterials the results may be better expressed as a function of surface area or particle number because particle size and specific area may play a main role in determining the toxicity of nanomaterials. This seems to be the case for many nanomaterials and there is a trend in toxicology to relate potential toxic effects of nanomaterials with these properties (e.g. inhalation) individually or in combination with each other. In addition, it appears that mass concentration is not sufficient for comparison of nanomaterials of the same chemical composition.

15. Conversion between the metrics of mass, number and surface area remains challenging. Accordingly, measurements that enable expression of data in terms of surface area and particle number should be made where and whenever possible (e.g. particle number counts in test media and surface area measurements on the dry nanomaterial).

16. For the environment, it seems too early to tell whether, for instance, dose by mass should and/or could be substituted with size or surface area. Too few studies have actually investigated alternative dose metrics at this point in time and correlated these with the observed effects.

17. Adequate characterisation of the material and consideration of the scope of applicability of the test is required, along with consideration of the design of the test (e.g. selected doses, sample preparation to minimise uncertainty/bias) and the selection of the most appropriate instrumentation/method. There are currently no definitive conclusions on the best metrics. However, there is growing consensus that when tests on nanomaterials are performed, there should be a sufficient characterisation allowing the dose-response to be expressed in the different metrics discussed: number, surface area and mass. It is also critical to report each of these metrics as well as the methods that were used to derive the measurements.

18. Particle size distributions are a function of particle mass concentration, and so any size distribution measurements or surface area measurements would need to be done at each dose. It is also recognised that surface area measurements are made in dry state and assuming that no aggregation but only agglomeration occurs. Agglomeration in the suspensions may be a slow process especially at low mass concentrations. Hence, it is recommended that the determination of the particle size be repeated at regular intervals to ensure that dynamic changes to the dose are detected and recorded.

19. Since specific surface area of particles in liquids cannot be measured directly, the derivation of surface area from size measurements has to be done with great care. Most sizing techniques will report a fraction of the outer diameter of existing or forming agglomerates (depending on which technique is used). The back-calculation from this diameter to a surface area is likely highly erroneous.

[2] Adapted from Miller, F.J. 2000. Dosimetry of particles in laboratory animals and humans in relationship to issues surrounding lung overload and human health risk assessment: a critical review. Inhalation Toxicology, V12:19-58.

SECTION IV: COMMON ISSUES REGARDING SAMPLE PREPARATION AND DOSIMETRY

20. There are some common features of sample preparation and dosimetry that apply to both toxicology and ecotoxicology testing, as well as different routes of exposure/delivery. These common aspects are outlined below, with deviations from these described in the relevant section of this document. One aspect that always deserves particular attention is the fact that small impurities can have a strong impact on the physical-chemical properties of nanomaterials. Therefore, it is important to pay attention to the presence of impurities. Substances with different impurities may behave very differently, even if the main constituent is the same.

21. Further, there is considerable interest in producing nanomaterials with specific surface functionalities/modifications. Such modifications can significantly affect the chemical reactivity of a nanomaterial, and thereby its potential effects on (or interactions with) living organisms and the environment. In addition, certain modifications (e.g. DNA or protein attachment) have been shown to have an effect on the uptake of nanoparticles into cells *in vitro*. Therefore, the surface functionality of a nanomaterial is likely to have a strong impact on its (eco) toxicological behaviour. Surface functionalisation is often employed to minimise nanomaterial agglomeration and/or to introduce functional groups or specific chemistry to the surface of the particle. But, the size of the material dispersed (as primary particles, agglomerates, or aggregates) may differ substantially for a given nanomaterial depending on whether or not it has been surface functionalised.

22. The issues described here apply to tests from all sections of the OECD Test Guidelines. It is critical to document in detail the steps used in sample preparation in order to understand the methodologies used, and allow for replication of test samples as needed. Keeping in mind that preparation protocols may be strongly dependent on the nature of the sample to be dispersed, it is suggested that media preparation methods be consistent among similar media types, wherever possible. For example, when a testing programme includes both tests for environmental behaviour and for ecological effects in aquatic media, an effort should be made to prepare suspensions using a common method (where this is feasible). This approach will help to minimize variation in material properties and foster strong linkage between results of fate and exposure testing and effects on organisms.

23. Other common features include:

1. Storage and stability of test material

24. Nanomaterials should be stored according to the manufacturer's recommendations, but some general issues are highlighted when manufacturer's information is limited. The usual considerations for storing chemicals will apply, including avoiding extremes of temperature, sunlight, and moisture. Nanomaterials that are supplied as dry powders or dispersions should be stored so that they remain dry or under liquid respectively. For any other variations on storage and re-dispersion, manufacturer's recommendations should be followed. Clearly, experimenters will need to make stock dispersions from the original material supplied by the manufacturer. These dispersions should be stored taking into account the usual considerations above for any chemical, but also considering the reactivity of the material. For example, photoreactive materials should be kept in the dark and precautions should be taken to avoid

adsorption of other chemicals from the laboratory atmosphere when handling the dry material (e.g. storage and handling under an inert gas atmosphere). Once stock dispersions are prepared, and a full characterisation of the freshly prepared stock dispersion has been made, additional checks should be done to assess the shelf life of the material or confirm the information given by the manufacturer. Two key aspects need to be investigated: (i) whether or not the nanomaterial gradually dissolves or transforms/degrades such that the solid material disappears (e.g. for metal particles that form free metal ions in the external medium) (ii) any temporal changes in the particle size distribution and surface charge in the stock dispersion. If changes occur, then protocols should be developed to restore the particle size distribution (e.g. re-sonicating the dispersion just before dosing in the case of agglomeration). If the stock dispersion cannot be restored, it should be made fresh from the same batch number of the test material and re-characterised. If a different batch number of test material is used, then additional physical-chemical characterisation will be required.

2. The chemical composition of the test media

25. The chemical composition of the test media will affect particle aggregation/agglomeration. The following parameters should therefore be measured for media used in ecotoxicology, mammalian, and *in* vitro studies (in addition to other routine measurements):

- **Ionic strength**- it is likely that many types of nanomaterials will agglomerate in very dilute brackish water, or any saline conditions, where studies have shown that even 2 % salt in seawater will do this. Thus, for any marine or estuarine studies the salinity and NaCl concentration in the water should be recorded. In natural fresh water or seawater, NaCl is likely to be the bulk electrolyte. Similarly, for salines or dispersion solutions used in cell-free, cell-based or mammalian studies, the composition should be given so that the ionic strength can be calculated. It is also highly likely that the typical salt concentrations in physiological salines (e.g. 0.9 % NaCl) and culture mediums would cause agglomeration of some nanomaterials.

- **Calcium concentration and hardness**- divalent metal ions can also have a significant effect on agglomeration, especially at low salinity (freshwater). Therefore, in all freshwater ecotoxicology studies the calcium (Ca) concentration of the water should be measured. In addition, magnesium (Mg) concentration, and total hardness would be useful. In mammalian studies, if drinking water is used to deliver nanomaterials then Ca, Mg and total hardness of the water should similarly be measured. These procedures are well known. In mammalian studies special attention should be given to reporting the Ca and Mg concentrations in salines, including the anion (e.g. whether $MgSO_4$ or $MgCl_2$ was used in their preparation).

- **pH**- this should be routinely determined in any experiment. pH affects agglomeration of charged nanomaterials. Physiological salines usually contain pH buffering, and the buffers should be reported precisely (e.g. the specific type of Tris buffer with the full chemical name, or exact details of phosphate buffers). Where commercially available buffer tablets or ready-made solutions are used, the full composition of the buffer should be reported.

- **Dissolved organic matter**- it is evident that the precise type of organic matter, and the ligands it presents, will have potentially large effects on agglomeration and dispersion of nanomaterials. It would therefore be prudent to have some general information about the organic matter in any water. This could be something simple like a measurement of total organic matter, or dissolved organic carbon. This would at least give an overview of the general type of water. For cell-free, cell-based, or mammalian studies, the addition of bovine serum albumin (BSA) and antibiotic preparations to salines represent a source of organic matter. It would be prudent to use high purity reagents in these cases (e.g. fatty acid-free BSA or similar) rather than cheaper reagents.

Since any charge-carrying organic substance which may be adsorbed on the surface of the nanoparticles will change the charge properties of the surface, and hence the dispersion behaviour, all the organic substances (proteins, antibiotics) added should be stated. It is important to also consider that different surface treatments on the same parent form (e.g.TiO_2) may interact quite differently with the dissolved organic matter.

- **Alkalinity**- this may affect agglomeration and similar arguments to pH apply. This is a routine measurement in ecotoxicology, but not in *in vitro* or mammalian toxicology. This will be especially important where bicarbonate buffers are used as the main method to control pH in salines.

- **Dispersing agents**- in case an added surfactant is used to stabilise the dispersion, it would normally be of high concentration, considering the high specific surface area presented by many nanomaterials. Distribution of the dispersing agents between the aqueous phase and the particle surfaces would occur. Therefore, information regarding structural formula and concentration of the agent should be provided. The use of strong dispersing or stabilising agents may modify the bioavailability of the nanomaterial and, in addition, if an added agent has been used to stabilise the stock dispersion, this may not be appropriate for studies that investigate the fate and behaviour of nanomaterials in natural conditions. Accordingly, care should be taken in the conduction of tests and the interpretation of the test results when the use of such agents is unavoidable. It should also be noticed that in most cases, in absence of dispersing agent(s) (e.g. pigments in paint application), particles will be present in form of aggregates.

3. Characterisation of stock dispersions

26. In addition to routine water quality measurements in ecotoxicity testing, or reporting saline quality in mammalian studies, or optimized solution characteristics for *in vitro* methodologies, there is some essential information required about the nanomaterials (discussed in Handy *et al*., 2008; Crane and Handy, 2007; Crane *et al*., 2008, Taurozzi *et al*., 2012, Ma, *et al*., 2012). The following would apply to stock dispersions and arguably, this list could be common to human health and ecotoxicology studies:

Any information from the manufacturer on the test material.

Measured mean primary particle size (for example by electron microscopy (EM)). The method of particle size determination should be described and the character of the mean (number, volume, z- or intensity) must be given. If a certain given mean/average value is calculated from a primary data (e.g. volume average derived from dynamic light scattering z-average) the calculation procedure should be described. In some cases, it may not be possible to measure particle size in the stock dispersion (e.g. the concentration may be too high, EM cannot measure directly in dispersion, etc…). In this case, it should be measured in other relevant media such as the diluted preparations used in the actual OECD test, further dilution may be necessary.

Particle size distribution and indications of mono or polydispersity (e.g. by dynamic light scattering or similar optical method)[3], or other attempt to describe aggregates, agglomerates or ranges of particle sizes in the stock dispersion, including distribution of primary particles. The methodology to derive this size distribution either must be standardised or must be described together with the applied procedures. If a buffer or saline is used to make the dispersion, then the exact composition of the medium, measured pH,

[3] Taking into account possible limitations of the applied technique, e.g. Calzolai L, Gilliland D, Garcìa CP, Rossi F. Separation and characterization of gold nanoparticle mixtures by flow-field-flow fractionation. *J Chromatogr A*. 2011 Jul 8;1218(27): 4234-9.

temperature and any aeration or gassing of the dispersion should also be reported as this may affect particle size distributions. The method of dispersion (stirring, sonication) should be fully described (duration, stir speed, sonication power (preferably in terms of energy input in J/L (joules/litre)), time, type of sonicator, probe and immersion depth if used, volume of sample, etc.[4]). Observations regarding e.g. concentration or limitations of EM as in ii) above also apply for size distribution.

Mass concentration (measured) in the stock dispersion (e.g. mg/l).Mass concentrations as such will not be sufficient for metal-based nanomaterials that dissolve. In such cases measuring the release of ions appears essential, e.g. by centrifuging suspensions and determine metal concentrations in the supernatant, in addition to determining the total mass of metal in suspension. In some cases ion specific electrodes may be used, or dialysis, etc.

Surface area measurements of the primary particles will allow results to be calculated on a surface area basis. When the nanoparticles are not highly aggregated, it is understood that the surface area of dry nanomaterial is a good estimate of free primary particles in the dispersion, however extrapolation from dry materials to the aqueous dispersions should be done cautiously.

For some charged particles, surface charge may be critical to the agglomeration process and so the surface charge may be indirectly assessed via measurements of zeta potential. Since the value of the zeta potential is a function of the ionic strength and composition of the dispersing medium, the conditions during determination should be standardised or reported. It would also be important to measure or fix other abiotic factors that might alter this, such as solution pH and ionic strength.

Any other measurement that is particularly relevant for a specific particle type, for example, aspect ratio for fibres, length of nanotubes, surface functionality.

27. A detailed analysis of the composition of the stock dispersion should be undertaken with special attention to the possible impurities in it. Contaminants can be incorporated into the nanomaterial at any point during production, handling and dispersing. Examples include iron contamination of carbon nanotubes during fabrication (Jurkschat *et al*., 2006), tetrahydrofurane (THF) (and its breakdown products) contamination of fullerene during solvent-exchange dispersion preparation (Markovic *et al*., 2007), and endotoxin contamination during manufacturing and handling (Vallhov *et al*., 2006). In some cases these "contaminants" are intrinsic components of the nanomaterial likely to be encountered during real world exposure, such as polyaromatic hydrocarbons on diesel exhaust particles (Xia *et al*., 2004), in which case their quantities should be measured to compare their impact across studies. In other cases, such contaminants may not be intrinsic to the original nanomaterial (such as contaminants in dispersing agents) and may be accounted for in controls. Ultrasonication processes sometimes produce contaminating particles by ablation of the probe tip and vessel. Alternatively, a purification step may need to be added to stock dispersion preparation.

28. While a test of the material for metal impurities is relatively easy to perform, a thorough test for unknown organic impurities may sometimes not be easy or feasible. Here the information from the manufacturers about additives and possible by-products is indispensable but encompasses both technological and policy implications.

29. The shape, volume and material type of the container using during the dispersion preparation may also have some influence on the size distribution and purity of the stock dispersion and should be considered and reported (Taurozzi, *et al*., 2012).

[4] See also next section

4. Characterisation of samples (prepared from stock dispersions) prior to administration/testing

30. The general recommendations about the characterisation of stock dispersions (above) should be followed.

31. The key point is to know the exact composition of the prepared sample, and to report how it was made. As particle size and concentration may vary with depth after stock dispersions are left to settle, a consistent sampling point for very heterogeneous samples over time could provide better precision (Ma and Bouchard 2009), also stirring or vortexing periodically and/or just before sampling can be helpful. The following information is required:

i) Volumes prepared, type of water or solvents used;

ii) pH and use of any buffers;

iii) Exact details of any sonication times (or preferably energy input in J/L) given in terms of durations/intensity/instrument used/frequency of ultrasound;

iv) Exact details of how long after sonication or mixing/stirring before the test dispersion was added to test vessels. Any extra (precautionary) period of mixing or sonication (e.g. 30 min or preferably energy input in J/L) immediately prior to dosing to the experimental model may be helpful and should be recorded. Re-characterisation of subsamples from stock suspensions after pH modification, sonication or other treatments should be considered;

v) Exact details of volumes added to tanks or test vessels, and how they were mixed in the tanks/test vessels. For example, passively by diffusion, stirred in, allowed to mix with air bubbling around the system. Details about the depth of the liquid under treatment in tanks or vessels or details on the depth of the probe that is inserted under the liquid surface should be recorded and kept constant in all related tests; and

vi) pH, ionic strength, dissolved organic matter.

5. References

Crane, M., Handy, R. D., Garrod J., and Owen, R. (2008), "Ecotoxicity test methods and environmental hazard assessment for engineered nanoparticles", *Ecotoxicology* 17, pp.421–437.

Crane, M. and Handy, R. D. (2007), "An assessment of regulatory testing strategies and methods for characterising the ecotoxicological hazards of nanomaterials", *Report for Defra*, London, UK. http://randd.defra.gov.uk/Document.aspx?DocumentID=2270/ (Accessed in September, 2012)

Handy, R. D., Kammer, F. v. d., Lead, J. R., Hassellöv, M., Owen, R., and Crane, M. (2008), "The ecotoxicology and chemistry of manufactured nanoparticles", *Ecotoxicology* 17, pp. 287-314.

IUPAC (1997), *IUPAC Compendium of Chemical Terminology*, 2nd Edition. IUPAC.

Jurkschat K., Ji X., Crossley A., Compton R.G., and Banks C.E. (2006), "Super-washing does not leave single walled carbon nanotubes iron-free", *Analyst* 132, pp. 21-3.

Ma H, Brennan A, Diamond SA. (2012), "Phototoxicity of TiO2 nanoparticles under solar radiation to two aquatic species: Daphnia magna and Japanese medaka", *Environmental Toxicology and Chemistry* 31, pp.1621

Ma, X., and Bouchard, D.(2009), "Formation of Aqueous Suspensions of Fullerenes", *Environmental Science and Technology* 43 (2), pp. 330-336

Markovic Z., Todorovic-Markovic B., Kleut D., Nikolic N., Vranjes-Djuric S., Misirkic M., Vucicevic L., Janjetovic K., Isakovic A., Harhaji L., Babic-Stojic B., Dramicanin M., and Trajkovic V. (2007), "The mechanism of cell-damaging reactive oxygen generation by colloidal fullerenes", *Biomaterials* 28, pp.5437-48.

Taurozzi J.S., Hackley V.A. and Wiesner M.R. (2012), "Reporting guidelines for the Preparation of Aqueous nanoparticle dispersions from dry materials", Ver.2.1. *CEINT/NIST Protocol*.

Vallhov H., Qin J., Johansson S.M., Ahlborg N., Muhammed M.A., and Scheynius A., and Gabrielsson S. (2006), "The importance of an endotoxin-free environment during the production of nanoparticles used in medical applications", *Nano Letters* 6, pp.1682-6.

Xia T., Korge P., Weiss J.N., Li N., Venkatesen M.I., Sioutas C., and Nel A. (2004), "Quinones and aromatic chemical compounds in particulate matter induce mitochondrial dysfunction: implications for ultrafine particle toxicity", *Environmental Health Perspectives*112, pp.1347-58.

SECTION V: SPECIFIC CONSIDERATIONS

A. PHYSICAL-CHEMICAL PROPERTIES [5]

32. Observations from the groups that have addressed the problem of sample preparation for physical-chemical characterisation often do not distinguish between characterisation appropriate in order to assess human health and environmental effects. Therefore, the term (eco) toxicology in this section is generally used unless a clear distinction is necessary.

33. As for any other materials or chemicals, the inherent ability of a nanomaterial to cause an effect (being it desirable or adverse) relates to its chemical and physical properties, including its impurities. Physical-chemical characterisation of a nanomaterial is fundamental both for determining its identity and to properly execute and interpret the results of (eco) toxicological tests, including comparisons with other experiments with the same or similar materials. Indeed, characterization of the nanomaterial's physical-chemical properties before (eco) toxicity testing ensures that the results are related to the nanomaterial intended for the testing. Generally, characterisation of the nanomaterial should be conducted at least 'as received' (that is, as sampled directly from the received package) and 'as administered' (that is, after preparing the material for introduction into the test system)[6] and 'after administration or *in situ*' (that is, once the material has been introduced into the (eco) toxicological test system, as appropriate).

34. In recognition of the unique properties of manufactured nanomaterials, several regional, national and international organisations formed Physical-Chemical expert panels for the purpose of issuing recommendations concerning the applicability of existing standardised test procedures (e.g. U.S. EPA Series 830 [U.S. EPA, 2007b] and the OECD Series 100 [OECD, 2007] test guidelines) to these materials. These workgroups identified a number of standardised test guidelines that are unlikely to be directly applicable to insoluble manufactured nanomaterials (for example, test guidelines for aqueous solubility and octanol/water partition coefficients); these same issues have ramifications for standardised test procedures in the areas of ecotoxicology, human health effects and in assessing the environmental fate (transport, degradation and accumulation) of these materials, The sample preparation step is crucial, so precise SOPs are required for every measurement technique.

35. In addition, development of some new physical-chemical characterisation procedures may be required, in order to allow nanomaterials to be adequately characterised and to assist in assessing the risks associated with intentional or unintentional exposure of humans or the environment to these materials. Characteristics requiring determination might include (but are not limited to): particle size, size distribution, aggregation, agglomeration state, shape, chemical composition, surface area, surface chemistry, dissociation constant, crystal structure, surface charge, zeta potential, Hamaker constant, interfacial tension,

[5] Note: It is recommended to characterising both stock dispersion and the diluted/prepared dispersions for dosing, as appropriate.

[6] The subject of this section A.

and porosity (Oberdörster *et al.*, 1994; Hunt *et al.*, 1996; Oberdörster *et al.*, 2002; Kreyling *et al.*, 2004; Oberdörster *et al.*, 2005; Nel *et al.*, 2006; Champion and Mitragotri, 2006; Elder and Oberdörster, 2006; Zhu *et al.,* 2006; Warheit *et al.*, 2007; Limbach *et al.*, 2007; Ji *et al.*, 2007; Teeguarden *et al.,* 2007; Murdock *et al.*, 2008; ISO, 2008; Utterback *et al*., 2008; OECD, 2008; Loux and Savage, 2008). A review highlights the difficulties associated with the characterisation of more complex nanomaterials that cannot be considered as simple colloids (Richman *et al*., 2009). The relevance of these characteristics will depend on the specific nanomaterial(s) considered. Moreover, the recommendation for a definition of nanomaterials, adopted by the European Commission (EC) on 18 October 2011, to be used for regulatory purposes[7]poses quite important questions. The answers to the questions certainly require additional work on the metrological aspects, to establish the methods and procedures to assess whether the substance under consideration corresponds to this definition and/or the presence of nanomaterials in consumer products.

36. Relevant findings are in three main areas:

Sample Preparation: Sample preparation is often unique to the characterization method to be employed (See also Section IV). When a procedure for generating nanomaterial preparations (such as dispersions) intended for (eco)toxicological studies is employed, a great attention should be paid to minimise any alteration of the physical, chemical or (eco)toxicological properties of the substrate (Crane *et al.*, 2008). For example, grinding agglomerates may lead to the fracturing of individual particles which in turn can expose new sites of enhanced reactivity. Preparing aqueous nanomaterial dispersions may require the use of surfactants, solvents or sonication, which in turn can alter the degree of agglomeration, fracture individual particles, change existing surface modifications, or alter the bioavailability and toxicity of the parent compound. Interaction with organic material or other constituents contained within the supporting medium also should be taken into account. In order to practically and meaningfully extrapolate laboratory findings to environmental and physiological systems, the difference between sample preparation techniques compatible with the test protocol and the anticipated environmental/physiological processes should be considered. It has to be recognised that the interaction of a nanomaterial with testing media will always influence, if not alter, its properties, as is the case for any other chemical. Another important point for the preparation of aqueous dispersions of nanomaterials is the disequilibrium after mixing and slow reaction (mainly surface chemistry) towards equilibrium. It could be helpful to allow some time for dispersions to equilibrate before they are dosed in an experiment. For example, distilled and/or deionised water is in disequilibrium with atmospheric CO_2, but CO_2 dissolution into the dispersion and adsorption to the surface of freshly dispersed nanomaterials is an important process altering the surface charge of many nanomaterials and the pH of the dispersion. The same holds true for all surface reactions of the nanomaterials with any substance in the used medium (e.g. Bovine Serum Albumin (BSA)). There are protocols for dispersing nanomaterials, which are specific to both the nanomaterial and the method by which it will be characterized (PROSPEcT, 2010a; PROSPEcT, 2010b; ISO/TR 13097; Wang *et al*., 2010; Keller *et al*., 2010; Ji *et al*., 2010; Chowdhury *et al*., 2010; Taurozzi *et al*., 2012a, b, c) and others intended to be more generally applicable (Jensen *et al.*, 2011). The preparation of pyroforic, not passivated, dry nanoparticles must be performed in argon atmosphere in fume hood due to the risk of ignition.

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[7] http://ec.europa.eu/environment/chemicals/nanotech/index.htm: Nanomaterial is *a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm – 100 nm*.

In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %. By derogation from the above, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials.

Dosing: (Eco) toxicological studies typically employ dosage procedures intended to be both reproducible and quantitative. However, aqueous nanomaterial dispersions may be very sensitive to the techniques employed in their preparation and they may not necessarily follow the principles of equilibrium partitioning. In particular, significantly more empirical data may be required in order to develop methods designed to ensure reproducible and quantitative dosimetry (especially with aqueous dispersions). Another aspect to be taken into account in dosing is the reduced (due e.g.to agglomeration or precipitation) or excessive delivery of nanomaterials into test systems, such as cells during the in vitro studies and to animals during in vivo studies. In fact high levels of exposure can give rise to overload effects that can be misinterpreted as evidence of cytotoxicity (Wittmaack, 2011a) and vice versa. In all cases selected doses of administered nanomaterials should ensure adequate exposure, need to be scientifically justified and must reflect possible exposure scenarios in eco- or human toxicology. On the other hand, as nanomaterials may change form not only after sample preparation but also during and after release to the test system (see previous paragraph and next section and specific chapters on environmental and human health sample preparation), dosing and exposure methodologies (including monitoring) may need to be adapted if material modifications are reasonably anticipated.

Physical-chemical characterisation for (eco)toxicology studies: Human and environmental toxicologists seek to develop rigorous mechanistic understandings of their findings for the purposes of elucidating: a) the toxicological response, b) possible antagonisms and synergisms with other toxicants, and c) predictive methodologies useful for assessing the risks posed by new products, for which there is limited knowledge available on certain characteristics. This last aspect is perhaps the least understood of these three main areas.

A.1 Tentative Guidance Relevant to Sample Preparation and Dosimetry for Physical-Chemical Characterisation

A.1.1 Particle size, shape, and size distribution

37. The Nanotechnology Committee of the International Organisation for Standardisation (ISO /TC 229) has developed a technical report entitled *'ISO/PDTR 13014: Nanotechnologies — Guidance on physicochemical characterization for manufactured nano-objects submitted for toxicological testing'* whose purpose is to assist health scientists and experts from other disciplines to understand, plan, identify, and address relevant physical-chemical characterization of such materials before conducting toxicological tests on them. Before the publication of the overview in ISO/PDTR 13014, a number of individual ISO and other organisations' standard documents were available, containing relevant information on Physical-Chemical characterization of nanomaterials.[8] The reader is also advised to consult the report issued by the European Commission's Joint Research Centre: *Requirements on measurements for the implementation of the European Commission definition of the term "nanomaterial"* (European Commission 2012). It describes the requirements for particle size measurements of nanomaterials based on the European Commission's Recommendation on the definition of nanomaterial (European Commission 2011b), it discusses the related generic measurement issues and reviews the capabilities of the measurement methods currently available. Moreover, it illustrates with practical examples the measurement issues that remain to be solved.

[8] There are several standards documents to be mentioned here:

- NIOSH/DUNE interlaboratory study: evaluation of a sample preparation technique for determination of TEM-based size distribution using NIST Reference Materials 8011, 8012, and 8013: gold nanoparticles
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38. An increasing number of publications on nanomaterials demonstrate that the particle size of a nanomaterial can strongly influence its toxicological properties (Carlson *et al*., 2008; Coi *et al*., 2008; Pan *et al*., 2007), as well as its dosimetric fate in the entire organism, including the organ of uptake, circulation and secondary organs of accumulation. The size characterization of nanomaterials, however, is not a trivial task, often nanomaterials show a distribution of sizes and the measurement of the particle size distribution (PSD) will be challenging, especially in dispersion (Carlson *et al*., 2008; Choi *et al*., 2008; Pan *et al*., 2007; Goodman *et al*., 2004; Chithrani *et al*., 2006; Walkey *et al*., 2011; Suresh *et al*., 2012). Particularly challenging is the discrimination between primary particles and their aggregates/agglomerates. Measurement of PSD also calls into question the metrics used for its reporting. PSD in principle can be expressed in mass, volume, or number of particles. Each one of these metrics presents its own issues and challenges. The European Commission has just recently published its definition of nanomaterials that require the measurement of the number PSD in the 1-100 nm size range (European Commission (2011/696/EU). At the moment, there is not a single technique able to satisfactorily measure the number particle size distribution of objects in the 1-100 nm size range. Techniques that can partially address this can be categorized as electron microscopy-based (Dudkiewicz *et al*., 2011, Buhr *et al*., 2009, Klein *et al*., 2011); laser diffraction-based (Brar *et al*., 2011); centrifugation-based (Camey *et al*., 2011) and separation-based (Baalousha *et al*., 2011). In any case more than just one technique (multi-method approach) is required for PSD measurements.

39. There exists a suite of standardised procedures (for example by EPA, OECD and other organisations) for physically characterising particles. However, many of them have minimum size cut off thresholds that exceed the 1 nm to 100 nm size range, although, agglomerates/aggregates may form from the primary particles to make secondary particles larger than 100 nm. Among all of the standardised characterisation procedures, these are perhaps the most easily modifiable through incorporation of more recent technological advances, which recognise the physical and analytical limits of those methods, and develop new methods, including also combinations of methods. These advances include areas such as transmission electron microscopy (TEM), scanning electron microscopy (SEM), confocal microscopy, Dynamic Light Scattering (DLS), atomic force microscopy (AFM), etc. Before quantitative measurements of nanomaterial characteristics are conducted, a qualitative TEM analysis is instrumental to judge the relevance/suitability of the below-mentioned quantitative analyses and to avoid/evaluate possible measurement artifacts or bias. To our knowledge no formal guidelines for the unambiguous and detailed description of a nanomaterial are available. Procedures can be based on methods described in the literature (Krumbein *et al*., 1963; ISO 9276-6, 2008; NIST 960-1, 2001). Such qualitative description includes at least: (i) representative and calibrated micrographs; (ii) the agglomeration- and aggregation status; (iii) the general morphology[9]; (iv) the surface topology; (v) the structure (crystalline, amorphous, …); (vi) and the presence of contaminants and aberrant particles.

40. Researchers also should recognise that dimensional values obtained from different measurement methods will differ with regard to the measurand, and for example from hydrodynamic estimates. Suitable methods for the measurement of particle shapes need to take into consideration the 'dimensionality' of a nanomaterial. The EC definition explicitly states 'in one dimension'. Techniques based on scattering, like DLS, and on the measurements of the hydrodynamic radius, like centrifugal sedimentation, reduce 3D information to 1D (e.g. radius of hypothetical sphere') which in unequiaxal nanomaterial, like fibers, might lead to erroneous conclusions. Taking into account that the aim is a 1-nm-resolution, choices are limited to a few techniques including SEM, TEM and, in specific cases, AFM; Yet, extreme care needs to be taken for the sample preparation for these microscopical techniques to avoid alterations like retrograde nanoparticle agglomeration and particle masking by drying debris contained in the solvent.

[9] Scientific Basis for the Definition of the Term "Nanomaterial"

[http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_030.pdf]

41. Thus, values for particle size, shape, size distribution and degree of agglomeration will depend both on the methodology used as well as on the properties of the medium supporting the sample under consideration. Although EM techniques are considered the standard in the peer reviewed literature, care should be exercised in extrapolating properties observed under high vacuum conditions (as with SEM and TEM measurements) to aqueous and physiological dispersions. Lastly, nanomaterial physical properties that may influence any of these properties, such as magnetisation, also should be characterised. The assessment of particle size, shape, size distribution and agglomeration is of a paramount importance in defining the dose metric applicable in *in vitro* investigation. In fact, a recent study on silica nanoparticles have demonstrated that in order to avoid overload of cells, it is fundamental to determine the relative magnitude of transport by diffusion and gravitational settling of nanoparticles (Wittmaack, 2011b).

A1.2 Particle Size Distribution

42. Number-based distributions of nanomaterial size, shape and surface characteristics can be obtained by quantitative (semi-automatic) analysis of aggregated/agglomerated nanomaterial based on TEM micrographs. Essential basic general principles are (i) the traceability of information, imaging and results, (ii) analysis and representation of results on the per-particle level, (iii) (for practicality) automating of repetitive tasks. To our knowledge, no suitable and validated procedure is available. The outline of in house procedure can follow NIST guide-lines (NIST 960-1, 2001). The different parts of such methods support on different guidelines. Subsampling and suspension of samples can be done according to ISO-14887(ISO 14887, 2000; ISO 14488, 2007; ISO 13322-1, 2004). Imaging and image analysis guidelines are given in ISO publications (ISO 13322-1, 2004; ISO 9276-2, 2001; Krumbein *et al*., 1963; ISO 9276-6, 2008). Data analysis and representation can be done in combination with the methods described in several ISO publications (ISO 13322-1, 2004; ISO 9276-1, 1998; ISO 9276-2, 2001; ISO 9276-6, 2008). It is important to note that particle size may change in the test media and liquid test media in particular, where smaller particles may aggregate/agglomerate or larger particles may deaggregate/deagglomerate.

A.1.3 Aggregation and Agglomeration

43. Primary particle size tends to be a relatively robust parameter as compared to aggregate/agglomerate size, less influenced by environmental conditions (pH, solvent, sonication, presence of proteins etc). In combination with other information on the nanomaterial, it may be correlated with Volume Specific Surface Area (VSSA) and nano-specific properties. Currently, no generally applicable guidelines for measurement of primary particle size and morphology are available. Primary particle size for metal based nanomaterials can be inferred from the unit crystal size as determined by powder x-ray diffraction. Otherwise, primary particle size is usually measured manually, more frequently by TEM. In specific cases, like suspensions of isolated nanoparticles, a semi-automatic measurement is feasible. Measurands are to be selected according to the method of measurement. In some research projects, including the EC's Nanogenotox Joint Action, TEM-based methodology is developed and explored for primary particle measurement focusing among others on the selection of the measurands and on the randomized selection of particles to be measured. For aggregated nanomaterial with a fractal-like morphology, fractal dimensions can be calculated by combining results as proposed by Brasil *et al*. (1999). Artifacts can be examined and interpreted using advanced TEM techniques including cryo-EM (Adrian *et al*., 1984; Adrian *et al*., 1998), and electron tomography (Van Doren *et al*., 2011)..

A.1.4 Chemical description (composition and identification)

44. A thorough chemical description of the nanomaterials comprising both purity and coating and/or surface modification(s) is essential. This issue encompasses both technological and policy implications. It is likely that nanomaterial preparations will contain impurities and the nanomaterials might receive surface treatments or coatings designed to generate desirable interfacial properties (Alexandre and Dubois, 2000).

From an (eco) toxicological point of view, impurities may be relevant if they are present in sufficient quantity to elicit (eco) toxicological effects of a significant magnitude. If technically feasible, relevant impurities should be chemically identified in detail to determine the exact composition of the sample used for toxicology testing. E.g. nanomaterials may contain residues of catalysts or other materials used in their production. Although there are a number of standardised test guidelines addressing the purity issue, it may be necessary to adjust them to focus more on the issue of surface coatings. Additional guidance also may be needed to specifically address surface coated nanomaterials. The composition and expected reactivity of coatings should be taken into account when preparing the samples for nanomaterials composition and purity analysis.

A.1.5 Specific surface area

45. Standardised procedures are available for measuring particle specific surface areas (the accessible surface of a sample when exposed to either gaseous or liquid adsorbate, generally normalised to the sample mass) that are likely to be applicable to manufactured nanomaterials (e.g. BET[10] procedures, dye adsorption, negative ion adsorption, particle morphology etc.). However, in many cases, specific surface areas are the derived quantities depending on parameters, such as the nature of the probe adsorbate molecule (Klobes *et al.,* 2006). In addition, investigators may wish to evaluate whether the particle size distributions (and surface areas) of sparingly soluble manufactured nanomaterials are altered through ripening and/or phase alteration phenomena (Ohman *et al*., 2006; Lefevre *et al.,* 2006). Lastly, according to Klobes *et al.* (2006), the measurement of the specific surface area might most efficiently be conducted concurrently with measurements of pore size, pore size distribution, porosity and perhaps even particle density as these properties will most probably have an important influence on the (eco) toxicological properties of the material. As a complement and altenative, the determination of the volumetric specific surface area (VSSA) is a versatile characteristic that may allow to reduce the variability caused by different specific densities of different nanomaterials (Kreyling *et al*., 2010).

A.1.6 Surface chemistry

46. The surface properties of nanoparticles are critically important with respect to the agglomeration, aggregation, and toxicity behaviours. The expression *surface chemistry* (generally speaking, the chemical nature and composition of the outermost layers of the nanomaterial) may need to be considered in more detail or perhaps in a hierarchical manner, including coatings, functional groups, capping agents potential surface reactions in different media (e.g. redox reactions, coordination chemistry, catalysis). Thorough characterisation of the surface chemistry of a nanomaterial requires analysis of spectroscopy, interfacial analysis (ISO, 2008), toxicology (reactive oxygen species generation; Oberdörster *et al.,* 2005; Nel *et al.,*2006; Balbus *et al.,* 2007), surface complexation modelling (Loux and Savage, 2008), and colloid chemistry (colloidal particle stability; Shaw, 1992, Richman and Hutchinson 2009). Moreover, there are several reports showing that nanoparticles of the same core material, but with different surface coatings have quite different properties with respect to cell uptake and toxicological profiles (Goodman *et al*., 2004; Chithrani *et al*., 2006; Walkey *et al*., 2011; Suresh *et al*., 2012).

47. In most applications (e.g. nanomedicine, drug delivery, teragnostic, fillers, rheological agents, inorganic and organic pigments, photocatalysts and anti-photocatalysts, fluorescence labelling, bio-sensors, diagnostics) controlling and tailoring the surface chemistry of nanomaterials is not only important but also required (Bui *et al*., 2010; Debouttière *et al*., 2006). As an example ZnO is surface treated for cosmetic applications to avoid photocatalytic activity. For instance, the possibility to synthesise core-shell nanostructures with well controlled surface functionalities will enable their broad application in the

[10] BET: Brunauer, Emmet and Teller. A classical method of determining surface area by measuring adsorption of a monolayer of an inert gas on the surface of a solid.

biomedical science. The high level of interest in these types of multilayer nanostructures means that high sensitivity spectroscopic methods are developed able to characterise their surface chemistry to gain better insight into the chemical composition and elemental distribution (Grainger *et al*., 2008). For instance, techniques such as X-ray Photoelectron Spectroscopy (XPS) and Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS) have been successfully applied to characterize core-shell quantum dots (Zorn *et al*., 2011). Moreover, XPS, coupled with other techniques such as TEM, HREM, Raman and SEM, has been also used to investigate the surface chemistry of several nanomaterials ranging from gold (Au) nanoparticles (Techane *et al*., 2011), to carbon nanotubes (Yang *et al*., 2005), and from polymers (Suna *et al*., 2008) to metal oxides (Baer *et al*., 2007).

48. The requirements for high-vacuum conditions of the conventional surface spectroscopy techniques impose a major limitation in analysing interfacial physicochemical processes at ambient conditions. Major efforts have been undertaken to overcome this so called "pressure-gap" to observe processes taking place at surfaces and interfaces under elevated or ambient gas pressure or liquid interfaces. For instance tremendous progress has been made in HRTEM and X-ray fluorescence spectroscopy (XRF) by means of the open environmental cells (E-cells) (Parson, 1974; de Jonge *et al*., 2011). Moreover, very recently, the E-cell has been adapted also to be used for the characterisation of nanomaterials by XPS. In particular, Kolmarov *et al*. (2011) have successfully used graphene oxide windows to study the surface composition of Au nanoparticles in liquid by means of XPS. Although this study has been conducted by using a synchrotron source, since the E-cell is compatible with the high vacuum, it can potentially be used with any commercial electron spectrometer, with no need of expensive differential pumped electron optics and high-flux synchrotron radiation. The sample can be kept in the cell after the XPS measurements and analysed also with other techniques such as SEM, TEM and Raman spectroscopy. This type of experiments, once standardized, will open a new chapter in the investigation of the surface and interface phenomena of nanomaterials. Another aspect of surface chemistry that may be particularly relevant is the surface acidity related to dissociation constants of surface ionisable sites. Ionisable sites may influence the surface charge which has been considered significant in toxicological studies (Oberdörster *et al.,* 2005; Nel *et al.,* 2006). Surface ionisation also may play a major role in colloidal particle stability (Shaw, 1992) and may even inhibit migration into hydrophobic phases (e.g. octanol/water partition coefficients). While surface chemistry is a complex property influenced by many parameters, surface ionisation will be discussed in more detail in this section, due to its significance.

49. Many metal oxide nanomaterials possess surface ionisable sites (e.g. >SOH groups) that exhibit surface complexation reactions with the hydronium ion (and other soluble ions) of the following forms (where >SOH designates a reactive bound site):

$$>SOH \Leftrightarrow >SO^- + H^+$$

$$>SOH_2^+ \Leftrightarrow >SOH + H^+$$

Historical mass action expressions that are sometimes used to describe these reactions are:

$$\frac{[>SOH][H^+]EXP(-e\Psi/kT]}{[>SOH_2^+]}$$

and

$$\frac{[>SOH][H^+]EXP(-e\Psi/kT]}{[>SOH_2^+]}$$

where the subscripted K's represent intrinsic acidity constants, the species in brackets represent the concentrations of reacting species, e is the charge of the proton, Ψ is the surface potential, k is the Boltzmann constant and T is the absolute temperature.

50. These reactions are analogous to the acidity behaviour of a diprotic acid in aqueous solution with two exceptions: 1) the use of concentrations instead of chemical activities, and 2) the exponential Boltzmann term which converts bulk solution hydronium ion concentrations into interfacial values. The interfacial potential (Ψ) can be related to the zeta potential obtained from electrokinetic studies which in turn can in part be used to develop predictions of colloidal particle stability. In addition, geochemical speciation model predictions of surfacial ionised site concentrations can be used to interpret (eco) toxicological findings when the surface charge is considered a significant variable.

51. There is a multitude of incompatible surface complexation models available for simulating these reactions (e.g. diffuse layer models; constant capacitance models; Gouy-Chapman-Stern-Graham models; triple layer models etc.), so although they display a vast potential for interpreting (eco)toxicological findings an agreed (set of) model(s) is yet to be developed. In addition, there is no general consensus within the environmental research community as to which model is demonstrably superior. The minimum dataset needed to utilise models of this nature then includes intrinsic acidity constants, site densities (requiring values for total concentrations of reactive sites and specific surface areas), solution composition and a multitude of binding constants useful for describing reactions with those dissolved ions commonly found in aqueous media. Some of these models (e.g. constant capacitance and triple layer models) may also require one or more capacitance terms. In summary, the area of surface complexation modelling would still need significant additional research and in the short term, it may be preferable to develop empirical datasets of the driving variables obtained from inquiries in this area: solution-composition-dependent surface charge densities and zeta potentials.

A.1.7 Surface charge, zeta potential and Hamaker constant

52. The toxicological role of surface charge is discussed in Oberdörster *et al.* (2005) and Nel *et al.* (2006). The surface charge of manufactured nanomaterials in aqueous suspension will likely result from two phenomena: 1) isomorphic substitution of an ion with one valence by an ion of a different valence in the crystalline structure and 2) surface site reactions with the proton and other ions in aqueous solution (Loux and Savage, 2008; Hemraj-Benny *et al.*, 2008). In particular, the surface charge of a given particle may be dependant both on pH and solution composition. Clearly, (eco) toxicologists conducting research in this area will need to ensure that this is measured and that the exact measurement conditions are given within the bounds of the fluid properties likely to occur in the medium of interest.

53. Associated with a surface charge is a surface potential (in volts). The surface potential plays a major role in such phenomena as: 1) surface complexation with other ions in solution (Loux and Savage, 2008), 2) interfacial pH and oxidation/reduction potentials (Loux and Anderson, 2001), and 3) the stability of colloidal particle dispersions in water (Shaw, 1992; Loux and Savage, 2008). Although it is difficult to measure the surface potential on nonconductive surfaces, it can be related to a zeta potential obtained from widely applicable electrokinetic procedures (Hunter, 1981; Delgado *et al.*, 2007). It should be noted, however, that such procedures are based on models, which simplify the system to a certain degree, thus relating the surface potential to the zeta potential implies some model assumptions. If one can obtain dispersion-composition-dependent zeta potentials for particles in aqueous dispersions, one can subsequently employ Poisson-Boltzmann charge/potential relationships to obtain estimates of the charge density at the beginning of the diffuse layer, within certain model assumptions. In conjunction with a specific surface area measurement, one can then estimate a total charge on the surface.

54. Along with the zeta potential, the Hamaker constant (which may be obtained from a variety of procedures; Visser, 1972; Bergstrom, 1997; Ackler *et al.,* 1996) can be used to predict whether manufactured nanomaterials are likely to agglomerate in natural waters (Nowack and Bucheli, 2007; Loux and Savage, 2008). Predictions of this type of agglomeration will be limited to homogeneous agglomeration of the particles since the data needed to predict the deposition with a heterogeneous set of natural surfaces is often not available (e.g. Hamaker constants and zeta potentials); in general the rate of homo-aggregation is lower than that of hetero-aggregation. The shape and possibly size of resulting aggregate structures may vary too. In this case homo-aggregate studies are not easily used for real world hetero-aggregations processes. Please note that concentration of salts and other chemicals could affect this process. Agglomeration is considered to play a role in (eco) toxicological phenomena; this property may also be useful for toxicological interpretations since agglomeration induces the passage from the nano size level to a micrometric size which does not allow, for instance, the entrance into the cells.

A.1.8 Influence of water chemistry on nanomaterial properties and dispersion behaviour

55. Although not rigorously tested yet, Derjaguin-Landau-Verwey-Overbeek (DLVO) based theories exist for predicting the agglomeration behaviour of nanomaterial dispersions in water. For example, Shaw (1992), Ross and Morrison (1988) and Overbeek (1952) derived expressions for predicting the minimum ionic strength in water (or the Critical Coagulation Concentration [CCC]) needed to lead to the onset of room temperature colloidal particle agglomeration (Loux and Savage, 2008):

Shaw (1992):

$$CCC = \frac{3.84x10^{-39}\,\gamma^4}{A^2 z^6} (\text{mol dm}^{-3})$$

Ross and Morrison (1988):

$$CCC = \frac{3.84x10^{-39} - 39\,\gamma^4}{A^2 z^6} (\text{mol dm}^{-3})$$

Overbeek (1952):

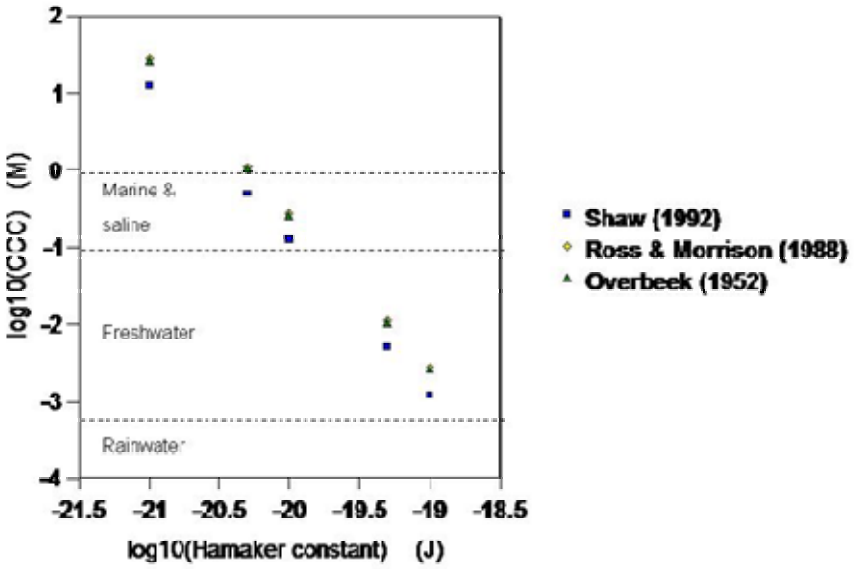
$$CCC = \frac{8.1x10^{-39}\gamma^4}{A^2 z^6} (\text{mol dm}^{-3})$$

CCC – Critical coagulation conc.
A – Hamaker constant (Joules)
k – Boltzmann constant
z – counter ion valence
Ψ – zeta potential
T – absolute temperature
e – proton charge
γ – (EXP(zeΨ/2kT)-1)/(EXP(zeΨ/2kT)+1)

56. Given values for both the zeta potential and Hamaker constant of a colloidal (or nano-) particle in aqueous dispersion, these calculated CCC values can be compared with the solution ionic strength (I; where $I = 1/2 \Sigma [c_i z_i^2]$; c_i is the concentration and z_i is the valence of dissolved ion i) and predictions can be made as to whether these colloidal particles are likely to form agglomerates. By definition, this approach requires knowledge of the concentration of the major ions in the aqueous medium (i.e., the *Water chemistry*). This is usually defined in most test guidelines.

57. Figure 1 compares estimated critical coagulation concentration (CCC) values obtained using the above three equations for particles with an absolute zeta potential of 0.025 V in a room temperature aquatic medium. Based on these simulations, particles with a Hamaker constant of 10^{-19} J (e.g. iron and aluminium oxides; Loux and Savage, 2008) are predicted to remain in a stable dispersion only in low ionic strength freshwaters and rainwater. In contrast, particles with a Hamaker constant of 10^{-21} J (e.g. some natural organic matter or organic matter coated particles; Loux and Savage, 2008) are predicted to remain in a stable dispersion even in hyper saline waters. Other possible influences on colloidal particle stability such as pH and organic matter are further discussed in parts C and D as well as in the introduction of this guidance document. It should be noted, however, that nanomaterials, which agglomerate – in agreement with DLVO-theory – can, under certain conditions, disagglomerate if the environment changes (Peters *et al*., 2012): silica nanoparticles aggregated at the simulated acid conditions of the human stomach, whereas disagglomeration occurred at simulated intestinal conditions at near neutral pH.

Figure 1. Estimated critical coagulation concentration (CCC) values for a room temperature system with particles possessing zeta potential of ca. 0.025 V



A.1.9 Preparation of liquid dispersions; octanol/water partition coefficients (K_{ow}s)

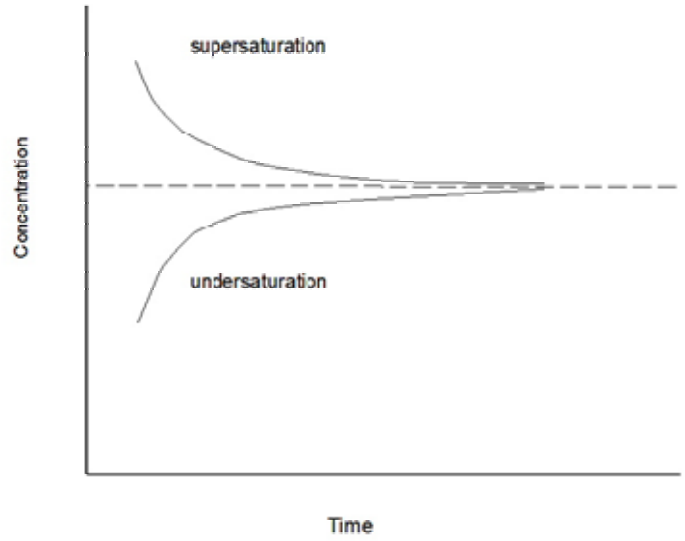
58. As will be discussed later, particles suspended in fluids may well be governed by kinetic limitations rather than thermodynamic considerations and therefore, under these circumstances, the application of a standardised concept such as an octanol/water partition coefficient may be inappropriate in this type of system. Nevertheless, K_{ow}s play a key role in assessing the hydrophobicity of truly dissolved chemicals and it may be necessary to develop surrogate procedures to acquire information of this nature with liquid dispersions.

59. It should be noted, however, that the partition coefficient has limitations for some materials, as has been shown for non-nanoscale materials, such as compounds that bind to proteins rather than to lipids. It should furthermore not be assumed that hydrophobicity is the only driving force behind accumulation and/or persistence; other mechanisms might dominate the effects on the nanometre scale.

60. A point raised within workgroup deliberations concerned the need to definitively characterize solutions/dispersions used in (eco) toxicological studies. Specifically, there is no evidence to support (or refute) a contention that solutions (derived from parent nanomaterials), insoluble nanomaterial dispersions or dispersions of insoluble nanomaterial agglomerates and/or aggregates are likely to engender the same toxicological response. Hence, the lack of information of this nature may compromise the interpretation of any subsequently obtained (eco) toxicological results.

A publication (Jafvert and Kulkarni, 2008) demonstrated great promise for extending the traditional concepts of equilibrium solubility/partitioning theory to buckminsterfullerene colloidal dispersions. It is not at all clear that similar successes will be achieved with larger, higher molecular weight manufactured nanomaterials. For example, Figure 2 illustrates the type of expected solubility behaviour for a compound immersed in a liquid as a function of time. Generally speaking, an undersaturated solution will dissolve more of the solute with time until it reaches a maximum value. In contrast, an oversaturated system (e.g. possibly obtained through the evaporation of solvent) will precipitate the solute onto particles with time until it reaches a minimum value. Therefore, with a compound exhibiting true thermodynamic solubility, one anticipates that one will observe a common equilibrium value in both under- and oversaturated systems given sufficient time. However, the required time to reach equilibrium may have implications for the environmental fate of these products because the kinetics of solubility phenomena is sensitive to the diffusive properties of the solute. In addition, it is known that very high molecular weight products can take extended time periods to reach equilibrium. Hence, even if a given liquid dispersion is being governed by thermodynamic equilibrium processes, the length of the equilibration period may be such that the local equilibrium assumption may be inappropriate in these systems, taking also into account that particles tend to accumulate at interfaces. On the other side, the surface treatment is a very important parameter when considering partitioning. There is literature on phase transfer of particles from aqueous to organic phases by changing the nature of the surface layer (Biondi *et al*., 2012). The particular case of Janus nanoparticles whose unique surface allows two different types of chemistry to occur on the same particle may pose additional challenges as well.

Figure 2. Expected concentrations as a function of time for a system exhibiting true thermodynamic solubility behaviour.



61. There are standardised methods for estimating particle size distributions via the preparation of time-dependent aquatic suspensions in the field of soil science (e.g. Amezketa, 1999; Fristensky and Grismer, 2008) and the adaptation of these existing methodologies may prove to be an efficient means of achieving this goal.

A.1.10 Crystal structure

62. Standardised powder X-ray diffraction (XRD) procedures exist for determining the crystal structures of colloidal particles (at least larger ones). Analysis of physical-chemical properties also may provide valuable information in this area. As well as basic crystalline structure, XRD may be used to determine average crystallite size of crystalline materials (which for mono-crystalline primary particles also means average primary particle size), and may also give indications on particle morphology. XRD is useful for distinguishing different crystal phases of materials of the same chemical composition. In turn, this can provide insight into whether historical data can be used to further characterize a given material. A major concern however is whether a given manufactured nanomaterial has been derivatised; in particular, an amorphous surface coating will not be revealed by the use of x-ray diffraction (unless a significantly high volume of the amorphous material can be detected). In that case TEM will enable the amorphous layer to be seen.

A.1.11 Interfacial tension

63. Thus far, the discussion has focused largely on water insoluble nanomaterials. Some manufactured nanomaterials will likely exhibit sparingly soluble behaviour. If the solubility or the transformation in the aqueous media of a sparingly soluble nanomaterial leads to aqueous toxicant concentrations in excess of (eco)toxic levels, then these products may also be of concern e.g. ions released into solution.

64. Due to their extremely small size, manufactured nanomaterials possess an extremely high specific surface area and, relatively speaking, also possess an extremely high fraction of atomic/molecular constituents on the surface (compared to the number of constituents contained internally). As these surface species have fewer bonds with adjacent species than do internal constituents, it takes less energy to remove

surface species from the particle. A consequence of this phenomenon is that in comparison with bulk material of the same composition, many of these nanoparticulate species will have lower melting points and enhanced solubility or degradation in solvents. For nanomaterials that can degrade in solution, e.g. metal nanoparticles, a quantitative expression relating aqueous "solubility" to the particle specific surface area (SSA) of the solid phase and the solid/water interfacial tension (γ) is given below (Stumm and Morgan, 1981):

$$\log(K_{sp,\,SSA}) = \log(K_{sp,\,SSA=0}) + (2/3)\,\gamma\,(SSA)/2.303RT$$

where $K_{sp,SSA}$ is the solubility product of a material with a specific surface area SSA, $K_{sp,SSA=0}$ is the solubility product of the bulk material, γ is the solid/water interfacial tension, R is the ideal gas constant and T is the absolute temperature.

65. It should be noted that the equation given above holds only for pure water as a solvent but other aqueous solvents have other solubility products which are partly determined in the literature; this equation needs to be generalized.

66. In most cases, finely divided materials are significantly more soluble than large, bulk products of the same composition or release ions into solution at a significantly faster rate. Similarly, with some materials, larger particles will grow larger at the expense of smaller particles in a given dispersion; the net result is that the aqueous solubility of a material also will decrease with time due to this phenomenon, also called Ostwald ripening. Within the context of the present discussion, many nanomaterials are likely to display enhanced aqueous solubilities when compared to the bulk material. Alternatively, given a value for the interfacial tension, one also can calculate the solubility of a nanomaterial provided that the temperature and solubility of the larger bulk material also is known.

A.1.12 Dustiness

67. The propensity of a material to become suspended in air is of interest when nanomaterials are manufactured or handled. The methods that are readily available were generally developed with an aim to assess the likelihood of workplace exposures to powders and were not designed with nanomaterials in mind (Hamelmann and Schmidt, 2003). An additional challenge is that many methods require a large mass of material which is often not available for nanomaterials (Ogura *et al.,* 2009). It has been shown that some methods alter the starting nanomaterials and can fracture aggregated/agglomerated nanomaterials into smaller entities (NanoCare, 2009). The methods chosen will need to take these factors into account. The Vortex Shaker method has been specifically developed for nanomaterials. Other specific methods related to tests of agglomerate stability of nanomaterials are a method based on shear forces in a nozzle (Stahlmecke *et al*., 2009), the rotating drum and continuous drop method (e.g. Kuhlbusch *et al*., 2009; Perosh, Nanodustiness Project, 2012). Standardization of dustiness of nanomaterials is still ongoing and e.g. on topic of the EU-FP7 Project MARINA.

A.1.13 Crystallite Size

68. For crystalline materials, microscopy methods have commonly been used to assess crystallite size (which for mono-crystalline primary particles also means average primary particle size) including AFM, SEM and TEM. These methods may have limited utility for materials that are aggregated and agglomerated. Alternatively, diffraction methods may be used. The techniques used to prepare samples that are suitable for analysis can affect the measurement so the microscopy methods are most useful for durable nanomaterials that can withstand the preparation conditions. Also, the measured particles typically are laid on a surface. Most microscopic techniques do not provide a good statistical assessment and the

methods generally measure the properties of tens or hundreds of particles. Nevertheless, if the particles measured can be demonstrated to be representative of the larger sample these methods can provide useful information about the shape and surface structure of the particles in the sample. (e.g. http://www.malvern.com/labeng/products/iwtm/particle_size_analysis.htm).

69. For nanomaterials that can be treated as spherical dynamic light scattering can be useful. DLS works best on particles in a medium, most frequently in a liquid medium. Thus medium effects have to be taken into account. The method should be used with caution for polydisperse nanomaterials (Calzolai *et al*., 2011). Due to the fact that the aggregation and agglomeration processes are time-dependent and that the method cannot discriminate between primary particles and aggregates, the measurements of samples in solutions under the DLS may display time-dependent character. It is important to state what condition the solution of particles is expected to be in. In most cases equilibrium state is required.

A.1.14 Electron Microscopy

70. Applicable methods may include ISO TS 10797 for TEM and ISO TS 10798 for SEM. These methods have been assessed for carbon nanotubes and may have more general applicability for nanomaterials in general. As noted above, the techniques used to prepare a sample that are suitable for EM analysis can affect the measurement so the microscopy methods are most useful for durable nanomaterials that can withstand the preparation conditions. Most microscopic techniques do not provide a good statistical assessment and the methods generally measure the properties of tens or hundreds of particles. Nevertheless, if the particles measured can be demonstrated to be representative of the larger sample these methods can provide useful information. Other more advanced electron microscopy techniques such as Electron Tomography (ET), Atomic Force Microscopy (AFM) or Scanning Transmission Electron Microscopy (STEM. A. Engel, Biozentrum Basel) should also be considered. Detailed protocols on how to measure 'Photocatalytic Activity' and 'Photocatalytic Radical Formation Potential' are given in JRC Report, 2011.

71. In some cases, SEM can be used to characterise/calibrate another technique that may be much more productive (DLS for example). The SEM would give a good indication of shape of particles and the second technique would give much more statistical account of the sample.

72. Recent developments in scanning electron microscopy applying the transmission mode (STEM) have demonstrated traceable measurements of number-based nanoparticle size distributions. The analysis is performed on per-particle level and (semi-)automatic image analysis tools help to improve statistics (Buhr *et al*., 2009, Klein *et al*., 2011).

A.1.15 Photocatalytic Activity

73. The photocatalytic activity gives an indication of the potential for transformations in the environment which in turn represents an important point of concern when evaluating the full life-cycle of the nanomaterial. On the other side, photocatalytic activity is an extremely important technical specification for nanomaterials that have this characteristic. The surface treatment is critical in this field and information on it is essential to understand the behaviour of the nanomaterial under consideration (See http://uscc.dreamscapesdesigners.net/documents/Photocatalytic.pdf). The photocatalytic activity of a substance can be characterized qualitatively by describing relevant observations or by reporting one or more specific photocatalytic chemical reaction equations. Photocatalytic activity can also be expressed by giving a quantitative value of the moles converted per g of sample per s of irradiation. It is also possible to give a microscopic description of the photocatalytic activity by reporting the turn-over frequency (TOF,

unit: 1/s), which is the maximum number of molecules converted per catalytic site per second. When reporting quantitative data it is necessary to specify the light source (wavelength) and the irradiation power in the method description or in the SOP. When comparing photocatalytic activities of different nanomaterials, it is advantageous to use an assay which is recognized also for other particulate substances, which are not necessarily nanomaterials. One example is the zero order rate of the photocatalytic oxidation (reactive oxygen species) of liquid propan-2-ol to propanone, under oxygenated conditions for a fixed mass of particulate, as has been done for particulate ZnO. See also: SCCNFP/0649/03 The Scientific Committee On Cosmetic Products And Non-Food Products Intended For Consumers Opinion Concerning Zinc Oxide [http://ec.europa.eu/health/ph_risk/committees/sccp/documents/out222_en.pdf and http://sourcedb.ipe.cas.cn/zw/lwlb/200908/P020090901337110041200.pdf].

A.1.16 Pour Density

74. The 'pour density' is a useful parameter for estimating the weight-to-volume ratio of a material; it is often used to estimate technical settings in industrial processes. It is defined as the apparent density of a bed of material formed in a container of standard dimensions when a specified amount of the material is introduced without settling.

75. Some information about pour density measurements (of carbon black) is available in the ASTM Standard D1513 – 05e2.

A.1.17 Porosity

76. Porosity may be determined using the Barrett-Joyner-Halenda (BJH) method of analysis of adsorption and desorption isotherms to determine pore area, specific pore volume and pore size distribution independent of external area due to the particle size of the sample. The t-plot method is commonly used to determine the external surface area, pore volume and pore surface area in microporous solids (European Commission, 2011).

A.1.18 Octanol-Water Partition Coefficient

77. The n-octanol/water partition coefficient (K_{OW}) is a key physico-chemical parameter that is used in numerous estimation models and algorithms for environmental partitioning, sorption, bioavailability, bioconcentration/bioaccumulation and as well for human toxicity and ecotoxicity. Traditional octanol-water partition coefficient methods might only be applicable under some circumstances to some classes of manufactured nanomaterials (OECD 2009,). However, traditional and alternative methods to address insoluble or partially soluble nanoparticles should continue to be monitored for their applicability to nanomaterials.

78. The octanol-water partition coefficient determination is based on a test that measures the distribution of a chemical between two immiscible liquid phases. Correlations are then used to predict the effects of a new chemical (using structure activity relationships, for example) for which a K_{OW} could be measured. K_{OW} is a key step in determining the fate of chemicals as well as assessing their (eco) toxicological risks (OECD, 1995, 2004, 2006). However, while these guidelines are applicable to water-soluble nanomaterials, there are caveats in applying these guidelines to insoluble nanomaterials due to factors such as the aggregation and accumulation of nanomaterials at phase interfaces. See also paragraph

92 and following as well as paragraphs 106, 109 (and, in general the general and especific sections on sample preparation) in these Guidances.

79. Petersen, *et al*. (2010) tested traditional K_{OW} methods, with modifications for carbon nanotubes, and concluded that these methods are not appropriate for generating K_{OW} values that would be predictive of MWCNT(Multi Wall Carbon Nano Tube) bioaccumulation in sediment species. A lack of MWCNT transport across interfacial boundaries between octanol and water, and other complications, were encountered. Hristovski *et al*. (2012) have suggested additional modifications of the traditional K_{OW} test methods that may make them predictive of fate, bioavailability and transport of some nanomaterials in the environment. They addressed the tendency of some nanomaterials to distribute to the octanol-water interface in test systems by developing additional distribution coefficients that increased the predictive nature of the tests on silver, fullerene, and hematite nanoparticles. Others (Xiao and Weisner, 2012) have examined methods that are not based on traditional approaches, such as those used by OECD, to measure nanoparticle hydrophobicity. They have applied several alternative methods developed, for example for nanoparticle pharmaceutical testing (including organic dye adsorption experiments), to fullerene- and silver-based nanoparticles.

A.1.19 Radical Formation Potential

80. The generation of radicals such as reactive oxygen species in vitro (ROS) has been associated with toxicity in in vivo experiments. ROS generation is correlated with oxidative stress, particularly via the inhalation route, and can lead to inflammation and cytotoxicity. ROS include singlet oxygen, superoxide, hydrogen peroxide, and hydroxyl radicals. These species can be generated at the particle surface due to factors including structural defects resulting in altered electronic properties, the presence of transition metals on the surface, and the photoactivation of electron hole pairs. Many of these points are summarized in Nel *et al.*, 2006.

81. There are at least four acellular assays for radical formation potential that have been tested for their applicability to nanomaterials, but they have not yet undergone extensive standardization. A vitamin C yellowing assay that measures the chemical reactivity of nanomaterials toward a vitamin C derivative as an antioxidant has been used to assess titanium dioxide, quartz and clay (Warheit, *et al*., 2010). A broad range of nanoparticles was assessed for intrinsic radical electron inducing capacity utilizing a dichlorofluorescin-based dye, which is used to detect ROS and reactive nitrogen species (Rushton, *et. al*, 2010). The FRAS (ferric reducing ability of serum) assay uses human blood serum as a reagent medium in an assay that measures changes in the total antioxidant capacity of the serum, and has been tested with both particulate nanoparticles and carbon nanotubes (Bello, *et al*., 2009). A fourth assay has been used to assess ROS formation in the presence of U.V. light: the thiobarbituric acid reactive substance assay was used to assess ROS formation of titania in the presence of U.V. light (Sanders, *et. al*. 2012).

A.2 References

Ackler, H.D., French, R.H., and Chiang, Y.M. (1996), "Comparison of Hamaker constants for ceramic systems with intervening vacuum or water: From force laws and physical properties". *Journal of Colloid and Interface Science* 179, pp.460-469.

Adrian M., Dubochet J., Lepault J., and McDowall A. W. (1984), "Cryo-electron microscopy of viruses". *Nature* 308(5954), pp.32-36.

Adrian M., Dubochet J., Fuller S.D. and Harris J.R. (1998), "Cryo-negative staining", *Micron* 29(2-3), pp.145-160.

Alexandre, M. and Dubois, P. (2000), "Polymer-layered silicate nanocomposites: preparation, properties and uses of a new class of materials", *Materials Science and Engineering* 28, pp.83-151.

American Society for Testing and Materials (2009), Standard Guide for Measurement of Particle Size Distribution of Nanomaterials in Suspension by Photon Correlation Spectroscopy (PCS), ASTM E2490-09

American Society for Testing and Materials (2011), Standard Test Method for Carbon Black—Morphological Characterization of Carbon Black Using Electron Microscopy, ASTM D3849 - 07

American Society for Testing and Materials (2012), Standard Practice for Calculation of Mean Sizes/Diameters and Standard Deviations of Particle Size Distributions, ASTM E2578-07

Amezketa, E. (1999), "Soil aggregate stability: A review", *Journal of Sustainable Agriculture*14, pp.83-151.

Baalousha, M., Stolpe, B.and Lead, J. R. (2011), "Flow field-flow fractionation for the analysis and characterization of natural colloids and manufactured nanoparticles in environmental systems: a critical review", *Journal of Chromatography* A 1218, pp.4078-103.

Baer, D. R., Amonette, J. E., Engelhard, M. H., Gaspar, D. J., Karakoti, A. S., Kuchibhatla, S., Nachimuthu, P., Nurmi, J. T., Qiang, Y., Sarathy, V., Seal, S., Sharma, A., Tratnyek, P. G. and Wang, C.-M. (2007), "Characterization challenges for nanomaterials", *Surface and Interface Analysis* 40, pp.529-537.

Balbus, J.M., Maynard, A.D., Colvin, V.L., Castranova, V., Daston, G.P., Denison, R.A., Dreher K.L., Goering, P.L., Goldberg, A.M., Kulinowski, K.M., Monteiro-Riviere, N.A., Oberdoster, G., Omenn, G.S., Pinkerton, K.E., Ramos, K.S., Rest, K.M., Sass, J.B., Silbergeld, E.K., and Wong, B.A. (2007), "Meeting report: Hazard assessment of nanoparticles– Report from an interdisciplinary workshop". *Environmental Health Perspectives* 115, pp.1654-1659.

Bergstrom, L. (1997), "Hamaker constants of inorganic materials". *Advances in Colloid and Interface Science* 70, pp.125-169.

Boyd, R D, Cuenat, A, Meli, F, Klein, T, Frase, C G, Gleber, G, Krumrey, M, Duta, A, Duta, S, Hogstrom, R, Prieto, E (2011), "Good practice guide for the determination of the size distributions of spherical nanoparticle samples", Measurement Good Practice Guide No. 119, National Physical Laboratory UK

Source: Measurement Good Practice Guide No. 119Ref: PDB: 6283 | DDB: 3618Brar, S. K. and Verma, M. (2011), "Measurement of nanoparticles by light-scattering techniques". *Trends in Analytical Chemistry* 30, pp.4-17.

Brasil A.M., Farias T.L. and Carvalho M. G. (1999), "A recipe for image characterization of fractal-like aggregates". *Journal of Aerosol Science* 30(10), pp.1379-1389.

Buhr E., Senftleben N., Klein T., Bergmann D., Gnieser D., Frase C. G., and Bosse H. (2009),"Charac¬terization of nanoparticles by scanning electron microscopy in transmission mode", *Measurement Science and Technology* 20, 084025 (9pp)

Bui, T., Stevenson, J., Hoekman, J., Zhang, S., Maravilla, K., Ho, R. J. Y. (2010), "Novel Gd Nanoparticles Enhance Vascular Contrast for High-Resolution Magnetic Resonance Imaging", *PlosOne* 5(9), pp.e13082-92

Calzolai, L., Gilliland, D., Garcia, C. P. and Rossi, F. (2011), "Separation and characterization of gold nanoparticle mixtures by flow-field-flow fractionation". *Journal of Chromatography* A 1218 (27), pp. 4234-9.

Carlson, C., Hussain, S. M., Schrand, A. M., Braydich-Stolle, L. K., Hess, K. L., Jones, R. L., and Schlager, J. J. (2008), "Unique Cellular Interaction of Silver Nanoparticles: Size-Dependent Generation of Reactive Oxygen Species". *The Journal of Physical Chemistry*, B 112, pp.13608-13619.

Carney, R. P., Kim, J. Y., Qian, H., Jin, R., Mehenni, H., Stellacci, F., and Bakr O. M. (2011), "Determination of nanoparticle size distribution together with density or molecular weight by 2D analytical ultracentrifugation", *Nature Communications* 2(335).

Champion, J.A. and Mitragotri, S. (2006), "Role of target geometry in phagocytosis", *Proceedings of the National Academy of Sciences USA*, 103, pp.4930-4934.

Chithrani, B. D., Ghazani, A. A. and Chan, W. C. W. (2006), "Determining the Size and Shape Dependence of Gold Nanoparticle Uptake into Mammalian Cells", *Nano Letters* 6, pp.662-668.

Choi, O. and Hu, Z. (2008), "Size Dependent and Reactive Oxygen Species Related Nanosilver Toxicity to Nitrifying Bacteria", *Environmental Science and Technology* 42, pp.4583-4588.

Chowdhury, I., Hong, Y. and Walker, S. L. (2010), "Container to Characterization: Impacts of Metal Oxide Handling, Preparation and Solution Chemistry on Particle Stability", *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 368 (1–3), pp.91-95.

Crane, M., Handy R.D., Garrod J. and Owen R. (2008), "Ecotoxicity test methods and environmental hazard assessment for engineered nanoparticles", *Ecotoxicolog*y 17, pp.421–437.

Debouttière, P.-J., Roux, S., Vocanson, F., Billotey, C., Beuf, O., Favre-Réguillon, A., Lin, Y., Pellet-Rostaing, S., Lamartine, R., Perriat, P. and Tillement, O. (2006), "Design of Gold Nanoparticles for Magnetic Resonance Imaging", *Advanced Functional Materials* 16, pp.2330–2339.

de Jonge, N.and Ross, F.M. (2011), "Electron microscopy of specimen in liquid", *Nature Nanotechnology* 6, pp.695-704.

Delgado, A.V., Gonzalez-Caballero, F., Hunter, R.J., Koopal, L.K. and Lyklema, J. (2007), "Measurement and interpretation of electrokinetic phenomena". *Journal of Colloid and Interface Science* 309, pp.194-224.

Dudkiewicz, A., Tiede, K., Loeschner, K., Soegaard Jensen, L. H., Jensen, E., Wierzbicki, R., Boxall, A. B. A., and Molhave, K. (2011), "Characterization of nanomaterials in food by electron microscopy", *Trends in Analytical Chemistry* 30, pp.28-43.

Elder, A., and Oberdörster, G. (2006), "Translocation and effects of ultrafine particles outside of the lung", *Clinics in Occupational and Environmental Medicine* 5, pp.785-796.

European Commission. (2011), "NM-Series of Representative Manufactured Nanomaterials - Zinc Oxide NM-110, NM-111, NM-112, NM-113 Characterisation and Test Item Preparation", JRC Scientific and Technical Report.

European Commission. (2012), "Requirements on measurements for the implementation of the European Commission definition of the term "nanomaterial", JRC reference report.

Fristensky, A. and Grismer, M.E. (2008), "A simultaneous model for ultrasonic aggregate stability assessment". *Catena* 74, pp.153-164.

Goodman, C. M., McCusker, C. D., Yilmaz, T. and Rotello, V. M. (2004), "Toxicity of Gold Nanoparticles Functionalized with Cationic and Anionic Side Chains", *Bioconjugate Chemistry* 15, pp.897-900.

Grainger, D. W. and Castner, D. G. (2008), "Nanobiomaterials and Nanoanalysis: Opportunities for Improving the Science to Benefit Biomedical Technologies", *Advanced Materials* 20, pp. 867-77.

Hemraj-Benny, T., Bandosz, T.J., and Wong, S.S. (2008), "Effect of ozonolysis on the pore structure, surface chemistry and bundling of single-walled carbon nanotubes", *Journal of Colloid and Interface Science 317*, pp.375-382.

Hirstovski, K, Westerhoff, P and Posner, J. (2012), "Octanol-water distribution of engineered nanomaterials", *Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering* 46(6), pp.636-647.

Hunt, J.A., Flanagan, B.F., McLaughlin, P.J., Strickland, I. and Williams, D.F. (1996), "Effect of biomaterial surface charge on the inflammatory response: evaluation of cellular infiltration and TNF alpha production". *Journal of Biomedical Materials Research* 31, pp.139-144.

Hunter, R.J. (1981), Zeta Potential in Colloid Science, Academic Press, London.

ISO (1994), ISO 5725-1: Accuracy (trueness and precision) of measurement methods and results -- Part 1: General principles and definitions, ISO, Geneva.

ISO/TR 13097 "Guide for the characterization of dispersion stability."

ISO (1996), ISO 13321:1996, Particle size analysis -- Photon correlation spectroscopy, ISO, Geneva.

ISO (1998), ISO 9276-1:1998, Representation of results of particle size analysis --Part 1: Graphical representation, ISO, Geneva.

ISO (1998), ISO 9276-1:1998/Cor 1:2004, Representation of results of particle size analysis -- Part 1: Graphical representation -- Technical Corrigendum 1, ISO, Geneva.

ISO (1998), ISO 9276-1: 1998. Graphical representation. In: Representation of results of particle size analysis, ISO, Geneva.

ISO (2001), ISO 14887: 2000. Dispersion procedures for powders in liquids. In: Sample preparation, ISO, Geneva.

ISO (2001), ISO 9276-2:2001, Representation of results of particle size analysis --Part 2: Calculation of average particle sizes/diameters and moments from particle size distributions, ISO, Geneva.

ISO (2001), ISO 9276-4:2001, Representation of results of particle size analysis --Part 4: Characterization of a classification process, ISO, Geneva.

ISO (2004), ISO 13322-1:2004, Particle size analysis -- Image analysis methods --Part 1: Static image analysis methods, ISO, Geneva.

ISO (2004), ISO 16700:2004: Guidelines for the calibration of Electron Microscopy image magnification, ISO, Geneva.

ISO (2005), ISO 9276-5:2005, Representation of results of particle size analysis --Part 5: Methods of calculation relating to particle size analyses using logarithmic normal probability distribution, ISO, Geneva.

ISO (2006), ISO 13322-2:2006 -Particle size analysis- Image analysis methods Part 2: Dynamic image analysis methods, ISO, Geneva.

ISO (2007), ISO 14488: 2007. Particulate materials -- Sampling and sample splitting for the determination of particulate properties, ISO, Geneva.

ISO (2008), ISO 9276-3:2008, Representation of results of particle size analysis --Part 3: Adjustment of an experimental curve to a reference model, ISO, Geneva.

ISO (2008), ISO 9276-6:2008, Representation of results of particle size analysis --Part 6: Descriptive and quantitative representation of particle shape and morphology, ISO, Geneva.

ISO (2008), ISO 22412:2008, Particle size analysis -- Dynamic light scattering (DLS) , ISO, Geneva.

Jafvert, C.T. and Kulkarni, P.P. (2008), "Buckkminsterfullerene's (C60) Octanol-Water Partition Coefficient (Kow) and Aqueous Solubility", Environmental Science and Technology 42, pp.5945-5950.

Jensen K.A., Kembouche Y., Christiansen E., Jacobsen N.R., Wallin H., Guiot C., Spalla O.and Witschger O. (2011), "The generic NANOGENOTOX dispersion protocol", Web report, The Nanogenotox third General Assembly meeting, Denmark, October, 2011, www.nanogenotox.eu/files/PDF/web%20nanogenotox%20dispersion%20protocol.pdf (Accessed September, 2012)

Ji, J.H., Jung, J.H., Kim, S.S., Yoon, J.U., Park, J.D., Choi, B.S., Chung, Y.H., Kwon, I.H., Jeong, J., Han, B.S., Shin, J.H., Sung, J.H., Song, K.S., and Yu, I.J. (2007), "Twenty-eight-day inhalation toxicity study of silver nanoparticles in Sprague-Dawley rats", *Inhalation Toxicology* 19, pp.857-871.

Ji, Z., Jin, X., George, S., Xia, T., Meng, H., Wang, X., Suarez, E., Zhang, H., Hoek, E.M.V., Godwin, H., Nel, A. E. and Zink, J. I. (2010), "Dispersion and Stability of TiO2 Nanoparticles in Cell Culture Media", *Environmental Science and Technology* 44(19), pp. 7309-7314.

Ji, Z. (2010), High Throughput Dynamic Light Scattering Analysis of Nanoparticle Dispersion in Cell Culture Media, University of California, Los Angeles www.cein.ucla.edu/research/UC_CEIN_research_Protocols.html

Jillavenkatesa, A., Dapkunas, S. J., and Lum, L-S. (2001), "Particle Size Characterization", NIST Special Publication 960-1

Klein, T., Buhr, E., Johnsen, K-P., and Frase, C. G. (2011),"Traceable measurement of nanoparticle size using a SEM in transmission mode (TSEM)", *Measurement Science and Technology* Vol.22, Number 9, 094002.

Klmarov, A., Dikin, D.A., Cole, L.J., Huang, J., Abyaneh, M. K., Amati, M., Gregoratti, L., Günther, S., and Kiskinova, M. (2011), "Graphene oxide windows for in situ environmental cell photoelectron spectroscopy", *Nature Nanotechnology* 6, pp. 651-57.

Keller, A., Wang, H., Zhou, D., Lenihan, H., Cherr, G., Cardinale, B., Miller, R., and Ji, Z. (2010), "Stability and aggregation of metal oxide nanoparticles in natural aqueous matrices", *Environmental Science and Technology* 44 (6), pp. 1962-1967.

Klobes, P., Meyer, K., and Munro, R.G. (2006), Porosity and Specific Surface Area Measurements for Solid Materials, National Institute of Standards and Technology, Nist recommended Practice guide, Special Publication 960-17, U.S. Department of Commerce, Washington, DC.

Kreyling, W.G., Semmler, M., and Moller, W. (2004), "Dosimetry and toxicology of ultrafine particles", *Journal of Aerosol Medicine* 17, pp.140-152.

Kreyling, W. G., Semmler-Behnke M., and Chaudhry, Q. (2010), "A complementary definition of nanomaterial", *Nano Today* 5(3), pp.165-168.

Krumbein, W.C. and Sloss, L.L. (1963), Stratigraphy and Sedimentation, W H Freeman and Co.

Kuhlbusch et al. (2009), "NanoCare Health related Aspects of Nanomaterials Final Scientific Report", at http://nanopartikel.info/files/content/dana/Dokumente/NanoCare/Publikationen/NanoCare_Final_Report.pdf (Accessed in September, 2012)

Lefevre, G., Duc., M. and Fedoroff, M. (2006), "Accuracy in the determination of acid-base properties and modelling of metal oxides surfaces", Chapter 2 in Luzenkirchen, J. (Ed.), *Surface Complexation Modeling*, Academic Press, Amsterdam.

Limbach, L.K., Wick, P., Manser, P., Grass, R.N., Bruinink, A., and Stark, W.J. (2007), "Exposure of engineered nanoparticles to human lung epithelial cells: influence of chemical composition and catalytic activity on oxidative stress", *Environmental Science and Technology* 41, pp.4158-4163.

Loux, N.T. and Anderson, M.A. (2001), "Mobile ion activities at charged interfaces", *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 177, pp.123-131.

Loux, N.T. and Savage, N. (2008), "An assessment of the fate of metal oxide nanomaterials in porous media", *Water, Air and Soil Pol*lution 194, pp.227-241.

Murdock, R.C., Braydich-Stolle, L., Schrand, A.M., Schlanger, J.J., and Hussain, S.M. (2008), "Characterisation of nanomaterial dispersion in solution prior to In Vitro exposure using dynamic light scattering techniques", *Toxicological Sciences* 101, pp.239-253.

Nel, A., Xia, T., Madler, L., and Li, N. (2006), "Toxic potential of materials at the nanolevel", *Science* 311, pp.622-627.

NIST (2010), Measuring the Size of Nanoparticles in Aqueous Media Using Batch-Mode Dynamic Light Scattering, NIST - NCL Joint Assay Protocol, PCC-1 Version 1.1, http://ncl.cancer.gov/NCL_Method_PCC-1.pdf (Accessed September, 2012)

NIST (2010), Measuring the Size of Nanoparticles Using Transmission Electron Microscopy (TEM), NIST - NCL Joint Assay Protocol, PCC-7 Version 1.1, http://ncl.cancer.gov/NCL_Method_PCC-7.pdf (Accessed September, 2012)

NIST (2011), Measuring the size of colloidal gold nano-particles using high-resolution scanning electron microscopy, NIST-NCL Joint Assay Protocol, PCC-15 Version 1.1, http://ncl.cancer.gov/NCL_Method_PCC-15.pdf (Accessed September, 2012)

Nowack, B. and Bucheli, T.D. (2007), "Occurrence, behavior and effects of nanoparticles in the environment", *Environmental Pollution* 150, pp.5-22.

Oberdörster, G., Ferin, J., and Lehnert, B.D. (1994), "Correlation between particle size, in vivo particle persistence and lung injury", *Environmental Health Perspectives* 102 Suppl 5, pp.173-179.

Oberdörster, G., Sharp, Z., Atudori, V., Elder, A., Gelein, R., Lunts, A., Kreylilng, W., and Cox, C. (2002), "Extrapulmonary translocation of ultrafine carbon particles following whole body inhalation exposure of rats", *Journal of Toxicology and Environmental Health* A. 65, pp.1531-1543.

Oberdörster, G., Maynard, A., Donaldson, K., Castranova, V., Fitzpatrick, J., Ausman, K., Carter, J., Karn, B., Kreyling, W., Lai, D., Olin, S., Monteiro-Riviere, N., Warheit, D., and Yang, H. (2005), "Principles for characterising the potential human health effects from exposure to nanomaterials: elements of a screening strategy", *Particle and Fibre Toxicology* 2(8).

OECD (1995), OECD Test Guideline 107- Partition Coefficient (n-octanol/water): Shake Flask Method, OECD, Paris, http://browse.oecdbookshop.org/oecd/pdfs/free/9710701e.pdf. (Accessed in September, 2012)

OECD (2004), OECD Test Guideline 117 - Partition coefficient (n-octal/water) - high performance liquid chromatography method, OECD, Paris.

OECD (2006), OECD Test Guideline 123 - Partition Coefficient (1-Octanol/Water): Slow-Stirring Method, OECD, Paris.

OECD (2007), "OECD Guidelines for the Testing of Chemicals, Section 1: PHYSICAL-CHEMICAL Properties", www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-1-physical-chemical-properties_20745753;jsessionid=19t0jlq3uv6gl.delta (Accessed in September, 2012)

Ogura, I., Sakurai, H, and Gamo, M. (2009), "Dustiness testing of engineered nanomaterials". *Journal of Physics*: Conference Series, 170:012003.

Ohman, L.O., Lovgren, L., Hedland, T., and Sjoberg, S. (2006), "The ionic strength dependency of mineral solubility and chemical speciation in solution", Chapter 1 in Luzenkirchen, J. (Ed.), *Surface Complexation Modeling* Volume 11, pp.1-34.

Overbeek, J.Th.G. (1952), "Stability of hydrophobic colloids and emulsions", Chapter 8 In Kruyt, H.R. (Ed.), *Colloid Science*, Vol.I, Irreversible Systems, Elsevier Publishing Co., Amsterdam, Netherlands.

Pan, Y., Neuss, S., Leifert, A., Fischler, M., Wen, F., Simon, U., Schmid, G., Brandau, W., and Jahnen-Dechent, W. (2007), "Size-Dependent Cytotoxicity of Gold Nanoparticles". *Small* 3, pp.1941-1949.

Parson, D.F. (1974), "Structure of wet specimen in electron microscopy". *Science* 186, pp.407-14.

Perosh (2012), Nanodustiness Project, at www.perosh.eu/p/0B560A3BFF7F1D30C12576190043FA2B.

Peters, R., Kramer. E., Oomen, A.G., Herrera Rivera, Z.E., Oegema, G., Tromp, P.C., Fokkink, R., Rietveld, A., Marvin, H.J.P., Weigel, S., Peijnenburg, A.A.C.M., and Bouwmeester, H. (2012), "Presence of Nano-Sized Silica during In Vitro Digestion of Foods Containing Silica as a Food Additive". *ACS Nano*, 2012, 6 (3), pp 2441–2451.

Petersen, E., Huang, Q, Weber, W. (2010), "Relevance of octanol-water distribution measurements to the potential ecological update of muti-walled carbon nanotubes", *Environmental Toxicology and Chemistry* 29(5), pp.1106-1112.

PROSPEcT. (2010a), "Protocol for Nanoparticle Dispersion" at www.nanotechia-prospect.org/managed_assets/files/prospect_dispersion_protocol.pdf (Accessed in September, 2012)

PROSPEcT. (2010b), "Guidelines and Protocols for Sampling" atwww.nanotechia-prospect.org/managed_assets/files/prospect_sampling_protocol.pdf (Accessed in September, 2012)

Richman, E. K., and Hutchison, J. E. (2009), "The Nanomaterial Characterisation Bottleneck". *ACS Nano* 3 (9), pp.2441-2446.

Ross, S. and Morrison, I.D. (1988), Colloidal Systems and Interfaces, John Wiley and Sons, New York, New York, USA.

Sanders,K.., Degn,L.L., Mundy,W.R., Zucker,M.R., Dreher,K., Zhao,B., Roberts, J.,E., and Boyes, W.K.(2012), "In Vitro Phototoxicity and Hazard Identification of Nano-scale Titanium Dioxide", *Toxicology and Applied Pharmacology* 258, pp. 226–236

Shaw, D.J. (1992), Introduction to Colloid and Surface Chemistry4th Edition, Butterworth Heinemann Publishers, Oxford, United Kingdom.

Singh, C. et al. (2011), NM-Series of Representative Manufactured Nanomaterials - Zinc Oxide NM-110, NM-111, NM-112, NM-113 Characterisation and Test Item Preparation, JRC Scientific and Technical Report, European Union, Luxembour.

Stahlmecke, B., Wagener, S., Asbach, C., Kaminski, H., Fissan, H., and Kuhlbusch, T.A.J.(2009), "Investigation of airborne nanopowder agglomerate stability in an orifice under various differential pressure conditions", *Journal of Nanoparticle Research* 11(7), pp.1625-1635.

Stumm, W. and Morgan, JJ. (1981), Aquatic Chemistry, 2nd Edition, John Wiley and Sons, New York, New York.

Suna, B., Ranganathana, B. and Feng, Si-Shen. (2008), "Multifunctional poly (D,L-lactide-co-glycolide)/montmorillonite (PLGA/MMT) nanoparticles decorated by Trastuzumab for targeted chemotherapy of breast cancer". *Biomaterials* 29, pp.475-86.

Suresh, A. K., Pelletier, D.A., Wang, W., Morrell-Falvey, J.L., Gu, B., and Doktycz, M.J. (2012), "Cytotoxicity Induced by Engineered Silver Nanocrystallites is Dependent on Surface Coatings and Cell Types". *Langmuir*7 28(5), pp.2727-35.

Techane, S., Baer, D.R and Castner, D.G. (2011), "Simulation and modelling of self-assembled monolayers of carboxylic acid thiols on flat and nanoparticle gold surface*", Analytical Chemistry* 83, 6704-12.

Taurozzi J.S., Hackley V.A. and Wiesner M.R. (2012a), "Preparation of Nanosclae TiO2 Dispersions in an Environmental Matrix for Eco-Toxicological Assessment", Ver.1.1. CEINT/NIST Protocol

Taurozzi J.S., Hackley V.A. and Wiesner M.R. (2012b), "Preparation of a Nanoscale TiO2 Aqueous Dispersion for toxicological or Environmental Testing", Ver.1.2. CEINT/NIST Protocol

Taurozzi J.S., Hackley V.A. and Wiesner M.R. (2012c), "Reporting guidelines for the Preparation of Aqueous nanoparticle dispersions from dry materials", Ver.2.1. CEINT/NIST Protocol

Teeguarden, J.G., Hinderliter, P.M., Orr, G., Thrall, B.D., and Pounds, J.G. (2007), "REVIEW Particokinetics In Vitro: Dosimetry Considerations for In Vitro Nanoparticle Toxicity Assessments", *Toxicological Sciences* 95, pp.300-312.

Test Guidelines to Manufactured Nanomaterials: Recommendations from the ORD PHYSICAL-CHEMICAL Properties Workgroup, U.S. EPA internal report submitted to the U.S. EPA Office of Prevention, Pesticides and Toxic Substances.

U.S. EPA. (2007a), Nanotechnology White Paper. U.S. Environmental Protection Agency, Science Policy Council, Washington, DC. EPA 100/B-07/001.

U.S. EPA. (2007b), at www.epa.gov/opptsfrs/publications/OPPTS_Harmonised/830_Product_ Performance_Test_Guidelines/index.html (Series 830 Product Performance Test Guidelines–Finals)

Utterback, D., Loux, N., Hatto, P., Veronesi, B., Su, Y.S., Tolaymat, T., Diamond, S., Winchester, E., and Savage, N. (2008), *Final Report: Applicability of OPPTS 830 Series and OECD 100 Series Harmonised*

Van Doren E., De Temmerman P-J., Francisco M. and Mast J. (2011), "Determination of the volume-specific surface area by using transmission electron tomography for characterization and definition of nanomaterials". *Journal of Nanobiotechnology* 9(1), pp.17.

Visser, J. (1972), "On Hamaker Constants: A comparison between Hamaker constants and Lifshitz-Van der Waals constants". *Advances in Colloid and Interface Science* 3, pp.331-363.

Walkey, C. D., Olsen, J. B., Guo, H., Emili, A. and Chan, W. C. W. (2011), "A Combination of Nanoparticle Size and Surface Chemistry Determines Serum Protein Adsorption and Macrophage Uptake". *Journal of the American Chemical Society* 134(4), pp.2139-47.

Wang, X., Xia, T., Ntim, S. A., Ji, Z., George, S., Meng, H., Zhang, H., Castranova, V., Mitra, S., and Nel, A.E. (2010), "Quantitative techniques for assessing and controlling the dispersion state and biological effects of multi-walled carbon nanotubes in mammalian tissue culture cells", *ACS Nano*, 4(12), pp. 7241-7252.

Warheit, D.B., Borm, P.J.A., Hennes, C. And Lademann, J. (2007), "Testing strategies to establish the safety of nanomaterials: Conclusions of an ECETOC workshop", *Inhalation Technology* 19, pp.631-643.

Wittmaack, K. (2011a), "Eccessive delivery of nanostructured matter to submersed cells caused by rapid gravitational settling", *ACS Nano* 5(5), pp. 3766-3778.

Wittmaack, K. (2011b), "Novel dose metric for apparent cytotoxicity effects generated by in vitro cell exposure to silica nanoparticles", *Chemical Research in Toxicology* 24, pp.150-58.

Xiao, Y and Wiesner M. (2012), "Characterization of surface hydrophobicity of engineered nanoparticles", *Journal of Hazardous Materials* 215-216, pp.146-151.

Yang, D-Q., Rochette, J.F., and Sacher, E. (2005), "Controlled Chemical Functionalization of Multiwalled Carbon Nanotubes by Kiloelectronvolt Argon Ion Treatment and Air Exposure", *Langmuir* 21, pp.8539-45.

Zhu, S., Oberdorster, E., and Haasch, M.L. (2006), "Toxicity of engineered nanoparticle (fullerene, C60) in two aquatic species, Daphnia and fathead minnow", *Marine Environmental Research* 62 Suppl, pp.S5-S9.

Zorn, G., Dave, S. R., Gao, X. and Castner, D.G. (2011), "Method for determining the elemental composition and distribution in semiconductor core-shell quantum dots", *Analytical Chemistry* 83, pp.866-873.

B. GUIDANCE ON PREPARING SAMPLES OF NANOMATERIAL IN EXPOSURE MEDIA FOR ECOTOXICITY STUDIES

B.1 Introduction

82. There is currently no broad consensus on the best approaches for preparing nanomaterial samples in media for ecotoxicity studies. However, recent publications describe evaluations of preparation methods for specific nanomaterials (e.g. nano-silver), suggested operating procedures, and the use of natural dispersants rather than pure solvents as dispersion or suspension agents (El Badawy 2010, Allen 2010, Kennedy *et al.,* 2008). Review of the literature also reveals that a wide range of suspension methods continue to be used and include use of strong solvents (e.g. tetrahydrofuran, THF), dispersion or stabilising agents (e.g. TWEEN^{TM}, citrate, etc.), bath or probe ultrasonication, stirring for a broad range of time periods, bead milling, etc (Klaine *et al*., 2008, Handy *et al*., 2008, Hund-Rinke *et al*., 2010, Handy *et al*., 2011). This observation is confirmed by a recent survey of international nanomaterial researchers completed by Environment Canada (based on preliminary summary of responses, January 2012). Adding to the difficulty in evaluating these various methods is the evidence that some solvents may alter nanomaterial properties and toxicity (Smith *et al*., 2007) or even be toxic themselves (Henry *et al*., 2007). Moreover, in most cases, characterisation of the tested nanomaterials has been limited to the working or stock dispersions, rather than in the post-dilution exposure media or directly in the test vessels. There is also some probability that the methods reported might not produce similar results for all nanomaterials, or forms of a specific nanomaterial, for example anatase dominated or rutile dominated titanium dioxide, or surface and non-surface treated titanium dioxide. Finally, these evaluations will, in some cases, be strongly dependant on the characterization methods used, e.g. DLS might give different answers compared to TEM or other microscopy approaches. For all of these reasons, preparation of exposure media for ecotoxicity testing cannot yet be highly standardized and should continue to be viewed as experimental.

83. Variability in nanomaterial properties, most notably agglomerate or aggregate size, has been shown to depend consistently and significantly on media pH, ionic strength, and concentration and form of dissolved organic matter (El Badawy *et al*., 2010, Domingos *et al*., 2009 and French *et al*., 2009). Even within the narrow dissolved ion range of 3.4 to 13.3 mmol/L, well within the range typical of freshwaters, agglomerate or aggregate size can vary two to three fold (French *et al*., 2009, Stolpe and Hassellöv, 2007). Because total surface area for a given volume of material increases as a square function of decreasing particle size, and the interaction of nanomaterials and biotic systems occur at the particle surface, these ionic strength effects have clear implications for exposure in ecotoxicity testing of nanomaterials. For dissolved organic matter (DOM), the extensive literature describing its interactions with chemical contaminants (e.g. metals) suggests that nanomaterial properties, fate, and toxicity might be strongly affected by these substances (Chen *et al*., 2011). For example, DOM has been shown in several studies to stabilise particles in suspension, and to reduce the agglomeration or aggregation phenomena (Hyung *et al*., 2007, Loux and Savage 2008). The interaction of nanomaterials and DOM might also alter bioavailability and rates of uptake by test organisms (Duval and Qian, 2009). It is also important to recognise that DOM itself is highly variable among water sources, is difficult to characterize, and can contain a broad range of aromatic, lipid, protein, and other constituents; all factors that are likely to produce variation in their effects on nanomaterials.

84. An additional concern is the degradation of nanomaterials during testing, most importantly the release of soluble species (e.g. ions) from solid surfaces. This is particularly important where soluble

species (e.g. ions released) are especially toxic, or in close proximity to potential biological targets, as is the case with silver (Ag^+) ions released from silver nanoparticles. Ionicsilver is highly toxic to aquatic species, with effect levels in the low μg/L range; therefore even relatively slow release of Ag^+ from silver nanoparticles can results in toxic levels of these species. Degradation of both nano-scale silver and ZnO_2 has been well-documented (Allen *et al*., 2010, Kennedy *et al*., 2010, Liu and Hurt 2010, Franklin *et al*., 2007) and is likely an issue for nano-scale copper (Heilaan *et al*., 2008).

85. Control and measurement of these factors is highly desirable to assure that outcomes of research projects, i.e. data are as consistent and comparable as possible. Every effort should be made to quantify these factors at time intervals sufficient to fully describe exposure conditions, most importantly their effect on bulk concentration and particle size over the duration of testing. Where possible, it is also suggested that additional efforts be made to determine how these factors affect particle behaviour and properties. For example, simple beaker tests might be done over a range of pH values typical for a test system to quantify the effects on particle size (*See also the Guidance Manual for the Testing of Manufactured Nanomaterials: OECD's Sponsorship Programme: First Revision ENV/JM/MONO(2009)20/REV*). In the absence of broadly applicable methods for producing exposure media for biotic effects assessment, the following advice is generally intended to minimise intra- and inter-laboratory variability within sponsorship groups.

86. Finally, it is strongly suggested that where research involves both ecotoxicity testing and environmental behaviour, degradation, transformation, and bioaccumulation (Section C of this document) common methods for media preparation be used. This approach will maximize comparability and integration of exposure and effects studies by increasing the probability that nanomaterials have similar suspension properties, at least at the initiation of each type of test. There also may be circumstances where well-vetted methods for media preparation exist for fate-related tests, but not for ecotoxicity tests. Due to the variability and uncertainty in the real exposure levels, even under controlled laboratory conditions, measuring and reporting exposure in terms of internal doses and/or body burdens at the end of the exposure period should be considered as a complementary option. Section C. 2.3 Bioaccumulation offers recommendations for measuring the level of nanoparticles or its transformation products in biota.

B.2 Aquatic Media Preparation

B.2.1 Methods of suspension

87. Dispersion of nanomaterial might include stirring, sonication, grinding, use of solvents, and stabilising agents. The advantages and disadvantages of these methods are outlined in Crane *et al*. (2008) and Handy *et al*. (2011). This suggested that some nanomaterials are significantly altered by sonication and grinding (e.g. carbon nanotubes can be shortened, coatings can be removed, surfaces can be hydroxylated, etc.) and that the interaction of solvents with some nanomaterials might result in toxic by-products. The method of dispersion will also depend on the specific material to be tested and whether or not it has been surface treated. Best scientific judgment should be used in selecting the methods, and where there is evidence or an indication that a dispersion method might significantly alter toxicity, those effects should be controlled or quantified. The general goal of dispersion efforts is to produce consistent particle sizes with predictable polydispersity. It is unlikely that these properties will be stable over renewal periods (or the duration of a test); rather, the goal should be to have a repeatable initial test medium, and to monitor property changes with sufficient frequency to quantify these changes. In some cases, in order to remove the larger particles, filtration with 0.45 μm or 0.22 μm filters might be employed (e.g. Ma and Bouchard, 2009). This approach should be altered where information is available concerning environmentally relevant particle sizes. This might include manufacturer information on specific sizes produced and incorporated into products, and unlikely to undergo post-production processes that might alter particle size or distribution. It is assumed that tests will be conducted using periodic renewal approaches to avoid expense

and waste production. Test media quality (pH, ionic strength, DOM concentration) should be harmonised as much as possible between comparative studies. One source of Standard Operating Procedures is the US Center for the Environmental Implications Nanotechnology (CEINT). In collaboration with the US National Institute for Standards and Technology (NIST), SOPs for sonication and suspension of TiO_2 have been made available (CEINTa-d, http://www.ceint.duke.edu/research/transport-and-transformations, last accessed 07 November, 2011).

B.2.2 Media quality

88. It is essential that pH, ionic strength, and DOM concentrations be quantified and made as uniform as possible among tests (and replicates), endpoint measurements, and laboratories. Careful consideration should be given to assuring that physical-chemical properties determinations are representative of all other test conditions, e.g. a full suite of physicochemical determination of a material in de-ionised water are very unlikely to accurately predict many of those values in high ionic strength media. Accordingly, it is recommended that physico-chemical characterisation be made in the actual test media, whenever possible, (Antunovic *et al*., 2011). Media quality determinations should be made at intervals sufficient to determine their variability, both in stock solutions and in test media. Typically, intervals described (and the methods used) in current guidelines should be sufficient, however, more determinations should be made where there is evidence or indication of increased variability, perhaps due to tested nanomaterials, e.g. interaction of DOM with fullerenes or carbon nanotubes. See also the paragraph 87.

B.2.3 Physical-Chemical Characterisations

89. It is likely that agglomeration will occur during most toxicity tests and will alter and likely reduce test organism exposure either due to reduced particles counts, surface area, or loss of bulk concentration. For this reason, particle and/or agglomerate size distribution and material concentration should be assessed at intervals sufficient to quantify exposures. Because few nanomaterial studies have measured these parameters across dilution series or at intervals during exposure, it is difficult to prescribe a specific approach. However, at a minimum and where possible, these determinations should be made immediately prior to, and after, media renewal. It is also desirable to measure particle/aggregate/agglomerate size distribution using two or more methods, e.g. dynamic light scattering and SEM, TEM (possibly cryo-TEM), and other microscopy techniques. Where possible, it is also suggested that the size-determination methods differ in the approach used, e.g. DLS measures size based on hydrodynamic diameter and electrophoretic mobility (and can bias size determination due to charge layering), whereas microscopy provides for direct observation and visual measurement of physical size, but limits the size of particle population that can be measured. [See section A.1.1 regarding comparability of results using different methods and annex III *of the Guidance Manual for the Testing of Manufactured Nanomaterials: OECD's Sponsorship Programme ENV/JM/MONO(2009)20*]. These confirmatory measurements might be made in preliminary studies with the test media, or in a subset of tests or treatment levels to establish the comparability of measurement techniques. These measurements should also be taken in media in the presence of test organisms (and food when feeding is required) as they will likely alter particle behaviourconsiderably... Finally, it is recognised that many physicochemical properties cannot be determined in wet media, most notably surface area, which relies upon dry samples. Refer to Section A of this document for more in-depth discussion of characterization approaches.

B.2.4 Reporting results for media preparation approaches

90 bis. It is important that results of various media preparation approaches be reported in detail. Negative results, i.e. cases where preparation methods lead to excessive agglomeration or complete failure in exposure, are as important as positive results in providing the basis for future specific guidance for testing.

B.3 Non-Aqueous Media Preparation

B.3.1 Method of nanomaterial introduction

90. This section covers media preparation for all non-aqueous tests, including sediment (e.g. OECD TG 218), soil (e.g. OECD TG 222), dung (e.g. OECD TG 228), and direct application (e.g. OECD TG 214) testing. Given the lack of methods for detecting or quantifying many nanomaterials in these more complex media, these tests will necessarily be relatively more exploratory compared with aquatic testing. Materials may be delivered to test media in the form of water-based dispersions or mixed as dry material. An advantage of using wet suspensions is that the starting point for material addition can be made uniform between aquatic and sediment or soil testing. If the nanomaterial is introduced and homogenised directly in solid form to the media, care should be taken in homogenisation so that the test material is not unintentionally damaged, and detailed description of the methods should include homogeniser type, speeds or force applied, and duration. In either wet or dry application, the problem of characterizing materials in complex media such as soil, sediment, or sludge, has yet to be resolved. However, one exception is extraction and analysis of SWCNTs using solvents and near infrared fluorescence spectroscopy (Schierz *et al*., 2011). TEM has also been used to characterize materials in solid test matrices (e.g. Kool *et al*., 2011).

B.3.2 Media quality

91. All of the media quality issues discussed for aquatic tests apply mutatis mutandi as well to preparation of dispersions for delivery to other media. In addition, all guidance on characterising these media described in appropriate OECD test guidelines should be followed. Best scientific judgment should be used in determining whether nanomaterial testing might require additional or more frequent measurement. All efforts should be made to minimise variation in these media variables between tests and sponsor laboratories. One approach to address this issue is to homogenise and distribute natural media among all researchers, or to use a single batch of laboratory-constructed media, following test guideline recipes and procedures.

B.3.3 Physical-Chemical Characterisations

92. It is recognised that methods for many physical-chemical properties, most importantly, particle size, have yet to be developed for complex media. However, where possible, and using best scientific judgment, methods for doing so should be investigated, e.g. identifying and perhaps measuring carbon nanotubes using microscopy techniques. Where methods exist for digesting or extracting materials for determination of bulk concentration, these measurements should be made. The intervals for such measurement should be sufficient to document accurate and consistent delivery of materials to test media.

B.3.4 Reporting results for media preparation approaches

93. It is important that results of various media preparation approaches be reported in detail. Negative results, i.e. cases where preparation methods lead to excessive agglomeration or complete failure in exposure, are as important as positive results in providing the basis for future specific guidance for testing.

B.4 References

Allen, H.J., Impellitteri, C.A., Macke, D.A., Heckman, J.L., Poynton, H.C., Lazorchak, J.M., Govindaswamy, S., Roose, D.L., and Nadagouda, M. (2011), “Effects from filtration, capping agents,

and presence/absence of food on the toxicity of silver nanoparticles to Daphnia magna", *Environmental Toxicology and Chemistry* 29, pp.2742–2750.

Andrievsky, G.V., Kosevich, M.V., Vovk, O.M., Shelkovsky, V.S., and Vashenko, L.A. (1995), "On the production of an aqueous colloidal solution of fullerenes", *Journal of the Chemical Society, Chemical Communications* 12, pp.1281–1282.

Antunović, B., Barlow, S., Chesson, A., Flynn, A., Hardy, A., Jany, K-D., Jeger, M-J., Knaap, A., Kuiper, H., Larsen, J-C., Lovell, D., Noerrung, B., Schlatter, J., Silano, V., Smulders, S. and Vannier, P. (2011), "Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain", *EFSA Journal* 9, pp.2140.

Brant, J.A., Labille, J., Bottero, J-Y., and Wiesner, M.R. (2006), "Characterising the impact of preparation method on fullerene cluster structure and chemistry", *Langmuir* 22, pp.3878–3885.

Brant, J., Lecoanet, H., Hotze, M., and Wiesner, M. (2005), "Comparison of electrokinetic properties of colloidal fullerenes (n-C60) formed using two procedures", *Environmental Science and Technology* 39, pp.6343–6351.

CEINT/ NIST (a). Protocol for Preparation of Nanoparticle Dispersions from Powdered Material Using Ultrasonic Disruption, Ver.1.1, www.ceint.duke.edu/sites/ceint.duke.edu/files/NIST.SP_.1200-2.pdf .(Accessd on 07 November, 2011)

CEINT/ NIST (b). Reporting Guidelines for the Prepartion of Aqueous Nanoparticle Dispersions from Dry Nanomaterials, Ver.2.1, www.ceint.duke.edu/sites/ceint.duke.edu/files/NIST.SP_.1200-1.pdf .(Accessed in September, 2012).

CEINT/ NIST (c). Protocol for Preparation of Nanoscale TiO2 Aqueous Dispersion for Toxicological or Environmental Testing, Ver.1.2, www.ceint.duke.edu/sites/ceint.duke.edu/files/NIST.SP_.1200-3.pdf. (Accessed in September, 2012)

CEINT/ NIST(d). Protocol for Preparation of Nanoscale TiO2 Dispersions in Biological Test Media for Toxicological Assessment, Ver.1.1, www.ceint.duke.edu/sites/ceint.duke.edu/files/NIST.SP_.1200-3.pdf. (Accessed in September, 2012)

Crane, M., Handy, R.D., Garrod, J., and Owen, R. (2008), "Ecotoxicity test methods and environmental hazard assessment for engineered nanoparticles", *Ecotoxicology* 17(5), pp.421-37.

Domingos, R.F., Tufenkji, N., and Wilkinson, K.J. (2009), "Aggregation of titanium dioxide nanoparticles: Role of fulvic acid", *Environmental Science and Technology* 43, pp.1282-1286.

El Badawy, A.M., Luxton, T.P., Silva, R.G., Scheckel, K.G., Suidan, M.T., and Tolaymat, T.M. (2010), "Impact of environmental conditions (pH, ionic strength, and electrolyte type) on the surface charge and aggregation of silver nanoparticles suspensions", *Environmental Science and Technology* 44: pp.1260–1266.

EPA (2010), "State of the Science Literature Review: Everything Nanosilver and More", Sientific, Technical Research, Engineering and Modeling Support Final Report, Contract No. EP-C-05-057 Task Order No. 95, Washington, D.C.

Franklin, N.M., Rogers, N.J., Apte, S.C., Batley, G.E., Gadd, G.E., and Casey, P.S. (2007), "Comparative Toxicity of Nanoparticulate ZnO, Bulk ZnO, and ZnCl2 to a Freshwater Microalga

(*Pseudokirchneriella subcapitata*): The Importance of Particle Solubility", *Environmental Science and Technology* 41 (24), pp. 8484–8490.

French, R.A., Jacobson, A.R., Kim, B., Isley, S.L., Penn, R.L., and Baveye, P.C. (2009), "Influence of ionic strength, pH, and cation valence on aggregation kinetics of titanium dioxide nanoparticles", *Environmental Science and Technology* 43,pp.1354–1359.

Handy, R.D., Cornelis, G., Fernandes, T., Tsyusko, O., Decho, A., Sabo-Attwood, T., Metcalfe, C., Steevens, J.A., Klaine, S.J., Koelmans, A.A., and Horne, N. (2012), "Ecotoxicity test methods for engineered nanomaterials: Practical experience and recommendations from the bench", *Environmental Toxicology and Chemistry* 31, pp.15–31.

Handy, R.D., Kammer, F., Lead, J.R., Hassellöv, M., Owen, R., and Crane, M. (2008), "The ecotoxicology and chemistry of manufactured nanoparticles", *Ecotoxicology* 17, pp.287-314.

Heinlaan, M., Ivask, A., Blinova, I., and Dubourguier, H-C. (2008), "Toxicity of nanosized and bulk ZnO, CuO and TiO2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus"*, *Chemosphere* 71, pp.1308–1316.

Henry, T.B., Menn, F., Fleming, J.T., Wilgus, J., Compton, R.N., and Sayler, G.S. (2007), "Attributing effects of aqueous C60 nano-aggregates to tetrahydrofuran decomposition products in larval zebrafish by assessment of gene expression", *Environmental Health Perspectives*115, pp.1059-1065

Hund-Rinke, K., Schlich, K., and Wenzel, A. (2010), "TiO2 nanoparticles - Relationship between dispersion preparation method and ecotoxicity in the algal growth test", *Umweltwiss Schadst Forsch* 22, pp.517 - 528.

Hyung, H., Fortner, J.D., Hughes, J.B., and Kim, J-H. (2007), "Natural organic matter stabilises carbon nanotubes in the aqueous phase", *Environmental Science and Technology* 41, pp.179–184.

Kennedy, A.J.H., Hull, M.S., Steevens, J.A., Dontsova, K.M., Chappell, M.A., Gunter, J.C., and Weiss, C.A. Jr. (2008), "Factors influencing the partitioning and toxicity of nanotubes in the aquatic environment", *Environmental Toxicology and Chemistry* 27, pp.1932–1941.

Kennedy, A.J., Hull, M.S., Bednar, A.J., Goss, J.D., and Gunter, J.C. (2010), "Fractionating nanosilver: importance for determining toxicity to aquatic test organisms", *Environmental Science and Technology* 44, pp. 9571-9577.

Klaine, S.J., Avarez, P.J.J., Batley, G.E., Fernandes, T.F., Handy, R.D., Lyon, D.Y., Mahendra, McLaughlin, M.J., and Lead, J.R. (2008), "Nanomaterials in the environment: Behavior, fate, bioavilability, and effects", *Environmental Toxicology and Chemistry* 27, pp.1825–1851.

Liu, J., and Hurt, R.H. (2010), "Ion release kinetics and particle persistence in aqueous nano-silver colloids". *Environmental Science and Technology* 44, pp. 2169-2175.

Loux, N.T., and Savage, N. (2008), "An assessment of the fate of metal oxide nanomaterials in porous media", *Water, Air, and Soil Pollution* 194, pp.227-241.

Ma, X., and Bouchard, D. (2009), "Formation of aqueous suspensions of fullerenes", *Environmental Science and Technology* 43, pp.330-336.

Schierz, P., Parks, A.N., and Ferguson, P.L. (2011), *Abstract: Novel Approaches for the Detection of Single Walled Carbon Nanotubes in Environmental Matrices,* Society for Environmental Toxicology and Checmistry, North America 32nd Annual Meeting.

Smith, C.J., Shaw, B.J., and Handy, R.D. (2007), "Toxicity of single-walled carbon nanotubes on rainbow trout (Oncorhyncus mykiss): Respiratory toxicity, organ pathologies, and other physiological effects", *Aquatic Toxicology* 82, pp.94-109

Stolpe, B., and 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, pp. 3292–3301

C. GUIDANCE ON PREPARING NANOMATERIAL SAMPLES FOR DEGRADATION, TRANSFORMATION AND ACCUMULATION STUDIES

C. 1 Introduction

94. This part presents a scan of the existing published literature on environmental behaviour and fate studies.

C. 1.1 Environmental behaviour

95. Regarding the currently tested nanomaterials it is highly likely that they will not stay dispersed in **natural freshwaters** (Klaine *et al.*, 2008). The tendency to undergo agglomeration or aggregation is however dependent on so many parameters that particles need to be tested for their agglomeration or aggregation behaviour until methods are available which are able to assist or replace the testing by modelling based on first principles. However, there have also been recent indications that nanomaterial agglomerates or aggregates may stay dispersed in other aquatic conditions and in high concentrations of OM some nanomaterial can de-agglomerate. Moreover also the settled nanoparticles attached to the suspended particulate matter may not be deposited but will travel along with the other particulate material e.g. to river estuaries. Depending on their chemistry and the receiving environment, interaction between the nanomaterials and the natural organic matter (NOM, which may enhance agglomeration, and thus sedimentation, or lead to dispersion) and sediment is likely. Depending on the nanomaterial type and receiving environment, the interaction with NOM may lead to enhanced (e.g. Hyung *et al*., 2007) or reduced (Baalousha *et al*., 2008) dispersibility and hence possible bioavailability for pelagic organisms.

96. The fate of nanomaterials in **the marine environment** is likely to be characterised by enhanced agglomeration and thus sedimentation (Klaine *et al.*, 2008, Stolpe and Hassellöv 2007), due to the high ionic strength of seawater. Although bioavailability could be diminished, it is possible that biological systems may become clogged by e.g. agglomerated nanomaterial and other particles, and thus their activity impaired (e.g. Nielsen *et al*., 2008). This considers only nanoparticles without specific surface functionalization and only possible electrostatic interaction based on surface charge and the variation of the surface charge by interaction with e.g. NOM. Non-charge effects as steric/entropic stabilisation through e.g. polymers attached to the surface, will make the dispersion stability more independent from simple electrostatic effects, hence more independent from ionic strength or the presence of NOM. It is not yet clear if those NPs particle types will be prone to quick aggregation in e.g. salt water / marine conditions. Reactions like bridging flocculation caused by e.g. natural polysaccharides, seem to be very effective (more effective than simple increase in ionic strength) and still not quantitatively understood for complex systems such as **the marine environment**. High energy environmental and biological processes, e.g. waves and turbulent stream conditions or flow through the fish opercula-gill system may re-suspend the nanomaterials and affect the agglomeration status and bioavailability.

97. Aggregation of nanomaterials and their tendency to concentrate at interfaces violate the assumption that the test substance has free diffusion through the phases, making log K_{OW} difficult to determine in practice. Jafvert and Kulkarni (2008) have studied the octanol-water partition coefficient (log K_{OW}) of fullerene (C_{60}) and its aqueous dispersability. They obtained a value for log K_{OW} of 6.7, and a value for the solubility of C_{60} in water-saturated octanol of 8 ng/L. Hence based on this high K_{OW}- value, it is expected that C_{60} has high affinity for lipids and organic matter. This indicates that in the natural

environments, C_{60} will tend to adsorb to solid phases. C_{60} behaves like an intermediate: partly as nanoparticle, partly as a large organic molecule. Hence some of the classical techniques (like a toluene extraction and LC-MS) work for C_{60}. Despite its hydrophobic characteristic, fullerene C_{60} has been dispersed in water by stirring or sonicating or via solvent exchange (e.g. Pycke *et al*., 2011; Ma and Bouchard, 2009). The fullerene agglomerates are able to form stable water suspensions (nC_{60} or aqu/C_{60}; e.g. Isaacson *et al*., 2011; Scharff *et al*., 2004).

98. Some modelling work has been published for titanium oxide (TiO_2), silver nanoparticles and carbon nanotubes (Mueller and Nowack, 2008; Boxhall *et al*., 2007, Gottschalk *et al*., 2010, Arvidsson *et al*., 2011, Quick *et al*., 2011). However, current knowledge of the behaviour of nanoparticles in natural waters does not provide sufficient basis for the full assessment of environmental exposure concentrations or amounts of dispersed nanomaterials. Many of these early modelling studies were only based on estimates and calculations and not on environmental measurements of nanomaterial content.

99. In terrestrial systems some nanomaterials may preferentially bind to NOM and thus become less bioavailable (e.g. Li *et al.,* 2008), although sediment and soil ingesters (e.g. oligochaetes) may be able to take up these nanomaterials. In fact they may preferentially ingest nanomaterial if they are associated with NOM, e.g. see Roberts *et al.* (2007) and they may be released and bound to the tissues (strip de-associate) within the gastrointestinal tract (GIT). The binding to decaying organic matter, detritus and the associated microorganisms may facilitate the dietary exposure of detritivorous and omnivorous organisms. The organisms selected for toxicity tests should address both types of exposure, i.e. in liquid matrix (in dispersion/solution) or associated to solid matrix (particle bound). It would be preferable to know the distribution of nanomaterial and the relevant type of exposure in advance.

100. Determining the agglomeration/aggregation and sorption characteristics of the nanomaterials can provide valuable information when developing new test guidelines, using existing test guidelines with modifications or interpreting the results from existing test guidelines. Comprehensive tests to assess the agglomeration behaviour of nanomaterial in natural waters are currently only describing the agglomeration of the nanomaterial with themselves, however these tests reveal some general behavioural characteristics, which could be proven to describe quite well the real-worlds behaviour (Kammer *et al*., 2010; Ottofuelling *et al.,* 2011). Also the potential capacity of nanomaterials to adsorb substances and work as toxicant carriers should be taken into account regarding the environmental behaviour of nanomaterials. In fact for nanomaterial to become relevant carriers for toxicants is relatively unlikely since some critical prerequisites must be met. One is the mobility of the particle in surface-, ground- and seepage waters, another is the strong binding of the toxicant to the nanomaterial and finally the nanomaterial must be present in a relevant concentration, compared to other particles which compete for the binding of the toxicant (Hofmann and Kammer 2009).

101. Methods for environmental analyses are now in development for various materials and environmental matrices (Hassellöv *et al*., 2008) and these methods could provide the basis for environmental fate testing. (See also Section V, part A of the document)

C. 1.2 Degradation and transformation

102. Degradation, transformation and persistence of nanomaterials in the environment depend on their chemical composition, of both core and surface material as well as environmental conditions including pH, natural organic matter, ionic concentration and composition and content of dissolved oxygen. It is likely that most nanomaterials which are currently available will stay in their original particulate form, though levels of agglomeration/aggregation can be expected to be different. Some nanomaterials might have biocidal effects on microorganisms and hence affect the biodegradation. There is lack of data in this area, although, the organic coatings could be biodegraded or transformed by environmental factors. Hartmann *et*

al. (2011) have studied degradability of aged aquatic suspensions of C_{60} nanoparticles. They observed no biodegradation of C_{60} in 28 days. However, when additional organic substrate (sodium acetate) was added, C_{60} nanoparticles (20 mg/L) did not inhibit the biodegradation as this other substrate which was completely mineralized.For metal containing particles, the UN GHS protocol for dissolution/transformation of metals and sparingly soluble metal compounds can be adapted to determine the rate and extent to which metallic nanomaterials can produce soluble available ionic and other metal-bearing species in aqueous media under a set of standard laboratory conditions representative of those generally occurring in the environment.

C. 1.3 Bioaccumulation

103. Current work assessing uptake has focussed on exposures in media with different nanomaterial loads over a specific time interval, followed by total body burden assessment, especially if individuals are small, such as *Daphnia* species, copepods, *Lumbriculus* or *Eisenia* species (Roberts *et al.*, 2007, Fernandes *et al.,* 2007a, Petersen *et al.*, 2008). For larger organisms, specific studies have focussed on detection, e.g. by electron microscopic methods, of loads within specific organs, such as liver, kidney, muscle, gills (e.g. titanium in trout, Federici *et al*., 2007). In terms of detection, it may not always be possible to identify the form of such material. This may be particularly important for materials that tend to transform and/or dissolve readily such as silver or for nanomaterial of chemical compositions based on elements that are already highly abundant in the environment or in the media used for exposure tests (e.g. Zn, Cu, etc).

104. The first step in the uptake and possible accumulation of a substance, at least in the aquatic environment, is often the adsorption and attachment and possible accumulation of the material onto the surface of the organism (Handy and Eddy 2004; Fernandes *et al.,* 2007b, Nielsen *et al.,* 2008). This has also been shown by the agglomeration of single wall carbon nanotubes on the gill mucus of rainbow trout (Smith *et al*., 2007).

105. As first generation nanomaterials tend to follow colloidal chemistry and colloids can eventually agglomerate, these nanomaterial agglomerates will end up in the sediments (Klaine *et al*., 2008). Thus, bioaccumulation studies on sediment organisms (e.g. OECD TG 317) would be especially important.

106. Petersen *et al.* (2008) have indicated that carbon nanotubes (CNTs) were not readily accumulated by the earthworm *Eisenia foetida* with results indicating bioaccumulation factors 2 orders of magnitude smaller than those measured for pyrene. Lee *et al.* (2008) have detected bioaccumulation of insoluble copper nanoparticles in cells of emerging and growing plants when tested on agar plates.

107. Not much work has been published on potential food chain effects of nanomaterials, although fish that drink water containing nanomaterials show gut pathology (Federici *et al*., 2007; Smith *et al*., 2007). A study (Holbrook *et al*., 2008) on the possible transfer of quantum dots in a simplified aquatic food chain has found that these materials can be transferred to rotifers through dietary uptake of ciliated protozoans. Although there was transfer across these levels, bioconcentration (accumulation from surrounding environment) in the ciliates was limited and no biomagnification (enrichment across trophic levels) in the rotifers was detected. This study indicates potential for transfer across food chain levels but this would depend on material type and food chain, as it is mostly the case for other studies of chemicals. Also Fortner *et al.* (2005) have observed that fullerene nanoparticles accumulate in microbial cells, in worms eating those microbes and possibly in animals higher up the food chain. The bioaccumulation assessment should consider the special uptake pathways, such as endocytosis (Iversen *et al*., 2011).

C. 2 Test Method Applicability and Dosimetry

108. Generally, it should be noted that when research and testing involves both ecotoxicology and environmental behaviour, degradation, transformation, and especially bioaccumulation, harmonized methods for media preparation are recommended. This approach will maximize comparability and integration of exposure and effects studies by increasing the probability that nanomaterials have similar suspension properties, at least at the initiation of each type of test. There also may be circumstances where well-vetted methods for media preparation and material detection exist for fate-related tests, but not for ecotoxicity tests.

C. 2.1. Environmental behaviour

C. 2.1.1 Methods

109. It is likely that the OECD test methods for a number of physical-chemical properties for environmental distribution are applicable, and their applicability has been assessed (OECD 2009). However, there is also a lack of several methods which can address nano-relevant physical-chemical endpoints or measurands (see Section V, part A of this document). Furthermore, as with other test methods, dosage for the testing and the detection, analysis and quantification of the nanomaterials are the most challenging issues. Methods for the characterisation of key properties of nanomaterials have been identified in several publications (e.g. Klaine *et al*., 2008, Hasselov *et al*., 2008, Tiede *et al*., 2008).

Dispersion and solubility

110. Dispersion and possible solubility/transformation of nanomaterials are important properties that have been already addressed in the general part of this document (section 3). It is unclear to date to what extent can the effects observed be attributed mostly to the soluble form or to a combination of soluble and particle form, and to the size of the particulate form or to degradation products such as metal ions from metal based nanomaterials (Franklin *et al*., 2007, Navarro *et al*., 2008). Although work in this area is increasing now, the results will depend on the material under consideration. The OECD assay on water solubility (OECD TG 105) may be useful in this context, but many of the carbon based materials (such as fullerenes) are so water insoluble that specialised methods are likely to be needed in order to measure or estimate solubility. For example, fullerene solubility is usually estimated by measuring solubility in alcohols and extrapolating to a zero carbon alcohol, i.e. water (Jafvert and Kulkarni, 2008). On the other hand, organic based nanomaterials have the advantage of possibly being labelled with radioactive isotope ^{14}C, which would allow easy quantitative determination but may not allow detection of the number of the particles and characterisation of e.g. agglomeration.

Water/octanol partitioning

111. The measurement of the K_{OW} (OECD 107, 117, 123) is problematic given that many organic nanomaterials have such low water solubility that measuring their concentration in the aqueous phase is not a straightforward procedure as nanomaterial are insoluble but they may stay attached in the liquid interphase. However, Jafvert and Kulkarni (2008) have studied the octanol-water partition coefficient (log K_{OW}) of fullerene C_{60} with method modifications.

C. 2.1.2 Dosage and sample preparation for studies on physicochemical properties

112. The methods presented in Section V, part A on assessing the physicochemical properties of nanomaterials could be generally followed for a dispersion.

C. 2.2 Degradation and transformation

C. 2.2.1 Methods for degradation

Biodegradation

113. In this document **biodegradation** means degradation of organic substances and material by microorganisms resulting ultimately carbon dioxide, water and increase of microbial biomass in aerobic conditions. In anaerobic conditions biodegradation may result in formation of methane and other reduced substances.

114. Again, the main technical challenges in degradation and transformation studies are the detection and characterisation of nanomaterials in the various environmental media. The existing test protocols (e.g. OECD test guidelines) seem to be as appropriate for nanomaterials as for organic substances.

115. Performing biotic degradation tests for purely inorganic nanomaterials is unnecessaryt. Therefore, it is necessary to examine first whether the nanomaterial contains carbon that can be used as an energy and nutrient source for microorganisms. Secondly, the physicochemical and compartmentalisation properties of the material can provide insight into whether some of the simulation tests are necessary. For example, if the material is unlikely to reside in the water column or if it is not soluble in water (e.g. fullerenes and carbon nanotubes), any testing in surface water may be unnecessary. However, current OECD guidelines for testing of pesticides (e.g. Hydrolysis TG 111, Photolysis TG 316 and others) do not give any lower limit for water solubility. According to current guidelines one has to deal with each substance no matter how low solubility might be.

116. OECD methods on biodegradation have been developed and validated principally for assessment of organic compounds. Today's nanomaterials, however, are principally inorganic; indeed even carbon-based nanomaterials tend to be of an inorganic nature. Hence, they will be most probably considered persistent against biodegradation and testing for biodegradation is not relevant. In principle, the methods measuring carbon dioxide production or oxygen uptake are applicable, but they require large amounts of test material. It is also important to consider whether carbon based nanomaterials such as fullerenes and nanotubes can be degraded at all under any conditions. However, limited data have indicated that fullerenes could be taken up by wood decay fungi, suggesting that the carbon from fullerenes could be metabolised (Filley *et al*., 2005). This is usually assumed for organic polymers, too. However, in combination with UV-treatment (simulated sunlight) a certain degradation of polymers can be determined. If several conclusive aerobic degradation tests indicate very low or negligible degradation, then other aerobic degradation tests will most likely also be negative and it may be useless to proceed with additional tests. For example, if the result of a ready biodegradation test is below 10%, then the chances are that the results of the biodegradation simulation test in surface water will also be very low and it may be better to decide to skip the more elaborate test, and conclude that the substance is not biodegradable. For biodegradation screening purposes, whether any biodegradation can happen, modified test systems may serve better than standard OECD test guidelines. The test conditions in the inherent biodegradability test e.g. TG 302 series are favourable for degradation. One possibility would be to have the OECD TG 310 headspace ready biodegradability test with CO_2 measurement, and enhance the test media and conditions to be more favourable for degradation (like in inherent biodegradability tests). In closed system with the measurement of carbon dioxide production of TG 310 or TG 301B the test material does not have to be soluble (like nanomaterials), and rather small amounts of test material is needed.

117. Simulation tests for biological degradation in various environmental compartments are also in principle applicable, but again the detection of the nanomaterials is the challenge. The possible degradation to carbon dioxide (mineralization), integration into biomass or other partition could be followed by labelled

test material. The advantage of using labelled substances would be to allow the testing of low concentrations and to provide degradation kinetics and mass balance on the fate of the carbon from the tested material. However, for mass balance studies radio-labelled nanomaterials can only be used with great caution: the label must be distributed uniformly on the nanomaterial. This very complicated issue requires further input from radiochemistry experts. Yet, non-uniformly labelled material could still help to decide if degradation occurs at all.

Abiotic degradation

118. **Abiotic degradation** means degradation of substances by physicochemical activity e.g. UV-light.

119. Like for biodegradation testing, for hydrolysis testing, the chemical structure of the material and whether it contains groups which could be subject to hydrolysis and/or release ions dictate whether this test is necessary or appropriate.

120. In view of the sometimes very long lifetime of nanomaterials in the environment, the photodegradation studies might be considered relevant. The OECD TG 316 for photodegradation and transformation in water could be an applicable method for this purpose, but again the characterization of the nanomaterial changes (specification) is essential.

C. 2.2.2 Dosage and sample preparation for degradation studies

121. Similarly to the testing of physicochemical properties or biotic effects, the dispersion methods to prepare the samples for degradation studies could include ultra-sonication and/or stirring for long periods e.g. weeks. Especially in biodegradation tests measuring carbon dioxide production or oxygen consumption, the use of organic solvents is not possible, as remnants of the solvent will interfere with the nanomaterial degradation. In the simulation tests using radiolabelled materials, the use of solvent carrier, dispersant or detergent could be possible. However, it should be noted that these will affect also nanomaterial characteristics.

122. The detection of biodegradation in standard **screening tests** is usually followed by measuring the carbon dioxide produced or oxygen consumed by the degraders. As carbon based or more organic nanomaterials are normally not soluble, the measurement of dissolved organic carbon might not be relevant. Of course, in certain test systems the decrease of total amount of carbon could be assessed. In **simulation testing** ^{14}C labelling and chemical analysis and characterisation of nanomaterials and possibly particle numbers by other methods (e.g. electron microscopy or Field Flow Fractionation analysis with ICP-MS) would be the means of detecting the degradation.

C. 2.3 Bioaccumulation

C. 2.3.1 Methods for bioconcentration and bioaccumulation

Aquatic studies

123. Many of the possible ideas for exposure for bioaccumulation studies originate from, and are informed by effect studies.

124. For simple organic chemicals, there is an established relationship between octanol-water partition coefficient (K_{OW}) and bioaccumulation or bioconcentration factor (BCF). However, this relationship may not hold true for many nanomaterials. The studies of Jafvert and Kulkarni (2008) have shown log K_{OW} of 6.7 for fullerene, and it is hence expected that C_{60} has high affinity for lipids and organic matter. Despite this hydrophobic characteristic, fullerene agglomerates are able to form a stable water suspensions (nC_{60} or

aqu/C_{60}; e.g. Isaacson *et al*., 2011; Scharff *et al*., 2004). To what extent such agglomerates can be taken up by organisms (and thus can bioaccumulate), however, is still uncertain.

125. The main challenge once again in testing the bioaccumulation of nanoparticles is their detection and characterisation in tissues and body fluids. Radiolabelling could make detection and quantification easy but it has also limitations, e.g. the labelled material can behave differently from the non-labelled particles (e.g. if a tethered label is used). One possibility could be neutron activation of metal and metal oxide nanoparticles (Oughton *et al.,* 2008). This enables both localisation and quantification within tissues or organisms. Also more traditional chemistry e.g. ICP-MS analysis for metals could provide valuable information on the total amounts of material accumulated by the organism. However, these methods can not define whether all the material found originates from nanomaterial. Recently field flow fractionation (FFF) techniques have been used in combination with ICP-MS. In this way metal content of individual particles can be measured. But first the nanomaterial has to be extracted from the animals and tissues.

126. Standard BCF testing protocols such as OECD 305 (OECD 1996) may have limitations for determining bioaccumulation of nanoparticles. It has been observed for substances dissolved in water that a large molecular size (MW > 600, or effectively a diameter size > 0.5 nm) effectively limits direct uptake. It is likely that in most cases the large size (1-100 nm) of nanoparticles compared to dissolved molecules limits their direct uptake by carrier-mediated transport in fish gills, but uptake by endocytosis cannot be excluded (Handy *et al*., 2008b). Fish dietary BAF testing (Fisk *et al.,* 1998; Stapleton *et al.,* 2004) is covered by the revised OECD 305 (adopted 2012). According to this Test Guideline dietary exposure tests are recommended for super-lipophilic substances, surfactants as well as complex mixtures. However, the spiked food method would also be suitable for testing of poorly soluble large molecules such as nanoparticles. It should be noted that the dietary approach yields a biomagnification factor (BMF) rather than a bioconcenttraion factor (BCF). Fish do eat diets contaminated with nanomaterials, and toxic effects have been observed (Ramsden *et al*., 2008). However, more data using the harmonised OECD dietary protocol, especially for testing nanomaterials, are needed. The selection of the water or dietary exposure route should be based on the expected relevance of the gill versus dietary exposure under relevant environmental conditions.

127. The testing results of human health endpoints e.g. toxicokinetics (ADME), if available, should also be taken into consideration when generating environmental testing plans for specific nanomaterials. Uptake studies from mammalian studies may give valuable basic information of uptake characteristics, rates and mechanisms of nanoparticles also for non-mammalian species.

128. Given the tendency of nanomaterials to agglomerate, and thus their likelihood to end up associated with sediments (Klaine *et al*., 2008), bioaccumulation studies on sediment organisms would be especially important. OECD adopted in 2008 a new method TG 315 (OECD 2008a) for the bioaccumulation in sediment worms e.g. using *Lumbriculus variegatus.* This method would be then probably relevant to be used in a test battery for risk assessment, as OECD has published recently a toxicity test OECD TG 225 (OECD 2008b), based on the same species, which would then also provide effects data.

Soil and terrestrial studies

129. Petersen *et al.* (2008) have indicated that CNTs were not readily accumulated by the earthworm *Eisenia foetida* with results indicating accumulation factors 2 orders of magnitude smaller than those measured for pyrene. Scott-Fordsmand *et al.* (2008) have detected effects on the reproduction of earthworms (*Eisenia veneta*) when the worms were exposed to double-walled carbon nanotubes in food. To assess bioaccumulation of chemicals in earthworms a validated OECD method (TG 317 on Bioaccumulation in Terrestrial Oligochaetes) is available.

130. Effects of ingested nanosized titanium dioxide on enzymatic activity of terrestrial isopods (*Porcellio scaber*) have been detected by Jemec *et al.,* 2008. The TiO_2 nanoparticles were dispersed in distilled water with and without sonication and pipetted on to homogenised hazelnut tree leaves. The isopods were then fed with the leaves. The particle location and its composition were analysed by transmission-electron diffraction pattern.

131. Lee *et al.* (2008) detected bioaccumulation of insoluble copper nanoparticles in cells of emerging and growing plants when tested on agar plates. The particles were well characterised and the homogenous distribution of Cu particles in the agar media was evaluated by SEM. The distribution and accumulation of Cu particles in the plant cells was characterised by TEM and energy-dispersive spectroscopy.

C. 2.3.2 Dosage, exposure and sample preparation for bioaccumulation studies

132. The methods of sample preparation and dosage for bioconcentration and bioaccumulation studies could be similar to those for assessing biotic effects. These might include ultra-sonication, stirring for various periods, use of solvents and introducing stabilising agents. There is still limited information to prioritise the dispersion methods for bioaccumulation studies, but probably the same preference could be valid as for testing biotic effects. The aim would be to achieve a stable and homogenous dispersion over the exposure period. Often the smallest particle size would be expected to provide high accumulation but the exposure conditions should be always decided depending on the nanomaterial tested and the aim of the study. Whatever the method of test material dispersion and dosage, the test media quality (pH, ionic strength, NOM concentration) should be as harmonised as possible between comparative studies. It is especially important that the conditions and the quality of the media are recorded throughout the study in order to enable possible retrospective analysis of the results.

133. Depending on the test, the exposure to the test nanomaterial could be via water, sediment and sediment pore water, soil and soil pore water or ingestion and food. The test design and dose selection should consider the relevance of dynamic and energy-dependent uptake processes to ensure that saturation does not occur, and the exposure is sufficient for reaching steady-state conditions. For aquatic studies, the methods for dosage mentioned above are relevant but e.g. for soil studies the nanomaterial can be introduced directly to the media in solid form and homogenised. Care should be taken in homogenisation so that the test material is not damaged, and details of homogenisers/velocities should be reported.

134. In bioconcentration and accumulation studies, both the accumulation and depuration phases are important. It must be noted that the nanomaterial could be excreted in a different form from which it was taken into the organism. Hence the characterisation methods for the test material are important, not just the total amount measured e.g. by total metal content of metallic nanomaterial.

135. It is unclear to date to what extent the effects observed can be attributed mostly to the soluble form or to a combination of soluble and particle form, and to the size of the particulate form or to degradation products such as metal ions from metal based nanomaterials (Franklin *et al*., 2007, Navarro *et al*., 2008). Solubility of silver nanoparticles and the resulting release of silver ions can occur in a variety of ways. The particles themselves can harm biota by direct interaction with biological targets. This release of ions (silver solubility) can happen outside the organisms and cells in the test media, but it has been proposed that the small silver particles can enter cells and organisms and release the ions there (the so called Trojan Horse effect). Definitely, the manufactured coatings (e.g. citrates, PVP) and also the natural coatings [natural organic material (NOM), protein corona] will affect the release kinetics of the silver ions from the particles depending on the environment of the particles. Some release of various metals ions or oxides can be expected also from other metallic nanomaterials.

i) Sample preparation

Soil studies

136. Direct mixing of dry nanomaterial into soil has proven to give more homogeneous distribution of test material than mixing an aquatic dispersion of nanomaterials into soil. This has been the case at least when Ag-nanoparticles were mixed into the OECD artificial test soil (Scott-Forsmand 2011). Systematic studies concerning spiking of soil for ecotoxicity tests (natural sandy soil (German reference Soil RefeSol 01-A spiked with powder using soil and silica sand resp. as carrier; soil / food spiked with aquatic dispersion; food spiked with powder) revealed that bioavailability differed between the different procedures, but replicates (6 samples) taken per spiking procedure used for chemical analyses revealed comparable standard deviations for all spiking procedures for soil.

Aquatic studies

Water phase

137. Dispersion of nanomaterial might include stirring, sonication [or preferably energy input in J/L (joules/litre)], grinding, use of solvents, and stabilising or dispersing agents. The advantages and disadvantages of these methods are outlined in Handy *et al*. (2011). This suggested that some nanomaterials are significantly altered by sonication [or preferably energy input in J/L (joules/litre)] and grinding (e.g. carbon nanotubes can be shortened) and that the interaction of solvents with some nanomaterials might result in toxic by-products. The method of dispersion will also depend on the specific material to be tested and whether or not it has been surface treated. Best scientific judgment should be used in selecting the methods, and where there is evidence or an indication that a dispersion method might significantly alter toxicity, those effects should be controlled or quantified. The operation conditions before and during the test should be described in detail.

138. It is unlikely that these properties will be stable over renewal periods (or the duration of a test), rather, the goal should be to have a repeatable initial test medium, and to monitor property changes with sufficient frequency to quantify these changes. In some cases, in order to remove the larger particles, filtration with 0.45 μm or 0.22 μm filters is employed (e.g. Ma and Bouchard, 2009). This approach should be altered where information is available concerning environmentally relevant particle sizes. This might include manufacturer information on specific sizes produced and incorporated into products, and unlikely to undergo post-production processes that might alter particle size or distribution.

139. It is assumed that tests will be often conducted using periodic renewal approaches to avoid expense and waste production of the flow through systems. Regardless of nanomaterial dispersion and dosage methods, the test media quality (pH, ionic strength, NOM concentration) should be as harmonised as far as possible between comparative studies. It is especially important that the conditions and the quality of the media are recorded throughout the study in order to enable possible retrospective analysis of the results.

Sediment

140. Direct mixing of dry nanomaterial into dry sediment has proven to give more homogeneous distribution of test material than mixing a water dispersion of nanomaterials in sediment. This has been the case when Ag-nanoparticles were mixed into the OECD artificial test soil (Scott-Forsmand 2011)

Ion release

141. When testing e.g. Ag-nanoparticles the ion solubility has to be taken into account. It is preferable to assess this by recent analytical methods measuring the solubilized ions by e.g. ICP-MS. However, in the

bioaccumulation studies of nanosilver it can be useful to test silver ions (e.g. silver nitrate) simultaneously in order to differentiate the accumulation and especially depuration kinetics of nanoform and ions.

Nanomaterial detection and characterization in the test media

Ion release

142. Ionic strength, pH, cation-anion composition, and dissolved organic material (NOM and e.g. organism exudates) can all affect agglomeration and degradation of primary particles, the fate of both free ions and particles, or their interaction with biological targets (Liu and Hurt 2010).

143. Methods that have been tested for separation of particles and ions in exposure media include dialysis, filtration, and ultracentrifugation. A related approach is to control or manipulate the type and level of chelators such as thiosulfate or cysteine, both of which strongly bind silver ions and reduce or eliminate their toxic potential. However, these chelating ligands can also increase the rate of oxidation and dissolution of Ag particles. Dialysis has not proven to be effective, largely due to adsorption of silver to membranes and the duration of testing; this has been demonstrated in mass balance studies where losses to membranes have been accounted for.

144. Filtration and ultracentrifugation are currently being researched. Their effectiveness and specific approaches have yet to be widely established, although the ease of use for filtration methods offers promising solutions

Detection in the solid matrix/porous media

145. The biggest challenges in the detection and quantification of nanomaterials from porous media e.g. soil or sediments lie in the pre-treatment of the samples. The nanomaterial has to be first extracted from the media and then separated from the extracted suspension. However, currently there are no harmonized ways of extraction. It seems that for separation of nanomaterial from the suspension centrifugation is better that filtration (Gimbert *et al*., 2006). It should be noted that, whatever these sample pre-treatment methods are there will most probably affects the characteristics of the nanomaterial.

146. The analysis of extracted and separated element, e.g. metals using ICP-MS (Geranio *et al*., 2009) or ICP-OES, is relatively simple. However, it is difficult to differentiate the manufactured material and its atoms from the natural background. Questions remain as to whether the isotopic fingerprint of a nanomaterial would be different from natural materials. This challenge may be addressed through labelling of the metal in nanomaterials by such methods as neutron activation, which provides for separation from the background.

147. With various electron microscopic techniques the NMs and their size and specification (state of agglomeration/aggregation) in a sample can be directly observed. However, only a limited number of microscopic fields can be screened which yields in statistical uncertainty. Hence, at the moment many microscopic methods are more or less qualitative methods (Doucet *et al.,* 2005). To some extent the same applies to XRF-methods.

148. Carbon nanomaterials like fullerenes and CNTs can be analysed from environmental samples by LC-MS chromatography (Isaacson *et al*., 2009)

149. Field flow fractionation combined with chemical analysis e.g. ICP-MS (Ranville personal communication 2011) to measure different amounts and sizes of the nanoparticles.

Nanomaterial detection in the biota

150. Detection of the overall amount of, e.g. ^{14}C labelled material is rather easy in the tissues and in the whole organism. In addition, neutron activation of metal and metal oxide nanoparticles (Ag, Co or Co_3O_4 and CeO_2) can be an option. These could enable both localisation and quantification within tissues or organisms. Of course, traditional chemical analysis ICP-MS for metals and various HPLC based techniques can be useful for the measurement of the total amount of the nanomaterial accumulated. But again these methods cannot separate accumulated nanomaterial from the bulk material or background metal concentrations. Electron microscopy provides means both for the detection and semi-quantitative analysis of the materials in the exposure media and inside the organism.

Note

151. Refer to Section V, Part C of this document for more in-depth discussion of characterization approaches.

C. 3 References

Arvidsson, R., Molander, S., Sandén, B. A., and Hassellov, M. (2011), "Challenges in exposure modeling of nanoparticles in aquatic environments", *Human and Ecological Risk Assessment* 17, (1).

Baalousha, M., Manciulea, A., Cumberland S., Kendall, K. and Lead, J.R. (2008), "Aggregation and surface properties of iron nanoparticles: influence of pH and natural organic matter", *Environmental Toxicology and Chemistry* 27, pp.1875-1882.

Boxall, A.B., Chaudhry, Q., Sinclair, C., Jones, A., Aitken, R., Jefferson, B., Watts, C. (2007), *Current and future predicted environmental exposure to engineered nanoparticles*, DEFRA Report, *Central Science Laboratory*, York.

Chistian, P., Von der Kammer F., Baalousha, M. and Hofmann, T. (2008), "Nanoparticles: structure, properties, preparation and behaviour in environmental media" *Ecotoxicology* 17, pp.326-343.

Doucet, F.J., Lead, J.R., Maguire, L., Achterberg, E.P., and Millward, G.E. (2005), "Visualisation of natural aquatic colloids and particles- a comparison of conventional high vacuum and environmental scanning microscopy", *Journal of Environmental Monitoring* 7, pp.115-121

Federici, G., Shaw, B. J. and Handy, R. D. (2007), "Toxicity of Titanium Dioxide Nanoparticles to Rainbow Trout, (*Oncorhynchus mykiss*): Gill Injury, Oxidative Stress, and Other Physiological Effects", *Aquatic Toxicology*, 84, 415-430.

Fernandes, T.F., Christofi, N., and Stone, V. (2007a), *The Environmental Implications of Nanomaterials. In: Nanotoxicology: Characterisation, Dosing and Health Effects* inNancy A. Monteiro-Riviere, and C.L. Tran (eds.), *Taylor and Francis*, CRC Press, USA.

Fernandes, T.F., Nielsen, H., Burridge, T. and Stone, V. (2007b), "Toxicity of nanoparticles to embryos of the marine macroalgae Fucus serratus", Paper presented at 2nd International Conference on the Environmental Effects of Nanoparticles and Nanomaterials, London, England

Filley, T.R., Ahn, M.M., Held, B.W., and Blanchette, R.A. (2005), "Investigations of fungal mediated (c60-C70) fullerene decomposition", Preprints of Extended abstract Presented at the ACS National Meeting, *American Chemical Society* 45(1), pp.446-450.

Fisk, A.T., Norstrom, R.J., Cymbalisty, C.D., and Muir, D.C.G. (1998), "Dietary accumulation and depuration of hydrophobic organochlorines: Bioaccumulation parameters and their relationship with the octanol/water partition coefficient", *Environmental Toxicology and Chemistry 17*, pp.951-961.

Fortner, J. D., Lyon, D. Y., Sayes, C. M., Boyd, A. M., Falkner, J. C., Hotze, E. M., Alemany, L. B., Tao, Y. J., Guo, W., Ausman, K. D., Colvin, V. L. and Hughes, J. B. (2005), "C60 in water: Nanocrystal formation and microbial response", *Environmental Science and Technology* 39, pp. 4307-4316.

Franklin, N.M., Rogers, N.J., Apte, S.C., Batley, G.E., Gadd, G.E. and Casey, P.S. (2007), "Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and ZnCl2, to freshwater microalga (Pseudokirchinella subcabitata): The importance of particle solubility", *Environmental Science and Technology* 41, pp.8484-8490.

Geranio, L., Heuberger, M. and Nowack, B. (2009), "The behavior of silver nanotextiles during washing", *Environmental Science and Technology*. 43, pp. 8113-8118.

Gimbert, L.J., Haygart, P.M., Beckett, R., Worsfold, P.J. (2006), "The influence of sample preparation on observed particle size distributions for contrasting soil suspensions using field-flow fractionation", *Environmental Chemistry* 3, pp.184-191.

Gottschalk, F., Sonderer, T., Scholz, R. W., and Nowack, B. (2010), "Possibilities and limitations of modeling environmental exposure to engineered nanomaterials by probabilistic material flow analysis", *Environmental Toxicology and Chemistry* 29 (5), pp.1036-1048.

Handy, R.D. and Eddy, F.B. (2004), "Transport of solutes across biological membranes in eukaryotes: an environmental perspective", in van Leeuwen, H.P. and Köster; W. (eds.) Physiological kinetics and transport at chemical-biological inter phases, *IUPAC series John Wiley,* Chichester, pp.337-356.

Handy, R.D., von der Kammer, F., Lead, J.R., Hassellov, M., Owen, R., and Crane, M.(2008),"The ecotoxicology and chemistry of manufactured nanoparticles", *Ecotoxicology* 17, pp.287–314

Handy, R.D., Henry, T.B., Scown, T.M., Johnston, B.D., and Tyler, C.R. (2008b), "Manufactured nanoparticles: their uptake and effects on fish--a mechanistic analysis", *Ecotoxicology* 17(5), pp.396-409.

Handy, R.D., Cornelis, G., Fernandes, T., Tsyusko, O., Decho, A., Sabo-Attwood, T., Metcalfe, C., Steevens, J.A., Klaine, S.J., Koelmans, A.A., and Horne, N. (2011), "Ecotoxicity test methods for engineered nanomaterials: Practical experience and recommendations from the bench", *Environmental Toxicology and Chemistry* 31(1), pp.15-31.

Hartmann, N., Buendia, I., and Baun, A. (2011), "Degradability of aged aquatic suspensions of C60 nanoparticles", *Environmental Pollution* 159(10), pp.3134-3137

Hassellöv, M., Readman, J.W., Ranville, J.F. and Tiedje K. (2008), "Nanoparticle analysis and characterisation methologies in environmental risk assessment of engineered nanoparticles", *Ecotoxicology* 17, pp.344-361.

Holbrook, R.D., Murphy, K.E., Morrow, J.B., and Cole, K.D. (2008), "Trophic transfer of nanoparticles in a simplified invertebrate food web", *Nature Nanotechnology* 3, pp.352 – 355.

Hyung, H., Fortner, J.D., Hughes, J.B. and Kim, J-H. (2007), "Natural organic matter stabilises carbon nanotubes in the aqueous phase", *Environmental Science and Technology* 41,pp.179-184.

Isaacson, C.W., Kleber, M. and Field, J.A. (2009), "Quantitative analysis of fullerene nanomaterials in environmental systems: a critical review", *Environmental Science and Technology* .43, pp.6463-6474.

Isaacson, C., Zhang, W., Powell, T., Ma, X., and Bouchard, D. (2011), "Temporal changes in aqu/C60 physical-chemical, deposition, and transport characteristics in aqueous systems", *Environmental Science and Technology* 45 (12), pp.5170–5177

Iversen T.-G., Skotland T., Sandvig K. (2011), "Endocytosis and intracellular transport of nanoparticles: Present knowledge and need for future studies", Nano Today 6 (2), pp. 176-185.

Jafvert, C., and Kulkarni, P. (2008), "Buckminsterfullerene's (C60) Octanol-Water Partition Coefficient (K_{OW}) and Aqueous Solubility", *Environmental Science and Technology 42*, pp.5945–5950.

Jemec, A., Drobne, D., Remskar, M., Sepcic, K. and Tisler, T. (2008), "Effects of ingested nano-sized titanium dioxide on terrestrial Isopods (*Porcellio scaber*)", *Environmental Toxicology and Chemistry* 27, pp.1904-1914.

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

Lee, W-M., An, Y-J, Yoon, H. and Kweon H-S. (2008) "Toxicity and bioavailability of copper nanoparticles to terrestrial plants mung bean (Phaseolus radiatus) and wheat (Triticum aestivum): Plant agar test for water-insoble nanoparticles", *Environmental Toxicology and Chemistry* 27, pp.1915-1921.

Li, D., Lyon, D.Y., Li, Q. and Alvarez, P.J.J. (2008), "Effect of soil sorption and aquatic natural organic matter on the antibacterial activity of a fullerene water suspension", *Environmental Toxicology and Chemistry* 27, pp.1888-1894.

Liu, J. and Hurt R.H. (2010), "Ion release kinetics and particle persistence in aqueous nano-silver colloids", *Environmental Science and Technology* 443, pp.2169-2175.

Ma, X. and Bouchard, D. (2008), "Formation of aquesous suspensions of fullerenes", *Environmental Science and Technology* 43, pp.330-336.

Mueller, N., Nowack, B. (2009), "Exposure Modeling of Engineered Nanoparticles in the Environment.", *Environmental Science and Technology* 42*,* pp.4447-4453.

Navarro, E., Baun, A., Behra, R., Hartmann, N.B., Filser, J., Miao, A. J., Quigg, A., Santschi, P.H. and Sigg, L. (2008), "Environmental behaviour and ecotoxicity of engineered nanoparticles to algae, plants, and fungi", *Ecotoxicology* 17, pp.372-386.

Nielsen, H.D., Berry, L.S., Stone, V., Burridge, T.R. and Fernandes, T.F. (2008), "Interactions between carbon black nanoparticles and the brown algae *Fucus serratus*: Inhibition of fertilization and zygotic development.", *Nanotechnology* 2, pp.88-97.

OECD (1996), *OECD Test Guideline 305. Bioconcentration: Flow-through Fish Test.* OECD Paris.

OECD (2005), *Revised OECD Test Guideline 305. Bioconcentration: Flow-through Fish Test.* OECD Paris.

OECD (2008a), *OECD Test Guideline 315. Bioaccumulation in Sediment-dwelling Benthic Oligochaetes*. OECD Paris.

OECD (2008b*), OECD Test Guideline 225. Sediment-water Lumbriculus toxicity test using spiked sediment.* OECD Paris.

OECD (2009), *OECD Environment, Health and Safety Publications – Series on the Safety of Manufactured Nanomaterials No. 15: Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials,* Report JT03267900 (ENV/JM/MONO(2009)21).

Oughton, D.H., Hertel-Aas, T., Pellicier, E., Mendoza, E.and Joner, E.J. (2008), "Neutron activation of engineered nanoparticles as a tool for tracing their environmental fate and uptake in organisms", *Environmental Toxicology and Chemistry* 27, pp.1883-1887.

Petersen, E.J., Huang, Q, and Weber, W.J. (2008), "Bioaccumulation of radio-labelled carbon nanotubes by *Eisenia foetida*", *Environmental Science and Technology* 42, pp.3090-3095.

Pycke, B.F.G, Benn, T.M. Herckes, P., Westerhoff, P., and Halden, R.U. (2011), "Strategies for quantifying C(60) fullerenes in environmental and biological samples and implications for studies in environmental health and ecotoxicology", *Trends in Analytical Chemistry* : TRAC. 30(1), pp.44-57.

Quik, J. T. K., Vonk, J. A., Hansen, S. F., Baun, A., and Van De Meent, D. (2011), "How to assess exposure of aquatic organisms to manufactured nanoparticles? ", *Environment International* 37, pp.1068-1077.

Ramsden, C. R., Smith, T.J., Shaw, B.J. and Handy, R.D. (2008), "Toxicology of dietary titanium dioxide nanoparticles to rainbow trout, (*Oncorhynchus mykiss*)", *Comparative Biochemistry and Physiology 150*(3) S60

Roberts, A.P., Mount, A.S., Seda, B. Souther, J., Qiao, R., Lin, S., Ke, P.C., Rao, A.M., and Kaine S.J. (2007), "In vivo biomodification of lipid-coated carbon nanotubes by *Daphnia magna", Environmental Science and Technology* 41, pp.3025-3029.

Scharff, P, Risch, K, Carta-Abelman, L, Dmytruk, I.M., Bilyi, M.M., Golub, O.A., Khavryuchenko, A.V. Buzaneva, E.V., Aksenov, V.L., Avdeed, M.V., Prylutskyy, Yu.I. and Durov, S.S. (2004), "Stucture of C_{60} fullerene in water: spectroscopic data", *Carbon* 42, pp.1203-1206

Scott-Forsmand, J.J., Krogh, P.H., Scaefer, M. and Johansen, A. (2008), "The toxicity testing of double-walled nanotubes-contaminated food to *Eisenia veneta* earthworms," *Ecotoxicology and Environmental Safety* 71, pp.616-625.

Scott-Forsmand J.J. (2011), Presentation at Nordic Nano network meeting, October 2011, Espoo, Finland

Smith, C.J, Shaw, B.J. and Handy, R.D. (2007), "Toxicity of single walled carbon nanotubes on rainbow trout, (Oncorhynchus mykiss): respiratory toxicity, organ pathologies, and other physiological effects," *Aquatic Toxicology* 82, pp.94-109

Stapleton, H.M., Letcher, R.J., Li, J., Baker, J.E. (2004), "Dietary accumulation and metabolism of polybrominated diphenyl ethers by juvenile carp (*Cyprinus carpio*)", *Environmental Toxicology and Chemistry 23*, pp.1939-1946.

Stolpe, B. and Hassellöv, M. (2007), "Changes in size distribution of fresh water nanoscale colloidal material and associated elements on mixing with seawater', *Geochimica et Cosmochimica Acta* 71, pp.3292-3301

Tiede, K., Boxall, A., Tear, S., Lewis, J., David, H. and Hassellöv, M. (2008), "Detection and characterisation of engineered nanoparticles in food and the environment", *Food Additives and Contaminants* 1(27).

D. HEALTH EFFECTS, PREPARATION OF A TEST SUBSTANCE AND DOSIMETRY

152. Material characterization for toxicity (screening) studies is most appropriately considered in the context of the studies being undertaken. Requirements for in vitro and in vivo screening studies will differ according to the material delivery, route or method. Additionally, understanding human exposures in the context of relevant studies might present a further set of characterization requirements. At least three main study contexts are proposed to be considered, and characterization recommendations are proposed to be considered within these contexts (Oberdörster *et al.,* 2005):

- Human exposure characterization
- Characterization of administered material
- Characterization of as-produced or supplied material

Precise and comparable measurement techniques as well as the provision of standards and standard operating procedures are a prerequisite for this. One other key component for comparable and high quality characterisation is appropriate reference materials.

153. Currently, standard materials include gold[11] (NIST), titanium dioxide[12], single wall carbon nanotubules[13] (NIST) and polystyrene nanomaterials as well as quality control material colloidal silica (IRMM-304[14]). These materials have assigned values for particle size distribution by various analytical techniques and may be useful for evaluating the influence of dispersion and dosing protocols on nanomaterial size, agglomeration state, and dispersability. The EU Commission Joint Research Centre (JRC) has established a repository of representative commercial nanomaterials (Joint Research Centre (JRC) nanomaterials repository http://ihcp.jrc.ec.europa.eu/our_activities/nanotechnology/nanomaterials-repository) that are characterised independently to provide confirmed reference samples.

154. Dosimetry, appropriate dose metrics and the absorption, distribution, metabolism and excretion processes are particularly relevant in relation to the actual internal dose. The value of this information for setting safety testing triggers and assessment factors should be addressed (Gangwal *et al.*, 2011, Oberdörster, 2012 in reply). However, this current version of guidance only deals with dosimetry briefly in some sections and overall content related to dosimetry requires expansion in future versions.

[11] See https://www-s.nist.gov/srmors/view_detail.cfm?srm=8011, https://www-s.nist.gov/srmors/view_detail.cfm?srm=8012, https://www-s.nist.gov/srmors/view_detail.cfm?srm=8013

[12] See https://www-s.nist.gov/srmors/view_detail.cfm?srm=1898

[13] See https://www-s.nist.gov/srmors/view_detail.cfm?srm=2483

[14] See https://web.jrc.ec.europa.eu/rmcatalogue/detailsrmcatalogue.do?referenceMaterial=I-0304%2B%2B%2B%2B%2B%2B

D.1 Knowledge transfer from environmental chemistry and stock dispersion preparation

155. The chemistry community has been working on colloids and particle chemistry for many decades, and this knowledge has been placed in context for nanomaterials ecotoxicology (Handy *et al*., 2008 and references therein). The generation of nanomaterials may be a general phenomenon for materials oxidised and reduced under common environmental conditions (Glover *et al.*, 2011). There is a clear list of abiotic factors that can have substantial effects on particle agglomeration (and therefore bioavailability), as outlined in Section V on specific considerations. Since these abiotic factors are fundamental properties, they should also be considered for the preparation of salines and other media for *in vitro* on mammalian studies. The characterisation suggested (Section IV) and information reporting precisely how a stock dispersion was made (volumes, sonication times [or preferably energy input in J/L], etc) should be provided. Therefore, a test or a combination of tests to confirm the nanoscale of the particles in dispersion is absolutely necessary. Dispersability depends on mechanical treatment, surface chemical modifications and particle concentration to be dispersed.

156. In order to investigate particle size and/or particle size distribution, a combination of characterisation techniques (see Table 1 below) including fractioning and analytical methods is suggested, especially in the case of a wide range of particle size distribution. A suitable combination could be field flow fractionation (FFF) for separation (advantage: covers a wide range of particle sizes) and small angle x-ray scattering (SAXS) for actual particle size. Alternatively, small angle neutron scattering, multi angle light scattering or dynamic light scattering could be considered for size determination. Chromatographic methods (size exclusion, gel permeation) are different suitable fractionation methods; however, stronger forces than occurring with field flow fractionation might influence the agglomeration behaviour. A suggested combination of methods has been in use for polymer analysis in industry for quite some time.

157. Agglomeration is a critical factor in testing nanoparticles. Its role on health effects *in vitro* and *in vivo* has to be considered and cautions need to be taken in order to control clustering in sample preparation as what's been done in environmental testing. However, nanoparticles in health effects testing may behave quite differently from particles dispersed in environmental media, depending on the particular route of exposure (e.g. inhalation of aerosols). Furthermore, the exposure scenario (which in turn will influence the testing design) and its regulatory consequences have to be taken into account. Hence, a "realistic" exposure scenario needs to be differentiated from a "worst case" scenario. A "realistic" scenario refers to how nanoparticles may actually have been taken up by consumers or at the work place (normally agglomerated). A "worst case" scenario means maximum dispersal, with primary size being one major factor in testing (e.g. for barrier penetration, bioavailability, toxic effects). To assess the toxic potency of a nanomaterial in the environment (e.g. for classification and labelling) may thus differ from deriving a "nano-dust" limit value at the work place. Accordingly, the regulatory purpose may also direct sample preparation (see Table 2).

158. A number of analytical techniques may be applicable for measuring nanomaterials in dispersion. These techniques are summarized in several review articles (Oberdörster *et al*., 2005; Powers *et al*., 2006; Powers *et al*., 2007; Handy *et al*., 2008; Hassellöv *et al*., 2008; Sayes and Warheit, 2009, Domingos *et al*., 2009, Wilkinson KJ, 2009).

Table 1: Characterization techniques

Refer to Section B of this document for more in-depth discussion of characterization approaches.

Properties of as-produced nanoparticles (nanoparticle powder)

Size, size distribution, shape	Scanning electron microscopy (SEM) Transmission electron microscopy (TEM) Atomic force microscopy (AFM) X-ray diffraction (XRD) for crystalline nanoparticles Differential mobility analysis
Crystallinity, crystal structure	X-ray diffraction (XRD) Electron diffraction in a transmission electron microscope(ED)
Chemical composition and purity Of a nanoparticle ensemble (powder sample) Chemical properties of single nanoparticles	Inductively-coupled mass spectroscopy (ICP-MS) Inductively-coupled plasma atomic emission spectroscopy (ICP-AES) Atom-absorption spectroscopy (AAS) X-ray fluorescence spectroscopy (XRF) X-ray photoemission spectroscopy (XPS) Time-of-flight secondary ion mass spectroscopy (TOF-SIMS) Ultraviolet-visible spectroscopy (UV-Vis) Fourier-transform infrared spectroscopy (FTIR) Energy-dispersive (wave-length) dispersive X-ray spectroscopy in an electron microscope
Surface chemistry and surface reactivity	X-ray photoemission spectroscopy (XPS) Electron spin resonance (ESR) Auger electron spectroscopy (AES)
Surface area as indicator for agglomeration	Isothermal gas adsorption/BET

Properties of nanoparticles in liquid suspension

Size distribution	Dynamic light scattering (DLS) - Analytical ultracentrifugation (AUC) Cryo-transmission electron microscopy (cryo-TEM) Field flow fractionation (FFF) combined with SAXS
Surface charge, surface potential (yields also information on agglomeration state)	Zeta potential by light-scattering electrophoresis
Radical species	Electron spin resonance (ESR)
Solubility (of a material in a liquid)	Conductance Visual conformation over time
Surface activity	Photocatalytic degradation Vitamin C assay
Tracking of single autofluorescent or fluorophore-functionalized nanoparticles in liquid suspension	Fluorescence microscopy
Sedimentation of nanoparticles	Computationel dosimetry [3]
Protein corona of single nanoparticles	Fluorescence correlation spectroscopy (FCS) [4]

Nanoparticles on the surface of cells (in-vitro experiments)

Averaging techniques, i.e. techniques without spatial resolution, like ICP-MS and others without spatial resolution do not allow distinction between nanoparticles on cell surfaces and in cells	
Number density, size distribution	Liquid atomic force microscopy (AFM) (Environmental) Scanning electron microscopy (ESEM)
Chemical composition and purity of single nanoparticles	Energy dispersive X-ray analysis in a scanning electron microscope (EDXS)
Aggregation of nanoparticles	Fluorescence-activated cell sorting (FACS) [5]

Nanoparticles in tissues and cells

Averaging techniques, i.e. techniques without spatial resolution, like ICP-MS and others without spatial resolution do not allow distinction between nanoparticles on cell surfaces and in cells	
Number density, size distribution and location of nanoparticles (spatially resolving techniques)	Fluorescence microscopy Confocal light microscopy X-ray fluorescence imaging (XFM) Liquid atomic force microscopy (AFM) (Environmental) Scanning electron microscopy (ESEM) Magnetic resonance imaging (MRI) of superpara-magnetic nanoparticles
Chemical composition and purity Total mass of nanoparticles (Usage of radioactively labelled nanomaterials avoids effects of potential contamination from culture vessels etc.)	Inductively-coupled mass spectroscopy (ICP-MS)

D.2 Recommended control measurements on test dispersions and test vehicles during experiments

159. The following recommended measurements are in addition to routine checks on pH, temperature etc (see also section IV of this document):

Exposure concentrations for test dispersions:

i) **Confirm initial exposure concentration** where techniques are currently available e.g. the concentration of dissolved metal and metal oxide nanomaterials. When ion release is expected, such as for ZnO, CuO and other metal and metal oxide nanomaterials, the measured ion concentration in the dispersant is also desired. This will aid distinguishing nano-specific effects and substance-specific effects.

This is problematic for carbon-based materials as the methodologies are not necessarily sensitive enough to measure environmentally relevant low microgram concentrations in water and the background levels of dissolved organic carbon (DOC) may overwhelm the actual concentration of carbon based nanomaterials. In salines containing protein (e.g. Bovine Serum Albumin) any carbon measurement is likely to be overwhelmed.

Maintain a consistent exposure concentration by changing the test media if needed (as would be the case if a semi-static test method is used). The frequency of media changes may need to be derived empirically, but target exposure concentrations should be maintained. Flow through methods can create a

waste disposal problem and semi-static methods reduce this and the occupational exposure risk. Nevertheless, in particular for in vitro methods, attention needs to be paid to possible detachment of cells and to possible removal of cells grown in suspension during test media change and the possibility of waste induced occupational exposure risk. Inhalation studies present additional challenges of measuring dose over time, and require both on-line and off-line analysis.

Particle number, size distribution and agglomeration: The characteristaion of these properties in the test dispersions is a fundamental problem (see collision theory in Handy *et al*., 2008). It must be accepted that agglomeration and dispersion will vary with each concentration in the test system. The total surface area available to the organisms will therefore not be the same at each concentration in the test design. Similarly, the total surface area and number concentration available to the organisms might not follow the predicted, mathematical dependency on the doses in the test design, but will be also influenced by dispersion taking place at each dose level. The rate of agglomeration/aggregation in culture media should be also checked over time especially for treatments longer than 24h. It is a huge amount of work to measure these changes in every test vessel or test vehicle and in each biological system like tissue, cell, cell type, organelle type, nucleus, and one may not have control of this because of different rates of ligand secretion by the organisms at each dose (e.g. mucus production). The stability of any nanoparticle suspension over time should be determined using techniques such as dynamic light scattering, differential centrifugal sedimentation, modified light microscopy, ultraviolet–visible spectroscopy (i.e., UV-Vis). This might be especially important in mammalian studies in vitro where saline media are re-circulated or re-used, and cellular secretions inevitably build up in the media. This is also very important for in vivo studies using inhalation route of exposure and direct delivery techniques. In order to control the particle agglomeration behaviour absolute quantification of the particle number or determination of agglomerate size, using appropriate methods, is necessary. Real dose may also depend on (a) sedimentation effects (size-dependent fractionation may result from sedimentation), or (b) interaction of particles with vessel wall for different materials (e.g. glass or plastic). Permanent online analysis of dispersion is recommended to control dispersion stability. The surface chemistry of the particles should also be investigated as dispersion behaviour strongly depends on the chemical surface properties of the particles.

> It is anticipated that it might not be possible to strictly adhere to some OECD Guidelines for in vivo tests requiring specifically high mass concentrations to be tested, because of limited dispersability of some nanomaterial. In such cases it is recommended to test a dose range up to the dispersability limit.

Control of vehicle (including dispersant, if used) effects by including vehicle controls- this would be the same as any other experiment. These controls must be included. However, the dogmatic approach of standardising dispersant levels in all treatments should be avoided. Excess dispersant can change particle shape (see discussion in Smith *et al*., 2007), and so some thought needs to be given as to whether such an excess is appropriate or not, or if individual dispersant controls are needed for each dose (as this poses an ethical dilemma regarding reduction of animal usage, wherever possible, existing data should be used). If dispersion cannot be reasonably achieved, then some standard protocol of sonication or mixing, "immediately" before dosing may be required to at least give a known amount of nanomaterial /unit volume of dispersant. Any deviation from the standard sonication protocol should be carefully reported in terms of duration and energy input. A certain degree of uncertainty would be unavoidable in this case.

160. It is important to note that characterisation of a nanomaterial in suspension is just one component of an overall characterisation strategy for toxicity testing. Various groups (e.g. governments, treaty-based organizations, standards development organizations, research consortia) have created lists of characterisation requirements for understanding exposure and toxicity assessments, including requirements for minimum characterization of a material as produced or supplied, as administered to a test subject, and

after administration (see, for example, Bouwmeester *et al*., 2011; Boverhof and David, 2010; Warheit, 2008; Oberdörster *et al*., 2005, EFSA Journal 9, 2011).

D.3 Special considerations for vehicles e.g. physiological media used in cell-free, cell-based, and mammalian studies

161. In addition to the details on stock dispersions above, the high ionic strength of buffer salt solutions may present specific problems for dosimetry, including instantaneous agglomeration of the test material. The reason for using these salt solutions in the first place is to match the ionic environment (e.g. ionic strength, pH) in e.g. intravenous administration. The use of buffers therefore cannot be avoided. It may be better to make stock dispersions in ultrapure water, if the nanomaterial is dispersible in water, and then disperse smaller volumes in the saline or other buffers. Or if small volumes are used, consider dosing the nanomaterials in ultrapure water if that has proven to cause little disturbance of homeostasis and results in a good dispersion. This can be controlled by taken along ultrapure water as a vehicle control and compare the vehicle to no treatment at all. If this second step is taken, then all the characterisation may need to be done again for the saline or other buffers used. It may also be helpful to add dispersion agents (such as PEG, Tween, Triton or other similar surfactants or DMSO) to the saline to improve dispensability/dispersion handling of the test material. However, this must be fully justified for practical reasons (i.e., impossible to handle the nanomaterial in saline phase without it), reflected in the dispersant controls, and with some appreciation of how the dispersing agent is working (e.g. coating the surface of the nanomaterial). Dispersant similar to what would be found in the target tissue, such as bronchoalveolar lavage fluid (BALF) or mimic BALF (Porter *et al*., 2008) for inhalation studies should be considered first. Tween, triton, and other similar products should be obtained at the best available analytical grade to minimise spurious effects of contaminants in these products on the nanomaterial chemistry. In addition the inherent toxicity of the dispersion agent has to be kept in mind, in order to avoid high toxicity (Zhu, S. *et al*., 2006, Monteiro-Riviere, N. and Tran, L. 2007), which might interfere the interpretation of studies even if appropriate vehicle or blank controls are used.

162. Physiological salt solutions and culture media for use in in-vitro studies with mammalian cells or tissues are often gassed with high concentrations of oxygen and carbon dioxide (e.g. 95 % O_2: 5 % CO_2), and of course may be used at body temperature (37^{o}C). It is important to note that these conditions have not been employed in agglomeration chemistry studies/environmental colloid chemistry. Currently it is assumed that these conditions used in mammalian studies in the above-mentioned systems will not alter the chemistry. This assumption may need to be revised when results of research on the nanomaterial chemistry in 5% CO_2 at body temperature are available. However, in the meanwhile, it should be reported whether the particle characterisation was done in gassed or air-equilibrated saline, and at which temperature. It is noted that the level of oxygen affects levels of metabolising enzymes. Use of such high oxygen levels are not recommended in cell culture protocols, where use ambient levels, i.e., 21% O_2 are common practice.

163. Physiological salt solutions and culture media also contain additional substances that are specific to different types of test, e.g. the use of lipopolysaccharide (LPS) as an immune activator (i.e. adjuvant), or the addition of metabolic inhibitors in ADME[15] studies. It must be stated whether characterisation was done before or after adding these extra substances, and preferably with some checks to show this does not have an appreciable effect on particle dispersion and toxicity.

[15] ADME is an acronym in pharmacokinetics and pharmacology for absorption, distribution, metabolism, and excretion, and describes the disposition of a test substance within an organism. The four criteria all influence the test substance levels and kinetics of test substance exposure to the tissues and hence influence the performance and pharmacological activity of the test substance.

164. Protocols for the preparation of stable nanoscale titanium dioxide solutions in biologic media are available. One protocol uses a stock solution prepared by ultrasonication methods (Taurozzi *et al*., 2011a) followed by addition of the stock solution into either Phosphate Buffered Saline (PBS) and Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (DMEM-FBS) (Taurozzi *et al*., 2011b). Solutions of nanoscale titanium dioxide prepared according to this protocol were found to be stable under incubation conditions for up to 48 hours. A second technique involved stabilising nanoscale titanium dioxide with citrate (Ramirez-Garcia, 2011). These suspensions were shown to be stable in cell medium with up to 10% protein for up to 25 hours at 37 °C.

D.4 Routes of delivery and the behaviour of nanomaterials dispersions in mammalian studies

165. Mammalian tests can involve inhalation/respiratory tract, oral or dermal route of exposure, and some consideration of the physical behaviour of the test material is needed for each route. The following delivery methods have been employed:

i. Respiratory tract route:

Aspiration or instillation of nanoparticle suspensions in salines.

Exposure to nanomaterials in air or gas phase (inhalation studies for which there is no need to produce nanoparticles dispersions in physiological salines but in suitable low toxicity vehicles, if any, which prevent agglomeration as much as possible).). Exposure by inhalation of an aerosol of nano-sized particles: particles may be aggregated and/or agglomerated into (much) larger particles and should be assessed.

The selection of a vehicle favouring the dispersion of nanoparticles (e.g. phosphate buffer) can address adequately a hazard-driven testing approach, whereas the dry dispersion technique is more risk-driven (workplace scenario); both approaches are justified and can be selected for the intended purpose.

ii. Oral route: dosing of saline via gavage (acute or repeated toxicity testing). Phosphate buffer saline (PBS) may also be used to achieve better dispersion of the nanomaterial

iii. Dermal route: External application of salines, emulsions (e.g. ZnO nanoparticles in corn oil for a stable suspension), or creams

iv. Injection route: Test substance in appropriate buffered solution (ADME studies for example).

166. For a correct interpretation of biological effects after in vivo exposure, at least qualitative evidence should be provided that nanomaterial reached (or not) the organ under investigation (Blank *et al.*). Where techniques are available, some attempts could be made to measure effective mass doses to target organs, for instance, measuring metal concentrations in case of metal nanoparticles. . In inhalation studies, the aerodynamic data of the aerosol (mass median aerodynamic diameter[16] - MMAD and aerosol concentration) allow a prediction of the target mass doses using reliable models (MPPD model), other models are based on the CMD and a measure of the distribution shape, as often aerosols have wide particle size distributions. Alternatively, chemical analysis and in special cases radio labelling can be used to determine actual mass doses. In addition, body weights should be monitored throughout the experiments

[16] An important distinction is the difference between the geometry of nanomaterials and the aerodynamic diameter. The latter is important for the estimating where the dose will be deposited, the former may be important for the interaction between a nanomaterial and a biological system (cells).

and doses should be adjusted with body weights in order to maintain constant dosing. It is possible, however, that in some cases the important dose metric might be surface area or the particle number deposited in the lung.

D.4.1 Respiratory tract exposures

167. Although much research on nanoparticle toxicity have used saline to deliver test material to the lung via intra-tracheal instillation (Pauluhn, 2009) or aspiration, inhalation is the physiological process during which (nano)particles are deposited in the respiratory tract including the nose and lungs, allowing for a slow build up of the dose and for normal clearance processes to occur. In addition, it should be noted that intratracheal instillation or aspiration are not considered in the OECD Test Guidelines and Guidance Documents for the inhalation route but there may be exemptions e.g. due to costs or time where instillation or aspiration is used. If this is the case, it needs to be emphasised that use of these methods may be acceptable to evaluate the relative toxicity of the test material when backed up by an appropriate and representative inhalation study. In addition, these methods should be supported by quality assurance testing including reproducible delivery of the nanoparticle suspension to the lungs such that nanoparticle deposition on the airway epithelium can be distinguished from that of the alveolar epithelium. Fewer studies have used inhalation exposure (i.e. breathing of particles dispersed in the air of inhalation atmospheres). A comparison of advantages and limitations of different exposure methods to respiratory tract are presented in available papers including (Madl and Pinkerton, 2009). Documented *in vitro* to *in vivo* data correlation can be seen in several publications and could be considered in place of aspiration and instillation (Geiser *et al*., 2005), (Rothen-Rutishauser B *et al*., 2008), (Jones and Grainger, 2009).

168. Overall, inhalation of aerosols, gases or vapours is the first-choice route of exposure prescribed in standard OECD Test Guidelines and this is the only way to determine the NOEL for the airborne concentration of suspended (nano)particle dust (Ji JH, *et al.*, 2007). In fact, the gold standard is inhalation studies using aerosolized engineered nanoparticles and the performance of these studies should be given careful consideration. In inhalation studies, it is important to consider not only toxicity but also potential exposure data and interaction of nanoparticles (mechanisms) with fluids, cells and tissue to allow for full characterisation of a biological response as related to Physical-Chemical properties of the nanomaterials (Ma-Hock *et al.,* 2009), (Stebounoval *et al.*, 2011). Ideally, they would include quantitative data on organ burden and distribution (see below). This in turn may be used to help select out nanomaterials with the lowest risk for given applications and help to identify control materials. The OECD repeated-dose inhalation study (28 days or 90 days) or possibly longer-term studies, which should include specific investigations (e.g. lung toxicity and cardiovascular effects), in addition to a complete histopathology, have to be preferred over the acute studies, due to the kinetics of deposition of particles in the lungs and the possible progressive building up of dose. However, determination of the administered nanoparticle dose is complicated and its estimation requires careful monitoring of breathing, of the aerosol parameters and of tissue analysis (SCENIHR, 2007). The toxicity of particulates in general and nano-structures in particular depends on their differences in the displacement volumes and the earlier attainment of lung overload. Particokinetics determines the toxicodynamics in that it influences the distribution patterns in the whole organism and the dose at the target site (Solomon *et al.*). Therefore, inhalation studies need to be designed to verify the kinetic threshold from homeostasis to adversity (Geiser *et al.*). This can be done using radiolabelled materials, chemical elemental analysis to determine organ concentrations and transmission electron microscopy. Retention and clearance also have to be considered in repeat dose studies. Overall, due to many (complex) variables associated with exposure, lung dosimetry needs to focus on cumulative lung burdens and associated particokinetics. It is important to characterize the materials and the generated aerosols by other metrics in addition to mass, e.g. surface and size distribution. The test atmosphere, e.g. with nanoparticle dust should be characterised and data (primary particle size, particle size distribution, mass concentration and number concentration and surface area) should be reported carefully so that the results can be useful for hazard and risk assessment and characterisation. For hazard assessment, aerosol

generation of nanoparticles should produce particle size distributions that allow maximum lung burden (MMAD <3µm, including agglomerates). Because repeated exposure studies typically use lower concentrations than acute studies, emphasis should be given to generating particle size distributions amenable to preferentially depositing in the lower respiratory tract, particularly for particles deemed to be innocuous and biologically "inert" in terms of their chemical reactivity. For such materials it is recommended that appropriate MMADs ranges be used in repeated exposure rodent studies to maximize lung exposure. Additional information is available in OECD Guidelines 403, 412, 413 and 436 and the associated Guidance Document GD 39 [OECD (2009)]. The OECD workshop (OECD Expert Meeting on Inhalation Toxicity; ENV/CHEM/NANO(2011)23) recommended that both OECD Guidance Document 39 and the TG collection for inhalation toxicity be updated to take into account of nanomaterials testing. Accordingly, further guidance is to be expected for the near future.

169. All technical aspects of inhalation toxicology studies, including the use of the preferred dynamic nose-only inhalation systems, are addressed in OECD Guidelines 403, 412, 413 and 436 and the associated Guidance Document GD 39 [OECD (2009)]. For inhalatory hazard assessment, particles should have characteristics, in terms of their morphology and size distribution, similar to those experienced in human exposures (but adjusted for rodent inhalabilty and respirability) and relevant to realistic human exposure scenarios. Test concentrations should cover the expected Maximum Tolerated Dose (OECD Expert Meeting on Inhalation Toxicity, 2011). However, aggregation and agglomeration seriously hamper generation of an aerosol consisting of nano-sized particles from a powder. There is a clear correlation between the number of particles in air (per cm3), the residence time and the formation of aggregates/agglomerates. So for sample preparation it is preferable to generate the aerosol as close as possible to the breathing zone of a test animal. Maximum reasonable effort should be made to aerosolize a powder by a venturi (Cheng *et al*., 1989) and further deagglomeration of the particles in the aerosol with a jet-mill (Cheng *et al*., 1985; Castranova *et al*., 1996) (Kuhlbusch *et al.*, 2011). Due to agglomeration, attainable aerodynamic particle size distributions are generally expected to be in the 0.3 – 3 µm range, but aerosols consisting of single nanoparticles cannot be expected. If relevant, aerosols with smaller particles can be generated for sufficiently diluted dispersion of nanoparticles (preferable in water; Mahurin and Cheng, 2007, Jing-CAST Technology GmbH, 2002) or by vaporisation and subsequent condensation. Alternatively, nanoparticle suspensions in suitable vehicles (e.g. PBS) can be used to generate mixed interim particles that after deposition in the respiratory tract easily break down to release the insoluble nanoparticles.

Table 2: Examples of Techniques for Generation of Exposure Atmospheres

Dispersion technique	**Vehicle**	**Characterisation of exposure atmosphere**	**Devices**
Risk-oriented approach (agglomerates; >100nm)			
Dry dispersion with pressurised air	Clean air	Aerosol concentration (particle mass; particle number); MMAD	Jet mill, venturi, rotating brush generator, Wright Dust feeder, ceramic electrical heater
Nebulisation of a liquid formulation	e.g. phosphate buffer	Aerosol concentration (particle mass; particle number); MMAD; nanoparticle-specific chemical analysis of filter samples for determination of dose	Jet nebuliser
+CNT: acoustic feeder system (subacute, sub chronic testing)	Clean air	Aerosol concentration (particle mass); MMAD : to be calculated based on filter samples/SEM	Membrane system to bring the individual/respirable agglomerates of CNT

			into the airborne state
CNT: Nebulisation of a liquid formulation (acute testing)	DPPC, BSA, Glucose solution	Aerosol concentration (particle mass); MMAD : to be calculated based on filter samples/SEM	Ultrasonic finger facilitating proper dispersion; no vortexing
Hazard-oriented approach (small agglomerates; < 100nm)			
Spark generation (abrasion of a metal electrode)	Argon, clean air	Aerosol concentration (particle number) ; mean mobility diameter	Electrical mobility spectrometer

170. It is important to distinguish the influence of the geometry of nanomaterials from the aerodynamic diameter on the behaviour of the nanomaterials. The latter is important for estimating where the dose will be deposited, the former may be important for the interaction between a nanomaterial and a biological system (cells).

171. Dry powder generation methods are to be preferred and attention has to be paid to respirability of the aerosols by rats when translating workplace aerosol measures to experiments. Using a pristine aerosol, i.e., without alteration of the particle surface, can best mimic the occupational situation. The overall aim should be to avoid artefacts where modified surfaces exhibit a modified toxic response in the biological test. These large particles can be removed from the test atmosphere upstream of the exposure chamber using a pre-selector such as an impactor. Therefore, in general, the aerosol can be characterised with the usual instruments, i.e., cascade impactor or other instruments, based on mass and inertial forces. However, for the toxicity of nano-sized particles mass based dose metrics may not be optimal and the number of particles or the total surface area may be more relevant. Mass based instruments are insensitive to nano-sized particles because of their low weight (e.g. 1 million 10 nm particles may have the same weight as one 1μm particle). Therefore, if separate nano-sized particles can be expected to be present, mass based instruments to characterize the concentration and particle size distribution should have to be supplemented with instruments based on counting individual particles (e.g. Scanning Mobility Particle Sizer (SMPS), Electrical Low Pressure Impactor (ELPI)). The concentration and the size distribution of surface area can be estimated from the particle size number distribution, though such calculations are to be interpreted with caution as these approaches can underestimate surface area relative to direct measurement by gas adsorption (Weibel *et al.,* 2005). In addition, separate instruments are available to estimate the deposited particle surface area per part of the human respiratory system (Weibel *et al*., 2009). Direct measurement of particle surface using gas adsorption (so-called BET surface area) has historically been applied to the bulk powder material. However, LeBouf *et al.* (2011) has reported a method for quantification of airborne surface area of nanomaterials for inhalation toxicology exposures, which uses gas adsorption. It is also suggested to characterise the morphology of produced particles. In addition, separate integrative reading instruments based on diffusive charging of submicrometer particles are available to derive "active" or lung-deposited surface area concentrations. The readings are based on calibrations as well as some assumption as examples discussed in Asbach *et al*. (2009).

172. Prior to the study, the test substance should be characterised comprehensively as recommended in previous chapters.

173. If it is necessary to use a vehicle to generate an appropriate concentration and particle size, water or a physiological buffer e.g. PBS should be given preference as these formulation tools can help to de-agglomerate the initially bigger agglomerates. Constancy and homogeneity of atmospheric concentrations of the tested particles should be ensured. If a vehicle other than water is used, the concentration of the vehicle in the atmosphere should be determined by an appropriate method (e.g. gas chromatography).

174. The flow of filtered air through the exposure chamber/system should be carefully controlled, continuously monitored, and recorded at least hourly during each exposure. Details on this and on the exposure chamber conditions can be found in the inhalation toxicity test guidelines including ISO10801 (2010) Nanotechnologies – Generation of metal nanoparticles for inhalation toxicity testing using the evaporation/condensation method.

175. The nominal concentration is the mass of nanomaterial introduced into the test atmosphere generation divided by the total volume of air passed through the inhalation exposure system, and generally should not be used to characterise the animals' exposure since the nominal concentration will be higher than the actual concentration in the animal's breathing zone in test chamber due to factors such as material impaction and settling in the particle generator and/or on the surfaces in the test chamber, .

176. The actual concentration, which is the nanomaterial concentration as sampled from the animals breathing zones in an inhalation system should be measured and reported. For non-volatile single component nanoparticles, the actual concentrations can, in some cases, be obtained by non-specific gravimetric filter analysis. For multi-component aerosols, concentration may also, in some cases, be determined by gravimetric analysis. However, this requires analytical data, which demonstrate that the composition of airborne material is homogenous and similar to that of the starting material. The range of exposure concentrations should possibly cover a range from the Maximum Tolerated Dose (MTD) down to doses relevant for human exposures. The MTD is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. However, the concept of Maximum Tolerated Dose (MTD; based on mass) may not apply to nanomaterials, and it is recommended to use concentrations that do not exceed 2-3 order of magnitude of a worse case exposure scenario based on either mass or particle number per volume of air. Although it is unlikely that overload situations can be achieved for particle distribution in the nanometre range due to the fact that high concentrations will lead to a shift to the right of the particle size distribution due to aggregation, overload situations in rodents need to be avoided. (Levy (1995), Inhal. Toxicol. (2000)). It should be realised that for nanoparticle exposure the maximal dose may be limited by the maximum concentration that can be obtained in the dispersion solution.

177. The exposure atmosphere should be held as constant as possible. The methods for monitoring this and the allowed deviation ranges are described in the OECD inhalation toxicity test guidelines.

178. The particle size distribution of aerosols should be determined at least once during the study for each concentration level by using an appropriate measurement method. To improve this process the determination could be increased to once per day and before and after the study. The total mass concentration obtained by particle size analysis should be within reasonable limits of the mass concentration obtained during concentration control analysis. In addition to mass concentration, particle number concentration may be measured using instruments such as condensation particle counter (approximate range: 10 to 1000 nm). Size distribution may be measured using optical particle counters (approximate range: 300 to 20,000 nm) to assess presence of agglomerates and/or aggregates of nanoparticles and larger diameter particles of nanostructured materials. To further characterise the presence of primary nano-particles in the inhalation atmospheres a differential mobility analysing system (DMAS) should be used. Some experts have also mentioned condensation particle counting as a suitable possibility (CPC) in some cases.

179. Other exposure techniques involve direct delivery to the respiratory tract in a liquid, so there is a need to disperse the nanoparticles for intratracheal instillation or laryngeal aspiration or for delivery to other sites, such as peritoneal cavity, skin or gut. A number of approaches have been published for preparing salines, including those used for cells/tissues in culture. Some studies (Warheit *et al.,* 2007), including influential studies from the US National Institute for Occupational Safety and Health (Shvedova

et al., 2005; 2008) have not used any dispersant other than Ca2+ + Mg2+-free phosphate-buffered saline (PBS) with sonication. Other techniques for dispersion of nanoparticles in biocompatible fluids for in vitro and in vivo studies of the nanoparticle–biology interaction are also emerging (Ramirez-Garcia *et al.*, 2011). All mammals have albumin as a ubiquitous protein and it is well conserved in evolutionary terms. Dispersal in bovine serum albumin (BSA) has therefore been used for intraperitoneal injection of nanotubes (Poland *et al*., 2008), although some experts have indicated that it would be preferred to use serum of the exposed species to avoid risks of immune response. One study has used the first BALF (Bronchoalveolar Lavage Fluid) obtained from normal rats to suspend the nanoparticles in before injecting back into rats (Sager *et al*., 2007; 2008). Although BALF is an effective dispersion medium, its utility is constrained by several factors.

180. First, a separate set of naive animals must be used to obtain BALF and as such requires a significant economic investment. Secondly, intra- and inter-laboratory variability in BALF is a problem due to differences in many factors, including animal handling, anaesthesia, and BAL technique, as well as the inherent complex composition of BAL fluid. Some guidance is provided in the OECD Guidance Document no 39 on Inhalation Toxicity Testing. Because of these factors, an alternative nanoparticle dispersion medium based on BALF was developed. This alternative nanoparticle dispersion medium (DM) is essentially a BALF fluid mimic, i.e., Ca2+ and Mg2+-free phosphate buffered saline (PBS) containing serum albumin and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), a lung surfactant (Porter *et al*., 2008). It is important to note that DM mimics components of the lung alveolar lining fluid important to nanoparticle dispersion, i.e., protein and surfactant, but at concentrations much lower than those present in lung alveolar lining fluid (Schürch *et al*., 1990; Gehr *et al.*, 1990). The choice for the right approach highly depends on the physicochemical characteristics of the specific nanomaterial and the administration method. It should be recognized that every dispersant can have an influence on dispersion and toxicity and pros and cons should be weighed. Every dispersant can have an influence on dispersion and toxicity, and pros and cons should be weighed. Overall, permanent online control of dispersion state is strongly recommended. It is crucial to assure that dispersed particles are nanoscale and that dispersion rate is not lowered over time by agglomeration. A number of chemical surfactants have also been used to disperse nanoparticles prior to instilling into rat lungs, including Pluronic-F68 (Mangum *et al*., 2006) and Tween (Warheit *et al*., 2004).

181. Aspiration of fine sprays may present some practical problems in terms of blocking spray equipment and achieving a precise quality of spray (and dose). The alternative is to use an instillation (essentially a gavage-like "injection") of saline that gently delivers the dose to the bronchi and lung. There are disputes about the level of penetration of each direct delivery method into the alveolar region of the lung, but the latter direct delivery method has less practical problems, and may become a more pragmatic direct delivery method for comparative hazard assessment.

182. Nevertheless, exposure via instillation is not considered as physiological because it usually results in exceedingly high dose and application rate and, since the particles are suspended in saline, the lung surface receives particles contained in a liquid, which is likely to affect the defence systems of the lung. In addition, instillation produced a less even particle deposition pattern in the lung than the inhalation method (Driscoll *et al*., 2000) and efforts need to be made to disaggregate the nanomaterial suspended in vehicle (Driscoll *et al*., 2001). The advantage of instillation is the administration of a more precise nanoparticles dose. Pharyngeal aspiration is a variant of instillation, still with a high dose, but the particles are in suspension. In this case, the exposure is to suspension droplets that disperse in the lung more readily than with simple instillation. Results with instillation and pharyngeal aspiration are rather similar in terms of allowing comparison in toxic potency between particle types. Both can be used for the oropharyngeal region down to the sterile alveolar region in the context of screening purposes and for mechanistic studies. However, a concern with the pharyngeal aspiration technique for pulmonary exposure, in addition to ensuring disaggregation of the suspended material in the vehicle, is the unintentional aspiration of food particles from the oral cavity during this procedure (Rao *et al.* (2003)). There is anecdotal evidence, which

is not reported by all laboratories, that alveolar inflammation induced by bacterial rinsing has been an undesired effect of pharyngeal aspiration in rats. In the mouse, there are numerous published studies demonstrating a lack of alveolar inflammation in vehicle-exposed mice after pharyngeal aspiration. With regards to the "unusually high doses to bronchioles", this may likely be an observation related to lack of adequate nanoparticle dispersion and/or morphology of a particular nanoparticle, and not a general effect of pharyngeal aspiration. However, neither method can be used to determine NOEL (SCENIHR, 2007).

183. Data analysis should include information on number of particles, volume of vehicle, particle concentration (Krug, H.F. and P. Wick, 2011) and also interpretation of aerosol characteristics, NOAEL, risk assessment implications, mode of action and a strategy for dosimetric extrapolation to humans. In addition, the inclusion of kinetic data is important. Overall, caution is advised on interpreting results of studies in which exposure is made by intratracheal instillation, and these are not recommended as the primary source of information but in a WoE (Weight of Evidence) or categorisation approach.

D.4.2 Oral Exposure

184. The acute regulatory tests use a gut gavage dosing (instillation into the stomach) of typically 10 or 20 ml of saline/kg body weight of laboratory animal (rat from 20 ml/kg to 50 ml/kg; mouse 20 ml/kg or 30 ml/kg. [17]This volume is designed to deliver a dose comfortably to the stomach of the animal without dilatation of the stomach. Chronic studies of dietary exposure are best performed by feeding the nanoparticles in a diet to the animal. Because nanomaterials are known to be carriers of contaminants, it is possible that the results of feeding studies will be affected by the food composition. Few published studies describe exposure via the digestive tract by dietary intake of NP contaminated food, but the necessity to incorporate the nanoparticles into diet means that considerations of aggregation/agglomeration may be secondary.

185. However, the techniques for manufacturing food often include a step where the test substance is sprayed into the feed mixture as it is blended, or used as a topcoat on the feed. In either case, a stock dispersion would be required (as above) and the aim would be to ensure a uniform spread of the dose in the resulting food pellets. Where possible, the dose should be measured in the food produced, along with the usual nutritional analysis of the feed. Storage and degradation of the feed may be an issue, especially with oxidising NPs (rancid food). The palatability of the food should also be considered. It may also be possible that nanoparticles cause secondary toxic effects by reducing the bioavailability or digestibility of the feed ingredients by adsorption processes or other effects. Additionally, the chemistry within the digestive tract and enterohepatic circulation needs to be considered. The low pH of the stomach is likely to have effects on any protein pre-coating and the general effects of the stomach milieu on nanoparticles could be to disperse them or agglomerate them, regardless of pre-treatments. The digestive tract is also a high ionic strength environment, containing mucus and other soluble proteins and special microbial environment. The chemistry is likely to be very complex, and not likely to be easy to predict from theory alone, and observational experiments are needed on nanoparticle bioavailability from different food matrices. The influence on the amount of bile excreted and enterohepatic circulation also should be considered as this can influence the absorbed dose. In-vitro models simulating GI-tract environment can be used to evaluate the interaction of nanomaterials with GI-tract liquids.

186. Administration of nanomaterials via the drinking water has been reported for repeated dose toxicity testing (Trouiller *et al*., Cancer Res 69: 8784-8789, 2009). This method is in general accordance to

[17]OECD TG407 notes that the volumne should not exceed 1 ml/100g body weight except in the case of aqueous solutions where 2 ml/100g body weight may be used. In addition, OECDTG423 notes that in rodent, the volume should not normally excveed 1ml/100g of body weight: however in the case of aqueous solutions where 2 ml/100g body weight can be considered.

oral repeated dose toxicity OECD test guidelines (TG 407, 408). However, care has to be taken to prevent sedimentation. In addition, Ca, Mg and total hardness of water should be measured and stability of NP dispersion should be checked over time (Kim *et al*., 2010) (Park *et al.*, 2010) and stability should be checked. In case of photosensitive or photoactive materials - light exposure, in this instance capsules or other oral dosage forms could also be considered.

D.4.3 Dermal Exposure

187. Dermal exposure to nanoparticles may occur in the workplace environment or via consumer products (e.g. cleaning agents, cosmetics, personal care products, textiles). In consumer products such as personal care products and cosmetics, the nanoparticles are usually dispersed in some excipient, such as glycerol, which allows the particles to be applied to the skin (Mortensen *et al*., 2008). With respect to textiles, the question is whether the nanoparticles can be leaching out of the product during use (after contact with sweat or saliva). It has been argued that, if nanoparticles are considered to resemble macromolecules of high molecular weight, skin absorption is considered unlikely. This expectation was verified in the Nanoderm project especially for TiO2 or studies with nanoparticles in cosmetic formulations (Gamer *et al*., 2006). Nevertheless, it cannot be excluded that other nanomaterials might penetrate the skin (Mortensen, Oberdörster *et al*., 2008). According to the Scientific Committee on Cosmetic Products (SCCP, 2007) nanomaterial constituents may act as penetration enhancers by penetrating individually into the stratum corneum and subsequently altering the intercellular lipid lamellae within this skin layer. In addition, nanomaterials may serve as a depot for sustained release of dermally active compounds. In addition, openings of hair follicles are compatible with particulate dimensions. The discussion herein has focused on particle penetration through intact skin barrier; however, if the barrier is compromised, penetration may be a factor of four or more compared to intact skin (Filon-Larese *et al*., 2009). Therefore, it is not unreasonable to anticipate a size dependent phenomenon, whereby particles lodged within the appendageal openings may allow increased diffusion for ingredients. Additionally, nanoparticles may have increased substantivity in skin "furrows" and may not be efficiently removed by standard cleaning procedures. It has been demonstrated that spherical and elliptical quantum dots penetrate the stratum corneum and localize within the epidermal and dermal layers. If the skin is exposed to large nanoparticle doses, even small fractions may become important to accumulating secondary target organs.

188. Methods for performing skin absorption studies are given in OECD Guidelines 427 (in vivo) and 428 (in vitro) and Guidance document 28 as well as OECD Guidance Notes on Dermal Absorption 156. Related relevant information can also be found in ??? (Oberdörster *et al*., 2005). The state of agglomeration is not easily studied in the assessment of skin absorption in vivo. Because of this and additional scientific and ethical issues related to the in vivo skin absorption guideline, it is preferable to use OECD TG 428 (Skin Absorption: In vitro Method). OECD TG 428 uses human skin samples as a component of the skin absorption testing strategy. This in vitro technique is already being applied to nanomaterials using human skin in vitro (Mavon *et al*., 2007; Baroli *et al*., 2007; Wissing and Mueller 2002). The skin samples are used in vitro to assess nanoparticle absorption through the skin by using Franz-type diffusion cells or the Saarbruecken penetration model. If, despite the previous considerations about the preference for the in vitro method (TG 428), the in vivo test is performed (TG 427), it is necessary to consider that one assumption in the in vivo dermal test is that the test material has reasonably good access to the skin under the fur or hair of the animal. It would be up to the experimenter to ensure this is the case. For example, does the test material agglomerate on fur, and not reach the skin in appreciable quantities? Does shaving a small area of skin eliminate this problem, or would this add unnecessary skin sensibility? This issue may be important given the known inflammatory effects of some particles in epithelial tissues. On the other side, when hairy skin is shaved or depilated before treatment, there is an additional risk of damage to barrier function exacerbating further the problem of reliably assessing nanoparticle absorption (SCCP, 2007). The use of hairless animal models (genetically modified) might overcome these practical issues, but would also create a significant ethical issue. Moreover, hairless mouse

skin does contain abnormal hair follicles, which may give rise to false-positive results. If a dispersing agent or solvent has been used to make the test solution or cream (emulsion), then a solvent control should be included to account for any irritating effects of these reagents, or their ability to alter the intrinsic permeability of the skin. The precise location on the skin should be stipulated (e.g. ear, precise region on the abdomen or thorax) because the thickness and sensitivity of the skin will change at different locations. The same location must be used on all the test animals. Since licking of treated skin might affect exposure, location should be chosen taking this issue into account. For the same reason, animals should be individually caged.

189. Delivery of a material to skin is an important consideration for toxicity studies. Generally, a substance is dispersed in a vehicle to facilitate homogeneous delivery to the skin. A wide range of vehicles have been used in dermal toxicity studies including water, ethanol, toluene, olive oil, petrolatum, propylene glycol, acetone/olive oil co-solvents, etc. The effect of vehicle on penetration rate may be dependent upon the test substance. For example, Bonnist *et al*. (2011) reported that penetration rates of cinnamaldehyde varied depending on the vehicle. Only one study was identified that investigated the effect of vehicle on penetration of nanoparticles. Labouta *et al*. (2011) studied four model gold nanoparticles of diameter 6 and 15 nm and reported that the vehicle (toluene or water) had minimal effect on skin penetration. It is important to note that nanoparticles that contact skin may not be suspended in liquids such as water or toluene rather, upon deposition materials, will be immersed in the aqueous sweat/oily sebum mixture on the human skin surface. Filon-Larese *et al*. (2009) used an artificial human sweat to disperse silver nanoparticles for in vitro skin penetration studies, though the effect of vehicle was not investigated in this study. Hence, consideration should be given to the choice of vehicle and its biological relevance and to a realistic exposure scenario when performing penetration studies.

190. In a study to investigate the effect of skin barrier integrity on nanoparticle penetration, Filon-Larese *et al*. (2009) lightly scored human donor skin using the tip of a syringe needle. Penetration of silver nanoparticles was compared for this damaged skin relative to intact skin using an in vitro Franz cell technique. As noted above, penetration of nanoparticles was a factor of four higher through damaged skin relative to intact skin. It is important to note that the skin samples used in this study were only lightly scored with a syringe, which disrupted the stratum corneum. It is not unreasonable that nanoparticles (or their agglomerates) may come into contact with skin that is damaged deeply to the underlying viable epidermis (e.g. open cuts or abrasions) and thus easily cross the skin barrier.

191. The concept of particle penetration through intact skin has been highly controversial. An early pilot study by Tan *et al*. (1996) suggested TiO2 could penetrate intact skin. In a later study, Tinkle *et al*. (2003) observed in vitro that polystyrene latex spheres <1 μm in diameter, when coupled with flexing motion, could penetrate intact human skin. However, many subsequent studies, including a study using the highly sensitive Zn-65 label (Fraunhofer ITEM 2011, unpublished) have shown, in agreement, that penetration of TiO2 and ZnO nanoparticles into skin is not likely (Cross *et al*., 2007). The reason for the observed disagreement among studies may be due to methodological issues. Rouse *et al*. (2007) demonstrated, in a study with quantum dots, that if the dots were functionalized and the skin sample was subjected to a flexing motion of skin, the testing material penetrated the skin within several hours. However, only limited penetration was observed for non-functionalised quantum dots in the absence of skin flexing. This might indicate that quantum dots penetration may occur if skin is subjected to flexing motion but only to a limited degree or not at all in the absence of flexing, whether this result is extrapolable to other nanomaterial types is not yet clear.

192. The ability of particles to penetrate diseased skin has not been studied in depth. No literature on the topic could be found; however, it is well known that diseased skin has different barrier properties from healthy skin, which could potentially influence penetration. For example, passive diffusion of water through the stratum corneum is referred to as trans-epidermal water loss (TEWL) and provides information

on changes in the stratum corneum water barrier function, which can be used as a marker of skin integrity. Generally, TEWL values are higher for diseased skin from conditions such as icthyosis, psoriasis, erthoderma, atopic dermatitis, and allergic dermatitis compared to healthy skin (Tagami *et al*., 2002; Giorgini *et al*., 1996; Lavrijsen *et al*., 1993; Grice *et al*., 1967).

D.4.4 Injection routes

193. Injections into circulation, tissues, or body cavities are generally used as administration routes in ADME studies[18].. This is usually done with formulations dispersed in saline, or in the case of a very hydrophobic material, in lipophilic vehicles like corn oil. In addition to the considerations on salines above, one concern is the behaviour of the nanoparticles in the syringe. Micro bubbles in the syringe can act as precipitation surfaces, and so it would be important to avoid creating bubbles by good dispensing skills, and also not allow the syringes to sit for too long where micro bubbles may form on the inner surface of the syringe. It is also important to consider that the actual dose may depend on nanoparticle interaction with the wall material. The gauge of needle should be sufficient to enable a smooth injection without blockage of the syringe. It may be that larger needle sizes are needed for some nanomaterials preparations, depending on concentration/viscosity. Additionally, we must not exclude the possibility that nanoparticle injections may be very painful because the materials are reactive; therefore, animal welfare should be paramount. A precautionary anaesthesia may be advised regardless of needle size.

194. Lack of dermal penetration and hence false negative results have to be taken into account when performing regulatory testing such as skin sensitisation. For instance, the local lymph node assay (LLNA, OECD TG 429 requires access to dendritic cells in living skin layers.

D.4.5 Cell cultures and dispersion of NPs in culture media

195. In vitro techniques with nanomaterials are discussed in more detail in documents prepared for SG7 for the OECD Working party. There are a number of in vitro test systems including cytotoxicity tests, mutagenesis tests, cell/tissue culture screening assays, and immunotoxicity assays, etc. Most, if not all of these test systems, rely on using a saline solution or a much more complex culture medium. It is inevitable that test materials will agglomerate in some commonly used culture mediums (Vevers and Jha, 2008) and increase the direct contact of the cells with the test material (J.G. Teeguarden *et al.*, 2007). It might be possible to use soluble peptides or other organic ligands to act as dispersing agents in cell culture medium. While this may be a good idea from the perspective of nanomaterial dispersion, it may be very problematic from a biological perspective. Biological systems may see these added materials as "antigens" or the material may change the unstirred layer chemistry on the cell membrane that defines how the material interacts with the cell surface, or even adhesion of the cells to the culture plate (also used as an end point in some tests). Culture media often contain proteins, and it may not be necessary to add a "special" dispersing agent. For example foetal bovine serum (FBS) is commonly added to cell culture systems, and contains the ubiquitous mammalian protein albumin (bovine serum albumin; BSA) and so it is already present at high concentration in cell cultures that contain serum. BSA, because of its zwitterionic nature (contains both positive and negative charges), is a useful dispersant that changes the balance of protein in a cell culture very little (Bihari *et al.*, 2008; Poland *et al*., 2008). It is likely that dispersing in a protein like BSA will aid in prevention of false positive toxicity engendered by the adsorption of nutrients from the culture medium onto nanoparticle surfaces and which may cause toxicity by nutrient depletion (Casey *et al*., 2008, Orts-Gil *et al*, 2011). Once dispersed in BSA, the nanoparticle surface should be changed/altered

[18] In the case of nanomaterials, distribution may differ substantially from inhalation (not only quantitatively with respect to time and concentration). For instance, brain entry via the olfactory bulb is bypassed by injections as is the passage through different body compartment that may differentially contribute to the corona (Oberdörster, Journal of Internal Medicine 267; 89–105, 2010).

and much less able to absorb nutrient proteins from solution. Furthermore, interaction with biomolecules may mask or modify specific surface properties of environmental nanomaterials or - in turn – environmental nanomaterials binding may affect the structure of the biomolecule.

196. Another issue with using serum or BSA is that such medium components inevitably contain a number of unknown ingredients (peptides, fatty acids, sugars etc) that vary with the batch of serum or BSA used. It is possible to buy high purity BSA, or chemically defined media that have been manufactured from non-animal sources where all the components of the media are known. A dispersion approach in studies with lung epithelial cells uses a surfactant lipid found in the lung lining fluid, called dipalmitoylphosphatidylcholine (DPPC, Herzog *et al*., 2008; Wallace *et al*., 2007) usually as an addition to BSA or serum. This is obviously favoured for studies modelling the lung.

197. Whichever dispersing agent or method is used, a balance must be sought between using a specific dispersion method and finding a realistic in vitro system with quality. It can be argued that the effects of serum or tissue-specific natural surfactants like DPPC should be accepted as part of the particle behaviour, especially since body fluids consist of a myriad of proteins, peptides etc, and additions of serum to culture medium would merely give a better reflection of what would happen in vivo.

198. Though still in a developmental stage, air-liquid interphase (ALI) test systems may provide a promising alternative approach to conventional cell culture assays for specific investigations with regard to pulmonary effects (Savi *et al.*, 2008, Paur *et al*., 2011). ALI is based on aerosol exposure of lung cell cultures thus preventing unwanted interactions of nanomaterial with media components and allowing a more realistic dosing and maybe a better control of particle agglomeration. Characterisation of the aerosol in such a system remains important, availability of these systems may be limited, and exposure concentrations may be challenging to control

199. When handling photoactive nanomaterials measures should be taken to prevent light exposure in cell culture assays. Above a critical particle size threshold (hydrodynamic diameter > 40 nm) sedimentation will predominate over diffusion. Accordingly, a dose normalized to surface area of bottom-adherent cells - considering typical incubation periods of up to 24 h - may overestimate the actual cellular dose of nanomaterial (and hence particle-cell interactions) that partition primarily via diffusion(Cho *et al*., 2011).

D.5 References

Asbach, C., Fissan, H., Stahlmecke, B., Kuhlbusch, T.A.J., and Pui, D.Y.H. (2009), "Conceptual limitations and extensions of lung-deposited Nanoparticle Surface Area Monitor (NSAM)", *Journal of Nanoparticle Research* 11, pp. 101-109.

Baroli B, Ennas M, Loffredo F *et al*.(2007), "Penetration of metallic nanoparticles inhuman Full thickness skin", *Society for Investigative Dermatology* 127, pp. 1701-1712.

Barthazy, E., Stetzer, O., Derungs, C., Saathoff, H., and Lohmann, U. (2006), "Water Uptake Of Soot Particles Emitted From a Jing-CastSoot", 20ETH, Zurich, Switzerland; 12th Conference on Cloud Physics

Bihari, P., Vippola, M., Schultes, S., Praetner, M., Khandoga, A.G., Reichel, C.A., Coester, C., Tuomi, T., Rehberg, M., and Krombach, F.(2008), "Optimized dispersion of nanoparticles for biological in vitro and in vivo studies", *Particle and Fibre Toxicology* 5, pp. 14.

Blank, F., Rothen-Rutishauser, B.M., Schurch, S., Gehr, P. (2006), "An Optimized In Vitro Model of the Respiratory Tract Wall to Study Particle Cell Interactions", *Journal of Aerosol Medicine* Vol 19 (3), pp.392-405.

Blank, F., Rothen-Rutishauser, B., and Gehr, P. (2007), "Dendritic Cells and Macrophages Form a Transepithelial Network against Foreign Particulate Antigens", *American Journal of Respiratory Cell and Molecular Biology*Vol 36, pp. 669–677

Blank, F., von Garnier, C., Obregon, C., Rothen-Rutishauser, B., Gehr, P., and Nicod, L. (2008), "Role of dendritic cells in the lung: in vitro models, animal models and human studies"*, Expert Review of Respiratory Medicine* 2, pp.215–8.

Bonnist, E.Y., Gorce, J.P., Mackay, C., Pendlington, R.U., Pudney, P.D. (2011), "Measuring the penetration of a skin sensitizer and its delivery vehicles simultaneously with confocal Raman spectroscopy", *Skin Pharmacology and Physiology* 24(5), pp.274-283.

Bouwmeester, H., Lynch, I., Marvin, H.J.P., Dawson, K.A., Berges, M., Braguer, D., Byrne, H.J., Casey, A., Chambers, G., Clift, M.J.D., Elia, G., Fernandes, T.F., Fjellsbo, L.B., Hatto, P., Juillerat, L., Klein, C., Kreyling, W., Nickel, C., Riediker, M., Stone, V. (2011), "Minimal analytical characterization of engineered nanomaterials needed for hazard assessment in biological materials", *Nanotoxicology* 5, pp.1-11 .

Boverhof, D.R., David, R.M. (2010), "Nanomaterial characterization: Considerations and needs for hazard assessment and safety evaluation", *Analytical and Bioanalytical Chemistry*396, pp.953-961.

Casey, A., Herzog, E., Lyng, F.M., Byrne, H.J., Chambers, G., and Davoren, M. (2008), "Single walled carbon nanotubes induce indirect cytotoxicity by medium depletion in A549 lung cells" , *Toxicology Letters* 179, pp.78-84.

Castranova, V., Vallyathan, V., Ramsey, D.M., McLaurin, J.L., Pack, D., Leonard, S., Barger, M.W., Ma J.Y., Dalal N.S., and Teass, A. (1997), "Augmentation of Pulmonary Reactions to Quartz Inhalation by Trace Amounts of Iron-containing Particles", *Environmental Health Perspectives* Vol 105, Supplement 5.

Cho, E.C., Zhang, Q. and Xia, Y. (2011), "The effect of sedimentation and diffusion on cellular uptake of gold nanoparticles", *Nature Nanotechnology* 6, pp.385.

Cheng, Y.S., Marshall, T.C., Henderson, R.F., and Newton, G.J. (1985), "Use of a jet mill for dispersing dry powder for inhalation studies", *American Industrial Hygiene Association Journal* 46 pp.449-454.

Cheng, Y.-S., Barr, E.B., and Yeh, H.C. (1989), "A Venturi Disperser as a Dry Powder Generator for Inhalation Studies", *Inhalation Toxicology* 1(4), pp. 365-371

Clinton Jones, David W. Grainger. (2009), "*In vitro* assessments of nanomaterial toxicity", *Advanced Drug Delivery Reviews* 61(6) , pp. 438–456.

Crane, M., Handy, R. D., Garrod J., and Owen R. (2008), "Ecotoxicity test methods and environmental hazard assessment for engineered nanoparticles", *Ecotoxicology* 17, pp.421–437.

Crane, M., and Handy, R. D. (2007), "An assessment of regulatory testing strategies and methods for characterising the ecotoxicological hazards of nanomaterials", Report for Defra, London, UK. At: http://randd.defra.gov.uk/Document.aspx?DocumentID=2270/ (Accessed in September, 2012)

Domingos RF, Baalousha MA, Ju-Nam Y, Reid MM, Tufenkji N, Lead JR, Leppard GG and Wilkinson, K.J. (2009), "Characterizing manufactured nanoparticles in the environment: multimethod determination of particle sizes", *Environmental Science and Technology* 43(19), pp.7277-84.

Driscoll, K.E., Costa, D.L., Hatch G., Henderson, R., Oberdörster, G., Salem, H., and Schlesinger R.B. (2000), "Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: Uses and limitations", *Toxicological Sciences* 55, pp.24-35.

Driscoll KE, Howard BW, Carter JM, Janssen YM, Mossman BT, Isfort RJ (2001) "Mitochondrial-derived oxidants and quartz activation of chemokine gene expression", *Advances in Experimental Medicine and Biology* 500, pp.489-496.

European Food Safety Authority. (2011), "Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain", *EFSA Journal* 9, pp.2140.

Filon-Larese, F., D'Agostin, F., Crosera, M., Adami, G., Renzi, N., Bovenzi, M., and Maina, G. (2009), "Human skin penetration of silver nanoparticles through intact and damaged skin", *Toxicology* 255, pp.33-37.

Gamer, A.O., Leibold, E., and van Ravenzwaay, B. (2006), "The in vitro absorption of microfine zinc oxide and titanium dioxide through porcine skin", *Toxicology in Vitro* 20(3), pp.301-7.

Gangwal, S., Brown, J.S., Wang, A., Houck, K.A., Dix, D.J., Kavlock, R.J., Cohen Hubal E.A. (2011), "Informing selection of nanomaterial concentration", *Environmental Health Perspectives*119, pp.1539-46.

Gehr, P., Schurch, S., Berthiaume, Y., Im Hof, V., and Geiser M. (1990), "Particle retention in airways by surfactant", *Journal of Aerosol Medicine* 3 pp.27-43.

Gehr, P., Green, F.H.Y., Geriser, M., Im Hof, V., Lee, M.M., and Schürch, S. (1996), "Airway Surfactant, a Primary Defense Barrier: Mechanical and Immunological Aspects", *Journal of Aerosol Medicine* 9(2), pp.163-181.

Geiser, M., Rothen-Rutishauser, B., Kapp, N., Schürch, S., Kreyling, W., Schulz, H., Semmler, M., Hof, V.I., Heyder, J., Gehr, P. (2005), "Ultrafine Particles Cross Cellular Membranes by Nonphagocytic Mechanisms in Lungs and in Cultured Cells", *Environmental Health Perspectives*113, pp.1555-1560

Giorgini, S., Brusi, and C., Sertoli, A. (1996), "Evaporimetry in the differentiation of allergic, irritant and doubtful patch test reactions", *Skin Research and Technology* 2, pp.49-51.

Glover, R.D., Miller, J.M., Hutchinson, J.E. (2011), "Generation of metal nanoparticles from silver and copper objects: Nanoparticle dynamics on surfaces and potential sources of nanoparticles in the environment", *ACS Nano* 5, pp.8950-8957.

Grice, K.A., and Bettley, F.R. (1967), "Skin water loss and accidental hypothermia in psoriasis, ichthyosis, and erythroderma", *British Medical Journal* 4, pp.195-201.

Handy, R. D., Kammer, F. v. d., Lead, J. R., Hassellöv, M., Owen, R. and Crane, M. (2008), "The Ecotoxicology and chemistry of manufactured nanoparticles", *Ecotoxicology* 17, pp.287-314.

Hassellöv, M., Readman, J.W., Ranville, J.F., and Tiede, K. (2008), "Nanoparticle analysis and characterization methodologies in environmental risk assessment of engineered nanoparticles", *Ecotoxicology* 17, pp.344-361.

Herzog, E., Byrne, H.J., Casey, A., Davoren, M., Lenz, A.G., Maier, KL., Duschl, A., Oostingh, G.J. (2008), "SWCNT suppress inflammatory mediator responses in human lung epithelium in vitro", *Toxicology and Applied Pharmacology* 234(3) pp.378-90.

ILSI Risk Science Institute. (2000), "The relevance of the rat lung response to particle overload for human risk assessment: a workshop consensus report. ILSI Risk Science Institute Workshop Participants", *Inhalation Toxicology* 12(1-2), pp.1-17.

Ji, J.H., Jung, J.H., Kim, S.S., Yoon, J.U., Park, J.D., Choi, B.S., Chung, Y.H., Kwon, I.H., Jeong, J., Han, B.S., Shin, J.H., Sung, J.H., Song, K.S., and Yu, I.J. (2007), "Twenty-eight day inhalation toxicity study of silver nanoparticles in Sprague-Dawley rats", *Inhalation Toxicology* 19, pp.857-871.

Jing-CAST Technology GmbH (2002), Jing-CAST Burner Operation Handbook.

Kim, Y., Song, M., Park, J., Song, K., Ryu, H., Chung, Y., Chang, H., Lee, J., Oh, K., Kelman, B.J., Hwang, I., and Yu, I. (2010), "Subchronic oral toxicity of silver nanoparticles", *Particle and Fibre Toxicology* 7, pp.20.

Krug, H.F., and Wick, P. (2011), "Nanotoxicology: an interdisciplinary challenge", *Angewandte Chemie International Edition* 50(6), pp.1260-78.

Kuhlbusch1, T.A.J., Asbach, C., Fissan, H., Göhler, D., and Stintz, M. (2011), "Nanoparticle exposure at nanotechnology workplaces: A review", *Particle and Fibre Toxicology* 8, pp.22.

Labouta, H.I., El-Khordagui, L.K., Kraus, T., and Schneider, M. (2011), "Mechanism and determinants of nanoparticle penetration through human skin", *Nanoscale* 3(12), pp.4989-99.

Lavrijsen, A.P.M., Oestmann, E., Hermans, J., Boddé, H.E., Vermeer, B.J., and Ponec, M. (1993), "Barrier function parameters in various keratinization disorders: Transepidermal water loss and vascular response to heaxyl nicotinate", *British Journal of Dermatology* 129:547-554.

LeBouf, R.F., Stefaniak, A.B., Chen, B.T., Frazer, D.G., and Virji, M.A. (2011), "Measurement of airborne nanoparticle surface area using a filter-based gas adsorption method following inhalation toxicology experiments", *Nanotoxicology* 5, pp.687-699.

Levy, L.S. (1995), "Review: The 'Particle Overload' Phenomenon and Human Risk Assessment", *Indoor and Built Environment* September 1995 4: 254-262.

Madl, A.K. and Pinkerton, K.E. (2009), "Health effects of inhaled engineered and incidental nanoparticles", *Critical Reviews in Toxicolology* 39(8), pp.629-58.

Ma-Hock, L., Treumann, S., Strauss, V., Brill, S.,Luizi, F., Mertler, M., Wiench, K., Gamer, A.O., van Ravenzwaay, B., and Landsiedel, R. (2009), "Inhalation Toxicity of Multiwall Carbon Nanotubes in Rats Exposed for 3 Months", *Toxicological Sciences* 112(2), pp.468–481.

Mahurin, S. M. and Cheng, M.-D. (2007), "Generating nanoscale aggregates from colloidal nanoparticles by different aerosol spray techniques", *Nanotoxicology* 1(2), pp.130-138.

Mailänder, V., and Landfester, K. (2009), "Interaction of Nanoparticles with Cells", *Biomacromolecules* 10, pp.2379

Mangum, J.B., Turpin, E.A., Antao-Menezes, A., Cesta, M.F., Bermudez, E., and Bonner, J.C. (2006), "Single-walled carbon nanotube (SWCNT)-induced interstitial fibrosis in the lungs of rats is associated with increased levels of PDGF mRNA and the formation of unique intercellular carbon structures that bridge alveolar macrophages in situ", *Particle and Fibre Toxicology*3, pp.15.

Mavon A, Miquel C, Lejeune O, Payre B, Moretto P.(2007), "In vitro percutaneous absorption and in vivo stratum corneum distribution of an organic and mineral sunscreen". *Skin Pharmacology and Physiology* 20, pp.10-20.

Monteiro-Riviere, N.A., and Tran, C.L.(2007), "Chapter 11 Dispersion of Nanoparticles in pulmonary surfactants for in vitro toxicity studies: lessons from ultrafine diesel exhaust particles and fine mineral dusts. in 'Nanotoxicology: characterisation, dosing and health effects'", *Informa Healthcare,* New York, pp.153-172.

Monteiro-Riviere, N. and Tran, L. (2007), "Nanotoxicology: Characterisation, Dosing and Health Effects", *CRC Press*, 434 pp.

Mortensen, L.J., Oberdörster, G., Pentland, A.P., and Delouise, L.A. (2008), "In vivo skin penetration of quantum dot nanoparticles in the murine model: the effect of UVR", *Nano Letters* 8, pp.2779-2787.

Nikula, KJ, *et al*. (2001), "Influence of exposure concentration or dose on the distribution of particulate material in rat and human lungs", *Environmental Health Perspectives* 109(4), pp.311-318.

Oberdörster, G. (1995), "Lung particle overload: implications for occupational exposures to particles", *Regulatory Toxicology and Pharmacology* 21(1), pp.123-35.

Oberdörster, G., Maynard, A., Donaldson, K., Castranova, V., Fitzpatrick, J., Ausman, K., Carter, J., Karn, B., Kreyling, W., Lai, D., Olin, S., Monteiro-Riviere, N., Warheit, D., and Yang, H. (2005), "Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy", *Particle and Fibre Toxicology* 2(8), 35 pp.

Oberdörster G. (2010), "Safety assessment for nanotechnology and nanomedicine: concepts of nanotoxicology", *Journal of internal medicine*. 267(1), pp.89-105.

Oberdörster, G. (2012), "Nanotoxicology: in vitro-in vivo dosimetry", *Environmental Health Perspectives* 120(1),: pp.a13, 2012. (reply to Gangwal *et al*.)

OECD (2009), *Guidance Document on Acute Inhalation Toxicity testing, Series on Testing and assessment*, No. 39, OECD, Paris.

OECD (2011), *Recomendations from the Expert meeting on Inhalation Toxicity*, OECD, Paris. (ENV/CHEM/NANO(2011)23)

OECD (2011), *OECD Guidance Notes on Dermal Absorption*. *Series on Testing and assessment*, No. 156, OECD, Paris.

Orts-Gil, G., Natte, K., Drescher, D., Bresch, H., Mantion, A., Kneipp, J., Österle, W. (2011), "Characterization of Silica Nanoparticles prior to in vitro Studies: from Primary Particles to Agglomerates", *Journal of Nanoparticle Research* 13(4), pp.1593-1604

Osier, M. Baggs, RB, Oberdörster, G. (1997), "Intratrachael instillation versus intratracheal inhalation: influence of cytokins on inflammatory response", *Environmental Health Perspectives*105 (Suppl 5), pp.1265-71.

Park E.J., Bae, E., Yi, J., Kim, Y., Choi, K., Lee, S.H., Yoon, J., Lee B.C., and Park K. (2010), "Repeated-dose toxicity and inflammatory responses in mice by oral administration of silver nanoparticles". *Environmental Toxicology and Pharmacology* 30, pp.162.

Pauluhn, J. (2009), "Comparative pulmonary response to inhaled nanostructures: considerations on test design and endpoints", *Inhalation Toxicology* 21 (S1), pp.40-54

Paur, H.R., Cassee, F.R., Teeguarden, J., Fissan, H., Diabate, S., Aufderheide, M., Kreyling, W.G., Hänninen, O., Kasper, G., Riediker, M., Rothen-Rutishauser, B., and Schmid, O. (2011), "In-vitro cell exposure studies for the assessment of nanoparticle toxicity in the lung - A dialog between aerosol science and biology", *Journal of Aerosol Science* 42, pp.668

Poland, C.A., Duffin, R., Kinloch, I., Maynard, A., Wallace, W.A., Seaton, A., Stone, V., Brown, S., MacNee, W., and Donaldson, K. (2008), "Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study", *Nature Nanotechnology* 3, pp.423-428.

Porter, D.W., Sriram, K., Wolfarth, M., Jefferson, A., Schwegler-Berry1, D., Andrew, M. E., and Castranova, V. (2008), "A biocompatible medium for nanoparticle dispersion", *Nanotoxicology 2008.* 2, pp.144-154.

Powers, K.W., Brown, S.C., Krishna, V.B., Wasdo, S.C., Moudgil, B.M. and Roberts, S.M. (2006), "Research strategies for safety evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation", *Toxicological Sciences* 90, pp.296-303

Powers, K.W., Palazuelos, M., Moudgil, B.M., and Roberts, S.M. (2007), "Characterization of the size, shape, and state of dispersion of nanoparticles for toxicology studies", *Nanotoxicology* 1, pp.42-51.

Puri, R., Richardson, T.F., and Santoro, R.J. (1993), "Aerosol dynamics processes of soot aggregates in a laminar ethene diffusion flame", *Combustion and Flame* 92(3), pp.320

Ramirez-Garcia, S., Chen, L., Morris, M.A., and Dawson, K.A. (2011), "A new methodology for studying nanoparticle interactions in biological systems: Dispersing titania in biocompatible media using chemical stabilisers", *Nanoscale* 3(11), pp.4617-24.

Rao, G.V.S., Tinkle, S., Weissman, D. N., Antonini, J. M., Kashon, M. L., Salmen, R., Battelli, L. A., Willard, P. A., Hoover, M. D., and Hubbs, A. F. (2003), "Efficacy of a Technique for Exposing the Mouse Lung to Particles aspirated from the Pharynx", *Journal of Toxicology and Environmental Health* 66(15), pp.1441-1452.

Röcker, C., Pötzl, M., Zhang, F., Parak, W.J. and Nienhaus, G.U. (2009), "A quantitative fluorescence study of protein monolayer formation on colloidal nanoparticles", *Nature Nanotechnology* 4, pp.577.

Rothen-Rutishauser, B.M., Kiama, S.G., and Gehr, P. (2005), "A Three-Dimensional Cellular Model of the Human Respiratory Tract to Study the Interaction with Particles", *American Journal of Respiratory Cell and Molecular Biology* 32 (4), pp.281-289.

Rothen-Rutishauser B *et al*.(2008), "A newly developed in vitro model of the human epithelial airway barrier to study the toxic potential of nanoparticles", *ALTEX.*,25(3), pp.191-6.

Rothen-Rutishauser, B., Schurch, S., Gehr, P. (2007), "Interaction of particles with membranes. In The toxicology of particles". Donaldson K. andBorm P. (eds), *CRC Press LLC*, pp.139-160.

Rouse, J.G., Yang, J., Ryman-Rasmussen, J.P., Barron, A.R., and Monteiro-Riviere, N.A. (2007), "Effects of mechanical flexion on the penetration of fullerene amino acid-derivatized peptide nanoparticles through skin", *Nano Letters* 7, pp.155-60.

Sager, T.M., Porter, D.W., Robinson, V.A., Lindsley, W.G., Schwegler-Berry, D.E and Castranova, V. (2007), "Improved method to disperse nanoparticles for in vitro and in vivo investigation of toxicity", *Nanotoxicology* 1, pp.118-129.

Sager, T.M., Kommineni, C., Castranova, V. (2008), "Pulmonary response to intratracheal instillation of ultrafine versus fine titanium dioxide: role of particle surface area" *Particle and Fibre Toxicology* 5, pp.17.

Savi, M., Kalberer, M., Lang, D., Ryser, M., Fierz, M., Gaschen, A., Rička, J., and Geiser, M. (2008), "A novel exposure system for the efficient and controlled deposition of aerosol particles onto cell cultures", *Environmental Science and Technology* 42, pp.5667-5674.

Sayes, C.M., and Warheit, D.B. (2009), "Characterization of nanomaterials for toxicity assessment". *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology* 1(6), pp.660 -670.

S.E., Innes, B., Roberts, M.S., Tsuzuki, T., Robertson, T.A., and McCormick, P. (2007), "Human skin penetration of sunscreen nanoparticles: in-vitro assessment of a novel micronized zinc oxide formulation", *Skin Pharmacology and Physiology* 20, pp.148-154.

Schürch, S., Gehr, P., Im Hof, V., Geiser, M., Greene, F. (1990), "Surfactant displaces particles toward the epithelium in airways and alveoli", *Respiratory Physiology and Neurobiology* 80, pp. 17–32

Scientific Committee on Consumer Products (SCCP) (2007), "Opinion on safety of nanomaterials in cosmetic products", SCCP/1147/07, European Commission 2007, Brussels..

Scientific Committee on Emerging and Newly-Identified Health Risks (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", European Commission 2007, Brussels.

Semmler-Behnke, M., Takenaka, S., Fertsch, S., Wenk, A., Seitz, J., Mayer, P., Oberdörster, G., Kreyling, W.G. (2007), "Efficient elimination of inhaled nanoparticles from the alveolar region: evidence for interstitial uptake and subsequent reentrainment onto airways epithelium", *Environmental Health Perspectives* 115(5), pp.728-33.

Shvedova, A.A., Kisin, E.R., Mercer, R., Murray, A.R., Johnson, V.J., Potapovich, A.I., Tyurina, Y.Y., Gorelik, O., Arepalli, S., Schwegler-Berry, D., Hubbs A.F., Antonini, J., Evans, D.E., Ku, B.K., Ramsey, D., Maynard, A., Kagan, V.E., Castranova, V., and Baron, P. (2005), "Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice 1" *American Journal of Physiology - Lung Cellular and Molecular Physiology* 289, pp.L698-L708.

Shvedova, A.A., Kisin, E.R., Murray, A.R., Kommineni, C., Castranova, V., Fadeel, B., and Kagan V.E. (2008), "Increased accumulation of neutrophils and decreased fibrosis in the lung of NADPH oxidase-deficient C57BL/6 mice exposed to carbon nanotubes", *Toxicology And Applied Pharmacology* 231, pp.235-240.

Smith, C. J., Shaw, B. J. and Handy, R. D. (2007), "Toxicity of single walled carbon nanotubes on rainbow trout, (Oncorhynchus mykiss): Respiratory toxicity, organ pathologies, and other physiological effects", *Aquatic Toxicology* 82, pp.94-109.

Solomon, P.A., Gehr, P., Bennett, D.H., Phalen, R.F., Méndez, L.B., Rothen-Rutishauser, B., Clift, M., Brandenberger, C., and Mühlfeld, C.(2012), "Macroscopic to microscopic scales of nanoparticle dosimetry: from source to fate in the body", *Air Quality, Atmosphere and Health* 5(2) pp.169-187.

Stoeger, T., Reinhard, C., Takenaka, S., Schroeppel, A., Karg, E., Ritter, B., Heyder, J., and Schulzet, H. (2006), "Instillation of six different ultrafine carbon particles indicates a surface area threshold dose for acute lung inflammation in mice", *Environmental Health Perspectives* 114, pp.328–333.

Stebounova, L. V., Adamcakova-Dodd, A., Kim, J.S., Park, H., O'Shaughnessy, P.T., Grassian, V.H., and Thorne, P.S. (2011), "Nanosilver induces minimal lung toxicity or inflammation in a subacute murine inhalation model", *Particle and Fibre Toxicology* 8(5), at www.particleandfibretoxicology.com/content/8/1/5 (Accessed in September,2012)

Tagami, H., Kobayashi, H., and Kikuchi, K. (2002), "A portable device using a closed chamber system for measuring transepidermal water loss: Comparison with the conventional method", *Skin Research and Technology* 8(1), pp.7-12.

Tan, M.H., Commens, C.A., Burnett, L., and Snitch, P.J. (1996), "A pilot study on the percutaneous absorption of microfine titanium dioxide from sunscreens", *Australasian Journal of Dermatology 37*, pp.185-187.

Taurozzi J.S., Hackley V.A. and Wiesner M.R. (2012a), "Preparation of Nanosclae TiO2 Dispersions in an Environmental Matrix for Eco-Toxicological Assessment", Ver.1.1. *CEINT/NIST Protocol*

Taurozzi J.S., Hackley V.A. and Wiesner M.R. (2012b), "Preparation of a Nanoscale TiO2 Aqueous Dispersion for toxicological or Environmental Testing", Ver.1.2. *CEINT/NIST Protocol*

Teeguarden, J.G., Hinderliter, P.M.. Orr, G., Thrall, B.D., and Pounds, J.G. (2007), "Particokinetics in Vitro: Dosimetry considerations for in-vitro nanoparticle toxicity assessments", *Toxicological Sciences* 95, pp.300

Tinkle, S.S., Antonini, J.M., Rich, B.A., Roberts, J.R., Salmen, R., DePree, K., and Adkins, E.J. (2003), "Skin as a route of exposure and sensitization in chronic beryllium disease", *Environmental Health Perspectives* 111, pp.1202-1208.

Trouiller, B., Reliene, R., Westbrook, A., Solaimani, P., and Schiestl, R.H. (2009), "Titanium Dioxide Nanoparticles Induce DNA Damage and Genetic Instability In vivo in Mice", *Cancer Research* 69, pp.8784-8789.

Vevers, W. F. and Jha A. N. (2008), "Genotoxic and cytotoxic potential of titanium dioxide (TiO2) nanoparticles on fish cells in vitro", *Ecotoxicology* 17, pp.410-420.

Warheit, D.B., Laurence, B.R., Reed, K.L., Roach, D.H., Reynolds, G.A., Webb, T.R. (2004), "Comparative Pulmonary Toxicity Assessment of Single-wall Carbon Nanotubes in Rats", *Toxicological Sciences* 77, pp.117-125.

Warheit, D.B., Webb, T.R., Reed, K.L., Frerichs, S., Sayes, C.M. (2007), "Pulmonary toxicity study in rats with three forms of ultrafine-TiO2 particles: Differential responses related to surface properties", *Toxicology* 230, pp.90-104.

Warheit, D. (2004), "Nanoparticles Health Impacts?", *Nanomaterials Today* 7, pp.32-35.

Warheit, D.B. (2008), "How meaningful are the results of nanotoxicity studies in the absence of adequate material characterization?" *Toxicological Sciences* 101, pp.183-185.

Weibel, A., Bouchet, R., Boulc'h, F., and Knauth, P. (2005), "The big problem of small particles: A comparison of methods for determination of particle size in nanocrystalline anatase powders", *Chemistry of Materials* 17, pp.2378-2385.

Weibel, E.R. (2009), "What makes a good lung", *Swiss Med Weekly* 139, pp.375-386.

Wissing S, Mueller R. (2002), "Solid lipid nanoparticles as carrier for sunscreens: in vitro release and in vivo skin penetration", *Journal of Controlled Release* 81, pp.225-233.

Zhu, S., Oberdörster, E., Haasch, M.L. (2006), "Toxicity of an engineered nanoparticle (fullerene, C60) in two aquatic species, Daphnia and fathead minnow", *Marine Environmental Research* 62(1), pp.S5-S9.