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Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials

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Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials

IOMC
INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
A cooperative agreement among UNEP, ILO, FAO, WHO, UNIDO, UNITAR and OECD

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No. 1, *Report of the OECD Workshop on the Safety of Manufactured Nanomaterials: Building Co-operation, Co-ordination and Communication (2006)*

No. 2, *Current Developments/ Activities on the Safety of Manufactured Nanomaterials: Tour de table at the 1st Meeting of the Working Party on Manufactured Nanomaterials (2006)*

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No. 13, *Report of an OECD Workshop on Exposure Assessment and Exposure Mitigation: Manufactured Nanomaterials (2009)*

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ABOUT THE OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 30 industrialised countries in North America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

The Environment, Health and Safety Division publishes free-of-charge documents in ten different series: **Testing and Assessment**; **Good Laboratory Practice and Compliance Monitoring**; **Pesticides and Biocides**; **Risk Management**; **Harmonisation of Regulatory Oversight in Biotechnology**; **Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; and the Safety of Manufactured Nanomaterials.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (http://www.oecd.org/ehs).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The participating organisations are FAO, ILO, OECD, UNEP, UNIDO, UNITAR and WHO. The World Bank and UNDP are observers. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

FOREWORD

The OECD Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology (the Joint Meeting) held a Special Session on the Potential Implications of Manufactured Nanomaterials for Human Health and Environmental Safety (June 2005). This was the first opportunity for OECD member countries, together with observers and invited experts, to begin to identify human health and environmental safety related aspects of manufactured nanomaterials. The scope of this session was intended to address the chemicals sector.

As a follow-up, the Joint Meeting decided to hold a Workshop on the Safety of Manufactured Nanomaterials in December 2005, in Washington, D.C. The main objective was to determine the "state of the art" for the safety assessment of manufactured nanomaterials with a particular focus on identifying future needs for risk assessment within a regulatory context.

Based on the conclusions and recommendations of the Workshop [ENV/JM/MONO(2006)19] it was recognised as essential to ensure the efficient assessment of manufactured nanomaterials so as to avoid adverse effects from the use of these materials in the short, medium and longer term. With this in mind, the OECD Council established the OECD Working Party on Manufactured Nanomaterials (WPMN) as a subsidiary body of the OECD Chemicals Committee. This programme concentrates on human health and environmental safety implications of manufactured nanomaterials (limited mainly to the chemicals sector), and aims to ensure that the approach to hazard, exposure and risk assessment is of a high, science-based, and internationally harmonised standard. This programme promotes international co-operation on the human health and environmental safety of manufactured nanomaterials, and involves the safety testing and risk assessment of manufactured nanomaterials.

The Working Party endorsed this document at its 5th Meeting on March 2009. This document is published on the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology of the OECD.

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THE WORKING PARTY ON MANUFACTURED NANOMATERIALS (WPMN)

The Working Party on Manufactured Nanomaterials[1] was established in 2006 to help member countries efficiently and effectively address the safety challenges of nanomaterials. OECD has a wealth of experience in developing methods for the safety testing and assessment of chemical products.

The Working Party brings together more than 100 experts from governments and other stakeholders from: a) OECD Countries; b) non-member economies such as Brazil, China, the Russian Federation, Singapore, and Thailand; and **c**) observers and invited experts from UNEP, WHO, ISO, BIAC[2], TUAC[3], and environmental NGOs.

Although OECD member countries appreciate the many potential benefits from the use of nanomaterials, they wished to engage, at an early stage, in addressing the possible safety implications at the same time as research on new applications is being undertaken.

The Working Party is implementing its work through eight main areas of work to further develop appropriate methods and strategies to help ensure human health and environmental safety:

- Development of a Database on Human Health and Environmental Safety (EHS) Research;
- EHS Research Strategies on Manufactured Nanomaterials;
- Safety Testing of a Representative Set of Manufactured Nanomaterials;
- Manufactured Nanomaterials and Test Guidelines;
- Co-operation on Voluntary Schemes and Regulatory Programmes;
- Co-operation on Risk Assessment;
- The role of Alternative Methods in Nanotoxicology; and
- Exposure Measurement and Exposure Mitigation.

Each project is being managed by a steering group, which comprises members of the Working Party, with support from the Secretariat. Each steering group implements its respective "operational plans", each with their specific objectives and timelines. The results of each project are then evaluated and endorsed by the entire Working Party.

More information about the work of the WPMN, as well as publications and updates on efforts of governments and other stakeholders to address safety issues of nanomaterials is available at http://www.oecd.org/env/nanosafety.

[1] Updated information on the OECD's Programme on the Safety of Manufactured Nanomaterials is available at: www.oecd.org/env/nanosafety

[2] The Business and Industry Advisory Committee to the OECD

[3] Trade Union Advisory Committee to OECD.

PROJECT ON MANUFACTURED NANOMATERIALS AND TEST GUIDELINES

The OECD Guidelines for the Testing of Chemicals (Test Guidelines)[4] are a collection of the most relevant internationally agreed testing methods used by government, industry and independent laboratories to assess the safety of chemical products. To date, OECD has published 118 test guidelines, which are organized in five sections:

- Section 1 - Physical Chemical Properties
- Section 2 - Effects on Biotic Systems
- Section 3 - Degradation and Accumulation
- Section 4 - Health Effects
- Section 5 - Other Test Guidelines

These Guidelines are an important component of the system of Mutual Acceptance of Data (MAD) [5], which has legally binding implications for OECD member countries (and those non-members who have adhered to MAD). MAD is based on an original OECD Council Decision with subsequent additions.

As part of its Programme of Work, the Working Party on Manufactured Nanomaterials (WPMN) [ENV/MONO(2008)2] established a project entitled "**Manufactured Nanomaterials and Test Guidelines**" to review the published Test Guidelines to assess whether or not they are suitable for manufactured nanomaterials in 2006. The project might identify the need for new Test Guidelines or amendments to existing Test Guidelines or might develop guidance would describe how existing Test Guidelines might be applied to nanomaterials. This work involves close collaboration with OECD's Working Group of the National Coordinators of the Test Guidelines Programme (WNT). The project was carried out by a steering group which comprises members of the WPMN, with support from the Secretariat.

The first task of the project was to identify questions regarding dosimetry, including what new measurement techniques will be needed to understand internal doses, and how to prepare and administer dosing material for *in vivo*/ *in vitro* studies for toxicity as well as for ecotoxicity, and fate and behaviour in the environment. The project also gathered existing information about the unique characteristics of manufactured nanomaterials and how such characteristics could impact on testing approaches. This activity was supported by a review of "white papers" or reports published by 2007.

Based on the previous activities, the project undertook the review of OECD Test Guidelines for their applicability to manufactured nanomaterials (MN). The review was conducted by four sub-groups under the steering group corresponding to the four sections of OECD Test Guidelines: Section 1 - Physical Chemical Properties; Section 2 - Effects on Biotic Systems; Section 3 - Degradation and Accumulation; and Section 4 - Health Effects.

This *Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials* combines the results from the review of the four sections of the OECD Test Guidelines. The review was originally presented at the 4th meeting of the WPMN (June 2008) and revised taking into accounts comments from the WNT and the WPMN (after the 5th meeting of the WPMN, March 2009). It is worth to note that the inputs by the WNT were various and supporting the conclusions of the document.

[4] More about OECD's Test Guidelines Programme, see http://www.oecd.org/env/testguidelines.

[5] More about OECD's MAD system, see http://www.oecd.org/document/41/0,3343,en_2649_34377_1890473_1_1_1_1,00.html.

Some comments from the WNT which are not directly applicable to the document were shared with the WPMN for its future work in the relevant activities including the alternative methods in nano toxicology, as they might be pertinent to such work. Based on the recommendations derived from this review, the WPMN have been developing Guidance Notes on Sample Preparation and Dosimetry since its 4th meeting (June 2008) in co-ordination with the other work projects of the WPMN as well as the WNT.

EXECUTIVE SUMMARY

Many of the OECD Test Guidelines are applicable, with conditions in some cases, while some are inadequate for testing Manufactured Nanomaterials (MN) as measuring, dosing, delivery and tracking nanomaterials are not reliably accomplished at this stage. Therefore, the review of OECD Test Guidelines reinforced the need for a **guidance document(s) for sample preparation and dosimetry**. It suggests that the guidance document(s) be developed as a new document(s), and be independent from the existing OECD guidance documents.

In Physical chemical properties section, 4 of 22 test guidelines for physical chemical properties are applicable to MN. 16 guidelines might be applicable under some circumstances or to some classes of MN. Two guidelines are not applicable to MN or, if applicable, provide no useful information. 13 of 22 guidelines require further assessment before modifying these guidelines. Furthermore, the group suggests 17 physical chemical properties to be a necessary pre-requisite of toxicological assessment of MNs. Section I includes two annexes: Table of results from the review of the OECD Test Guidelines for physical chemical properties for their applicability to MNs (Annex I-1); and Table of standards developed by one or more of the leading national or international standards organisations for 17 physical chemical properties (Annex I-2).

For 24 OECD Ecotoxicity Test Guidelines, the subgroup for biotic effects section concluded that the guidance on preparation, delivery, measurement, and metrology is currently insufficient for testing of MN. It agreed that the basic toxicological practices are adequate for testing MNs. The group found that terminology used in the OECD test guidelines is not descriptive of, or specific to, the particulate or fibrous nature of MNs, and suggests terminology needs to be revised to be both more specific to MNs and to assure that test outcomes accurately reflect the potential hazard of MNs, based on the most predictive properties of MNs. Looking at the test endpoints, the current state of knowledge concerning nanomaterial toxicity, as well as possible routes of exposure, precludes reviewers from making specific recommendations for the development of such new test guidelines. The OECD Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixture was also reviewed. It is strongly recommended to develop a guidance document very similar to that. In addition, other testing methods developed by Canada, Japan and ISO were reviewed.

Some of OECD Test Guidelines for degradation and accumulations are not applicable for testing of MNs. In fact, many of them are applicable with limitations or specific conditions. The sub-group for degradation and accumulation provided a table listing up the OECD Test Guidelines and other relevant testing methods with applicability for MNs (Annex III-1), as well as conclusions specific to conduct testing of MNs. It was recommended that WPMN develop a general guidance document for testing the fate and degradation of nanomaterials. Section 2 also includes a detailed review of OECD bioaccumation methods (Annex III-2).

The review of health effects related test guidelines (Section 4) concluded that, in general the OECD guidelines are applicable for investigating the health effects of nanomaterials with the important proviso that additional consideration needs to be given to the physicochemical characteristics of the material tested, including such characteristics in the actual dosing solution. In some cases, there will be a need for further modification to the OECD guideline. This applies particularly to studies using the inhalation route and to toxicokinetic (ADME) studies. Finally, it is important to build upon current knowledge and practical solutions in relation to in-vitro test approaches. Section 4 includes the detailed review of current OECD health effects test guidelines (Annex IV).

SECTION 1

PHYSICAL CHEMICAL PROPERTIES

Review of the OECD Guidelines

This preliminary review of the 22 physical-chemical test guidelines, included in Section 1 of the *OECD Guidelines for the Testing of Chemicals* (TGs), has been undertaken by the WPMN (Annex I-1). It has examined their applicability to manufactured nanomaterials and identified, provisionally, three categories of guideline:

- Those that are applicable to manufactured nanomaterials;
- Those that might be applicable under some circumstances or to some classes of manufactured nanomaterials; and
- Those that are not applicable to manufactured nanomaterials or, if applicable, will provide no useful information.

Category one: OECD Test Guidelines that are applicable to manufactured nanomaterials

The following OECD Test Guidelines are considered applicable to manufactured nanomaterials:

- TG102 - Melting Point / Melting Range;
- TG109 - Density of Liquids and Solids;
- TG113 - Screening Test for Thermal Stability and Stability in Air; and
- TG116 - Fat Solubility of Solid and Liquid Substances.

Of the four guidelines included in category one**,** the first two are designed to provide data on standard physical properties. The third might, amongst other things, provide important information regarding the stability of manufactured nanomaterials in storage and use. It can be concluded that no further work is needed regarding the application of TGs 102, 109 and 113.

TG116, although considered an important potential test method for evaluating manufactured nanomaterials with respect to their possible toxicological impact, has significant issues regarding the selection and availability of a suitable fat for the test[6]. It is assumed that this guideline was designed to assess, at least, three aspects of chemicals: i) the possibility to be administered or applied to a subject in a "greasy" preparation; ii) the ability to cross lipidic membranes; and iii) the possibility to accumulate in fatty tissues. Many years experience with other chemicals show that these characteristics can be assessed by other means. Nevertheless, it should be kept in mind that the particular properties of nanomaterials and their potential applications might require such a guideline. Regarding TG 116, a further assessment of its necessity needs to be undertaken.

[6] TG116 has been recognized by the WNT as a Test Guideline which needs revision if it is to be applied to traditional chemicals. The WNT agreed not to delete this TG while it awaits a proposal to revise it.

Category two: OECD Test Guidelines that might be applicable under some circumstances or to some classes of manufactured nanomaterials

Category two contains 16 guidelines. Of these, TG104 -Vapour Pressure, could be applicable to manufactured nanomaterials though it is not clear at this stage what value the information would have *for solid materials*; it is recommended, nevertheless, not to carry out any additional work on it.

Of the remaining 15 guidelines, the following 11 are applicable to solutions and it is not known how the results might be impacted by the presence of a colloidal suspension, which might be present if the sample manufactured nanomaterial does not completely dissolve. Hence, further work is required to determine this and to modify the TGs, if necessary. Priority should be given to guidelines related to solubility in water, partition coefficient, absorption/desorption in solid matrices and dissociation constants:

- TG101 - UV-VIS Absorption Spectra - (Spectrophotometric Method);
- TG105 - Water Solubility;
- TG106 - Adsorption - Desorption Using a Batch Equilibrium Method;
- TG107 - Partition Coefficient (n-octanol/water): Shake Flask Method;
- TG108 - Complex Formation Ability in Water (Polarographic Method);
- TG111 - Hydrolysis as a function of pH;
- TG112 - Dissociation Constants in Water;
- TG115 - Surface Tension of Aqueous Solutions;
- TG117 - Partition coefficient (n-octal/water) - high performance liquid chromatography method;
- TG121 – Estimation of the Adsorption Coefficient (Koc) on Soil and on Sewage Sludge using High Performance Liquid Chromatography (HPLC); and
- TG123 - Partition Coefficient (1-Octanol/Water): Slow-Stirring Method.

TG110 - Particle Size Distribution/Fibre Length and Diameter Distributions, consists of two methods – A and B, the first of which is not applicable to nanomaterials, whilst the second would, with some modification (the inclusion of fibres of less than 5 microns in length and less than 100 nm in diameter), be applicable to nanoparticles as well as nanotubes and nano fibers. Studies should be carried out in order to extend its range of applicability to fibres with nano-scale dimensions. It is known that alternative methods for (nano) particle size distribution already exist, which should be taken into account if such studies are undertaken.

The following three guidelines can be applied only to polymeric manufactured nanomaterials. At the present time, it does not seem necessary to modify them.

- TG118 - Determination of the Number-Average Molecular Weight and the Molecular Weight Distribution of Polymers using Gel Permeation Chromatography;
- TG119 – Determination of the Low Molecular Weight Content of a Polymer Using Gel Permeation Chromatography; and
- TG120 – Solution/Extraction Behaviour of Polymers in Water.

Category three: OECD Test Guidelines that are not applicable to manufactured nanomaterials or, if applicable, provide no useful information

Class three contains two guidelines:

- TG103 – Boiling Point; and
- TG114 – Viscosity of Liquids.

TG 103, though applicable for determining the boiling point of manufactured nanomaterials, is probably not relevant to existing solid nanomaterials for two reasons. Firstly, for solid manufactured nanomaterials, the change in state from solid to liquid would destroy the structure of the (nano)material, which would not be expected to be re-established during the cooling process. Thus, the boiling point determination would be extremely unlikely to be a characteristic of the manufactured nanomaterial, *per se,* but of the generic material composition. In the case of liquid manufactured nanomaterials (nano-emulsions), the act of heating to the boiling point would again change and ultimately destroy the structure of the nanomaterial, which would also be unlikely to re-establish on condensation, hence the "boiling point" determination would be for a material in different form. Additionally, the multiphase nature of a nanoemulsion means that it would be most unlikely to have a characteristic boiling point but rather a boiling range.

TG 114 is only applicable to liquids and does not refer to solutions, suspensions or emulsions. Although the viscosity of a solution can be measured, standardised preparation procedures would need to be included but are not given in TG 114. Additionally, it is not known what impact a colloidal suspension would have on the results. It is not clear yet what the importance of this property might be for the behaviour of nanomaterials, both in the environment and in living organisms. At the same time, there would be the need to define the medium or media in which such suspensions should be assessed.

Review of existing standards for physical chemical test methods

Despite the comments above regarding the applicability or otherwise of the 22 Test Guidelines for use with manufactured nanomaterials, few of them are considered to provide information relevant to the potential toxicological impact of such materials. In fact, most of the properties above are for testing environmental effects. It is considered that the following set of physical chemical characteristics is a necessary pre-requisite of such toxicological assessment.

- Agglomeration/ aggregation
- Catalytic properties
- Composition
- Concentration
- Crystalline phase
- Dustiness[7]
- Fat solubility/ oleophilicity
- Grain size
- Hydrodynamic size/particle size measurement/ distribution
- Length

7. Japan has provided a reference for dustiness: 'Dustiness is defined as the propensity of a material to generate airborne dust during its handling, and provides a basis for estimating the potential health risk due to inhalation exposure.' Lidén, G., "Dustiness Testing of Materials Handled at Workplaces," Annals of Occupational Hygiene, 50(5), 437–439 (2006)

- Purity
- Shape
- Specific surface area
- Surface charge
- Surface chemistry
- Water solubility/ hydrophilicity
- Zeta potential

Several of these are the subject of standards developed by one or more of the leading national or international standards organizations. Such standards were identified (see Annex I-2) and are being reviewed with a view to understanding how such information might assist in reviewing the Test Guidelines for their applicability to Manufactured Nanomaterials. For example, such information might assist in the development or modification of a Test Guidelines.

ANNEX I-1
TABLE OF RESULTS FROM THE REVIEW OF OECD TEST GUIDELINES FOR PHYSICAL CHEMICAL PROPERTIES FOR THEIR APPLICABILITY TO MANUFACTURED NANOMATERIALS

Indication:

Category One: those that are applicable to manufactured nanomaterials;

Category Two: those that might be applicable under some circumstances or to some classes of manufactured nanomaterials; and

Category Three: those that are not applicable to manufactured nanomaterials or, though strictly applicable, provide no useful added value.

TG Number	Title	Identified Category
101	UV-VIS Absorption Spectra - (Spectrophotometric Method)	2
102	Melting Point / Melting Range	1
103	Boiling Point	3
104	Vapour Pressure	2
105	Water Solubility	2
106	Adsorption - Desorption Using a Batch Equilibrium Method	2
107	Partition Coefficient (n-octanol/water): Shake Flask Method	2
108	Complex Formation Ability in Water (Polarographic Method)	2
109	Density of Liquids and Solids	1
110	Particle Size Distribution/Fibre Length and Diameter Distributions	2
111	hydrolysis as a function of pH	2
112	Dissociation Constants in Water	2
113	Screening Test for Thermal Stability and Stability in Air	1
114	Viscosity of Liquids	3
115	Surface Tension of Aqueous Solutions	2
116	Fat Solubility of Solid and Liquid Substances	1
117	Partitiion coefficient (n-octal/water) - high performance liquid chromatography method	2
118	Determination of the Number-Average Molecular Weight and the Molecular Weight Distribution of Polymers using Gel Permeation Chromatography	2
119	Determination of the Low Molecular Weight Content of a Polymer Using Gel Permeation Chromatography	2
120	Solution/Extraction Behaviour of Polymers in Water	2
121	Estimation of the Adsorption Coefficient (Koc) on Soil and on Sewage Sludge using H igh Performance Liquid Chromatography (HPLC)	2
123	Partition Coefficient (1-Octanol/Water): Slow-Stirring Method	2

ANNEX I-2 PHYSICAL CHEMICAL PROPERTIES AND IDENTIFIED STANDARDS (DRAFT)

Physical chemical property	*suitable technique(s) - Dispersion: A – Airborne, S - Solid, L – Liquid*	Relevant OECD Test Guidelines	ISO[1]	ASTM[2]	CEN[3]	JIS[4]
agglomeration/ aggregation	*No easy methods – light scattering, small angle neutron and x-ray techniques. Critically dependant on surface charge/composition parameters) (L).*	None	None	None	None	None
composition	*Bulk: Elemental Analysis, ICPMS, EDX, EELS, dynamic-SIMS, 3D – Atom Probe (S).*		*ISO/AWI TS 10798 Scanning electron microscopy (SEM) and energy dispersive X-ray analysis (EDXA) in the charaterization of single walled carbon nanotubes (SWCNTs) *ISO/AWI TS 10929 Measurement methods for the characterization of multi-walled carbon nanotubes (MWCNTs) ISO 22309:2006: Microbeam analysis -- Quantitative analysis using energy-dispersive spectrometry (EDS)			

Physical chemical property	*suitable technique(s) - Dispersion: A – Airborne, S - Solid, L – Liquid*	Relevant OECD Test Guidelines	ISO[1]	ASTM[2]	CEN[3]	JIS[4]
			*ISO/AWI TS 10797: Use of Transmission Electron Microscopy (TEM) in the Characterization of Single Walled Carbon Nanotubes (SWCNTs)			
concentration						
crystalline phase	*Powder XRD, HR-TEM, Raman spectroscopy (S)*				None relevant	**JIS R 7651:2007** Measurement of lattice parameters and crystallite sizes of carbon materials
Dustiness				Evaluation of Airborne Dust Measurement Methods for Agricultural Chemical Carriers, Paper ID: **STP25370S**	**EN 15051:2006** Workplace atmospheres - Measurement of the dustiness of bulk materials - Requirements and reference test methods	
fat solubility/ oleophilicity		**TG116** Fat Solubility of Solid and Liquid Substances	-		None relevant	

Physical chemical property	*suitable technique(s) - Dispersion: A – Airborne, S - Solid, L – Liquid*	Relevant OECD Test Guidelines	ISO[1]	ASTM[2]	CEN[3]	JIS[4]
grain size	Powder XRD, SEM, TEM (S)		**ISO 16700:2004** Microbeam analysis -- Scanning electron microscopy -- Guidelines for calibrating image magnification	**ASTM E112-96(2004)e2** Standard Test Methods for Determining Average Grain Size	None relevant	**JIS H 7804:2005** Method for particle size determination in metal catalysts by electron microscope
			*ISO/AWI TS 10797 Use of transmission electron microscopy (TEM) in walled carbon nanotubes (SWCNTs)			
			ISO/CD 15900 Determination of particle size distribution -- Differential electrical mobility analysis for aerosol particles			
hydrodynamic size/particle size measurement/ distribution	*Scanning Mobility Analysis (SMPS) (7nm and above for airborne particles) coupled with Condensation counter or electrometer detection (A); Transmission/Scanning electron Microscopy (offline solid samples) (S); Line broadening phenomena in spectroscopies (A, S, L); Dynamic Light scattering (for liquids, but there are issues with non- spherical particles) (L).*		**ISO 9277:1995** Determination of the specific surface area of solids by gas adsorption using the BET method	**B859-03** Standard Practice for De-Agglomeration of Refractory Metal Powders and Their Compounds Prior to Particle Size Analysis	**EN 725-5:2007** Advanced technical ceramics - Methods of test for ceramic powders - Part 5: Determination of particle size distribution	
			ISO 13320-1:1999 Particle size analysis -- Laser diffraction methods -- Part 1: General principles	**WK1127** New Guide for Powder Particle Size Analysis		
			ISO 13321:1996 Particle size analysis -- Photon correlation spectroscopy	**ASTM E1919-07** Standard Guide for Worldwide Published Standards Relating to Particle and Spray Characterization		
			ISO/TS 13762:2001 Particle size analysis -- Small angle X-ray scattering method			

Physical chemical property	*suitable technique(s) - Dispersion: A – Airborne, S - Solid, L – Liquid*	Relevant OECD Test Guidelines	ISO[1]	ASTM[2]	CEN[3]	JIS[4]
			ISO 21501-2:2007 Determination of particle size distribution -- Single particle light interaction methods -- Part 2: Light scattering liquid-borne particle counter			
			ISO 21501-3:2007 Determination of particle size distribution -- Single particle light interaction methods -- Part 3: Light extinction liquid-borne particle counter			
			ISO 21501-4:2007 Determination of particle size distribution -- Single particle light interaction methods -- Part 4: Light scattering airborne particle counter for clean spaces			
			ISO 16700:2004 Microbeam analysis -- Scanning electron microscopy -- Guidelines for calibrating image magnification			

Physical chemical property	*suitable technique(s) - Dispersion: A – Airborne, S - Solid, L – Liquid*	Relevant OECD Test Guidelines	ISO[1]	ASTM[2]	CEN[3]	JIS[4]
length	*SEM*	**TG110 Particle Size Distribution/Fibre Length and Diameter Distributions**	**ISO 16700:2004** Microbeam analysis -- Scanning electron microscopy -- Guidelines for calibrating image magnification	**ASTM D6480-05** Standard Test Method for Wipe Sampling of Surfaces, Indirect Preparation, and Analysis for Asbestos Structure Number Concentration by Transmission Electron Microscopy - Might be useful for CNTs and other fibrous materials		
			*ISO/AWI TS 10929 Measurement methods for the characterization of multi-walled carbon nanotubes (MWCNTs)	**ASTM D6281-06** Standard Test Method for Airborne Asbestos Concentration in Ambient and Indoor Atmospheres as Determined by Transmission Electron Microscopy Direct Transfer (TEM)		
				ASTM D5755-03 Standard Test Method for Microvacuum Sampling and Indirect Analysis of Dust by Transmission Electron Microscopy for Asbestos Structure Number Surface Loading		
purity			*ISO/AWI TS 10929 Measurement methods for the characterization of multi-walled carbon nanotubes (MWCNTs)			

Physical chemical property	*suitable technique(s) - Dispersion: A – Airborne, S - Solid, L – Liquid*	Relevant OECD Test Guidelines	ISO[1]	ASTM[2]	CEN[3]	JIS[4]
			*ISO/AWI TS 11308 Nanotechnologies -- Use of thermo gravimetric analysis (TGA) in the purity evaluation of single-walled carbon nanotubes (SWCNT)			
			ISO 22309:2006: Microbeam analysis -- Quantitative analysis using energy-dispersive spectrometry (EDS)			
shape	*Electron Microscopies (TEM/SEM) (S).*		*ISO/AWI TS 10797 Use of transmission electron microscopy (TEM) in walled carbon nanotubes (SWCNTs)			
			*ISO/AWI TS 10798 Scanning electron microscopy (SEM) and energy dispersive X-ray analysis (EDXA) in the charaterization of single walled carbon nanotubes (SWCNTs)			
			*ISO/AWI TS 10929 Measurement methods for the characterization of multi-walled carbon nanotubes (MWCNTs)			

Physical chemical property	*suitable technique(s) - Dispersion: A – Airborne, S - Solid, L – Liquid*	Relevant OECD Test Guidelines	ISO[1]	ASTM[2]	CEN[3]	JIS[4]
Specific surface area	-*BET methods (Gas Isotherm – solid samples only, but can be used to determine porosity). Difficulty with nanoporosity (<5nm) due to comparable size of probe molecules with pores (S);*		ISO 9277: Determination of the specific surface area of solids by gas adsorption using the BET method	**B922-02** Standard Test Method for Metal Powder Specific Surface Area by Physical Adsorption	**EN ISO 18757:2005** Fine ceramics (advanced ceramics, advanced technical ceramics) - Determination of specific surface area of ceramic powders by gas adsorption using the BET method (ISO 18757:2003)	
surface charge	-*Electrometer measurements (difficult – no methods for mapping charge distribution on NPs) (A);*					
surface chemistry	-*Surface Analytical Techniques (XPS, Auger, SIMS – in Vacuo) (S)* -*Radiation beam methods – (IR, NIR, Raman, SERS) (S, possibly A)* -*Electron miscopies coupled to EDX and EELS analysers. (S)*		*ISO NP/TS 10867 Use of NIR -- Photoluminescence -- (NIR-PL) Spectroscopy in the characterization of single-walled carbon nanotubes (SWCNTs) (ISO/TC229) *ISO NP/TS 10868 Use of UV-Vis-NIR absorption spectroscopy in the characterization of single-walled carbon nanotubes (SWCNTs) (ISO/TC229)	Characteristics of Beryllium Oxide and Beryllium Metal Powders for Use as Reference Materials, Paper **ID: JAI13174**		

Physical chemical property	*suitable technique(s) - Dispersion: A – Airborne, S - Solid, L – Liquid*	Relevant OECD Test Guidelines	ISO[1]	ASTM[2]	CEN[3]	JIS[4]
			ISO 17974:2002 - Surface chemical analysis -- High-resolution Auger electron spectrometers -- Calibration of energy scales for elemental and chemical-state analysis			
			ISO 15472:2001 - Surface chemical analysis -- X-ray photoelectron spectrometers -- Calibration of energy scales			
			ISO/TR 18394:2006 - Surface chemical analysis -- Auger electron spectroscopy -- Derivation of chemical information			
			*ISO/WD 10810 - Surface chemical analysis -- X-ray photoelectron spectroscopy -- Guide to analysis			

Physical chemical property	*suitable technique(s) - Dispersion: A – Airborne, S - Solid, L – Liquid*	Relevant OECD Test Guidelines	ISO[1]	ASTM[2]	CEN[3]	JIS[4]
water solubility/ hydrophilicity	-*Traditional techniques (monitor solution and relate to residual mass of material or monitor opacity) (L)*				**EN 12457-1:2002** Characterisation of waste - Leaching - Compliance test for leaching of granular waste materials and sludges - Part 1: One stage batch test at a liquid to solid ratio of 2 l/kg for materials with high solid content and with particle size below 4 mm (without or with size reduction) - Parts 2, 3 and 4 related to this one and all called in 99/31/EC	
Zeta potential	-*Electrophoretic mobility – laser light scattering (L).*					**JIS R 1638:2000** Test methods of iso-electric point of fine ceramic powders
Catalytic properties						

* Standards in preparation

1. International Organization for Standardization
2. American Society for Testing and Materials
3. European Committee For Standardization
4. Japanese Industrial Standards

SECTION 2

EFFECTS ON BIOTIC SYSTEMS

INTRODUCTION

This section summarizes reviews of OECD ecotoxicity test guidelines for their applicability to manufactured nanomaterials. 24 OECD test guidelines were reviewed to evaluate whether they are applicable to testing for adverse effects of manufactured nanomaterials. The specific charge to this activity was to:

- Review existing OECD test guidelines for adequacy in addressing manufactured nanomaterials; and
- Identify the need for the development of new test guidelines or a revision of existing test guidelines.

The charge was not to actually develop new test guidelines or revise existing test guidelines, rather it was expected to make substantive proposals for the revision of test guidelines or development of new test guidelines. The test guidelines which have been reviewed are listed in Table 1.

Test guideline review process

There are currently 24 OECD guidelines for testing substances for adverse effects on biota. These test guidelines examine effects in all environmental media (aquatic, terrestrial, sediments, and sludges). They address a variety of vertebrate, invertebrate, and microbial taxa, and include both acute and chronic tests. The tests also include both mortality and non-lethal endpoints, e.g. growth, plant vigor, respiration. These guidelines have each been evaluated by at least one reviewer, and in many cases by two or three reviewers. The review process involved initial development of a template for review. This template was simply a section-by-section document that provided space for reviewers to describe inadequacies (for testing nanomaterials) of each test guideline section. In addition, and subsequent to completion of reviews, the OECD's guidance document on testing difficult substances (*Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures* [ENV/JM/MONO(2000)6]) was evaluated. This additional review was undertaken in response to a common finding in the test guideline reviews; that guidance on delivery of substances to test systems was, in all cases, inadequate for nanomaterials. One approach to addressing this shortcoming is to modify or develop a single document that describes approaches for delivering nanomaterials in a variety of media and test systems. A brief review, and suggestions for modification, of the *Difficult Substances* document is presented at the end of this document. Finally, five non-OECD test guidelines were also briefly reviewed in an effort to identify documents that might inform the nanomaterial-specific test guideline revision or development process. These reviews are also summarized at the end of this section.

Table 1. Reviewed OECD Ecotoxicity Test guidelines.

Guideline Identification	Description of test
201	Alga, Growth Inhibition Test
202	*Daphnia* sp. Acute Immobilisation Test
203	Fish, Acute Toxicity Test
204	Fish, Prolonged Toxicity Test
205	Avian Dietary Toxicity Test
206	Avian Reproduction Test
207	Earthworm, Acute Toxicity Tests
208	Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test
209	Activated Sludge, Respiration Inhibition Test
210	Fish, Early-Life Stage Toxicity Test
211	*Daphnia magna* Reproduction Test
212	Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages
213	Honeybees, Acute Oral Toxicity Test
214	Honeybees, Acute Contact Toxicity Test
215	Fish, Juvenile Growth Test
216	Soil Microorganisms: Nitrogen Transformation Test
217	Soil Microorganisms: Carbon Transformation Test
218	Sediment-Water Chironomid Toxicity Using Spiked Sediment
219	Sediment-Water Chironomid Toxicity Using Spiked Water
220	Enchytraeid Reproduction Test
221	*Lemna* sp. Growth Inhibition Test
222	Earthworm Reproduction Test (*Eisenia fetida/Eisenia andrei*)
224	Determination of the Inhibition of the Activity of Anaerobic Bacteria Reduction of Gas Production from Anaerobically Digesting (sewage) Sludge
227	Terrestrial Plant Test: Vegetative Vigour Test

Organization of Reviews Summary

The greatest concern of reviewers is that guidance on preparation, delivery, measurement, and metrology in all of the test guidelines is currently insufficient for testing of nanomaterials. As this opinion applied equally across all tests, independent of endpoint, media, target organisms, or duration, it seemed most expedient to summarize the reviews on a test component basis, as opposed to a test-by-test, or section-by-section summary. These test components include *terminology*, *material characterization*, *exposure preparation and delivery*, *stability and consistency*, and *metrics and measurement*.

1. ADEQUACY OF TEST GUIDELINES

1.1 Toxicological Principles

The basic toxicological practices on which these test guidelines are based are adequate for testing nanomaterials. These include, in part, assuring that test organisms are healthy and viable prior to exposure, use of reasonable dilution series based on needs for statistical analyses of exposure-response relationships, and full control of all preparation and exposure variables including positive controls for population responses to stress. However, review of all OECD biotic effects test guidelines revealed common inadequacies relative to their use in testing nanomaterials. Specifically, their guidance on reporting the

properties of substances, the delivery of substances to test systems, exposure quantification, and dose metrics are not adequate for nanomaterials.

1.2 Test Guideline Terminology

All of the current OECD test guidelines use terminology that is primarily applicable to chemical substances. In many cases, the term *substance* is used rather than the term c*hemical*, however neither term is fully descriptive of, or specific to, the particulate or fibrous nature of nanomaterials. It should be noted however, that the use of the term *chemical,* by itself does not preclude the applicability of test guidelines to nanomaterials. Other terms that are not applicable to nanomaterials are listed below. These inadequacies are more than semantic; they define, in the case of the term *concentration* the specific metric that is used in estimation of effect levels, or dose-response relationships. Such terminology will need to be revised to be both more specific to nanomaterials and to assure that test outcomes accurately reflect the potential hazard of nanomaterials, based on the most predictive properties of nanomaterials. The issues associated with these terms are discussed in more detail below.

1.2.1 Chemicals

All of the OECD biotic effects test guidelines use terminology specific to chemicals, or chemical preparations. If test guidelines are to be used for both chemical and nanomaterial substances, then the term *nanomaterial* should be defined and incorporated into all descriptions of their handling and testing. Many of the test guidelines refer to the testing of preparations or formulations (e.g. TG213 and TG214). This concept may be particularly applicable to some nanomaterials which may be dependant on surface treatments and coatings or specific solvents and emulsifiers to maintain their nano-scale characteristics.

1.2.2 Solution/ solubility

Nanomaterials are generally in particulate or fibrous forms and their preparation and delivery is best described in terms of preparation or suspension, rather than solution. Some thought should also be given to the use of closely related terms such as *solvent*, or *dissolved*, when terms such as *suspension agents* or *matrices* and *suspension* might be more descriptive of nanomaterials. Test guidelines 204, 205, 206 and 219 have *Prerequisite* sections that refer specifically to dissolved chemicals. Such terminology might be interpreted as precluding the testing of suspensions of nanomaterials.

1.2.3 Concentration

For soluble chemicals the term *concentration* is definitive and is a direct measure of exposure level and severity of potential adverse effects. This is not true for suspensions of nanomaterials unless particle size (and size distribution), surface area, and other properties are quantified in addition to mass concentration. This is of particular concern where effect levels are discussed. Current knowledge of the toxicity of nanomaterials suggests that particle size, surface area, number concentration or surface charge may altogether be more accurate predictors of adverse effects. For these reasons, other terminology has to be used when discussing exposure to nanomaterials and their relationship to observed adverse effects.

1.2.4 EC50, LC50, NOEC, LOEC, etc.

The corollary to the above comments concerning the use of the term concentration is that predictive exposure-response relationships will also require terminology that is not dependant on concentration. Effect level metrics may be required that incorporate several properties specific to nanomaterials including (but not limited to) particle size, surface area or surface charge.

1.3 Test Endpoints

There is little evidence to suggest that the endpoints described in the current test guidelines are not applicable to the testing of nanomaterials. These endpoints generally involve whole-organism responses that integrate many possible modes of toxicity and are thus also likely to be indicators of potential adverse effects of nanomaterials. In some cases, for example, respiration or gas production in microbial communities, the endpoints are also integrative of adverse effects across taxa and at the microbial community level.

Future research may reveal that nanomaterials have modes of action that are unique, relative to chemical stressors (for example, nanoparticles are of a scale that suggests possible interaction with DNA or RNA, resulting in effects that might be revealed only in multi-generation tests, and possibly involving novel endpoints). Because nanomaterials are particles or fibers, exposures and uptake are likely to involve processes not typical for soluble chemicals. This suggests that test endpoints may need to be developed that are more predictive of adverse effects compared with the current test endpoints addressed by OECD guidelines. In addition, because nanomaterials are currently in the early stages of development it is difficult to predict their fate or pathways of exposure for biota. The current state of knowledge concerning nanomaterial toxicity, as well as possible routes of exposure, precludes reviewers from making specific recommendation for the development of such new test guidelines.

1.4 Major Components of Test Guidelines

1.4.1 Material characterization

This component, in all of these test guidelines, is currently inadequate for nanomaterial testing. The particulate or fibrous nature of nanomaterials limits the usefulness of solubility or nominal or measured concentrations as properties useful for describing exposure-response relationships. At the current time research suggests that particle count, size distribution, surface area, charge and other surface characteristics might be better predictors of toxicity, and more accurate metrics to be used in statistical determinations of dose-response relationships. While (mass)concentration may be a useful parameter in modeling toxic effects, its usefulness will depend on knowledge of the state of the particles contributing to nominal or measured, e.g. ten 1-mg particles may be far more toxic than four 2.5-mg particles, given equal suspension volumes and yielding equal concentrations.

Section 1 of this document provides the review of current OECD Physical-Chemical test guidelines to assess their applicability to nanomaterials. As part of that process, Section 1 also made recommendations for characteristics that should be incorporated into new or existing physical-chemical test guidelines. It is recommended that these reviews and suggestions for nanomaterial-specific physical-chemical guidelines be carefully considered as the current ecotoxicity test guidelines are modified, or newly developed. In addition, some physical chemical properties should not be included if a test guideline is to be developed specifically for testing of nanomaterials. For example, several guidelines include vapor pressure as one of few identified physical-chemical properties to be identified for test substances; this property is unlikely to be applicable to nanomaterials. It is also expected that new research on the biotic effects of nanomaterials will also guide the process of the revision of test guidelines.

The physical-chemical characteristics of nanomaterials has also been identified by the review of test guidelines related to biotic effects as being of primary importance compared to the other major test guideline components, discussed below.

1.4.2 Exposure preparation and delivery

The test guidelines related to biotic effects involve several media, including soils, sediments, water, food, and direct application (Bee test, TG 213, albeit by application of suspensions). Testing in each of these media presents unique problems relative to the properties of nanomaterials. Concerns specific to water exposures include factors that can strongly affect nanomaterial aggregation and agglomeration, including pH and ionic strength. Early testing has also demonstrated that the characteristics of suspended nanomaterials can vary significantly (and predictably in some cases) depending on mixing method, e.g. stirring versus sonication, and even the rate at which a diluent is added to working suspensions. The presence of dissolved organic matter and suspended natural substances can affect the physical properties of nanomaterials, as well as the stability of suspension.

These concerns apply directly to test sediments and soils that are prepared using suspensions in water. Dry application of nanomaterials will preclude these suspension-related issues, however, the effect of soils and sediment composition and physical/chemical properties will affect the characteristics of nanomaterials. Similarly, when nanomaterials are mixed into food, the method of mixing and the composition of the food matrix will affect their characteristics. It should be noted here that dry application of nanomaterials may involve exposure of lab personnel; this issue should be addressed.

None of the test guidelines related to biotic effects provides information on how to measure, control for, or otherwise address these exposure preparation variables. It is recommended that such guidance be added to modified or newly-developed test guidelines to assure their applicability to nanomaterials.

1.4.3 Stability and consistency

All of the exposure preparation and delivery issues discussed above are complicated by the stability and consistency of the properties of nanomaterials in the various exposure matrices used. In general, the current test guidelines do not provide adequate direction for monitoring the characteristics of nanomaterials over the duration of tests. Many nanomaterials agglomerate or aggregate and settle from solution. Generally, achieving a fully stable suspension is not possible. Variability of exposure levels can occur with chemical test substances as well, and many test guidelines describe allowable limits for chemical stability in test chambers. However, both the frequency of analysis, and specific characteristics to be analyzed, are inadequate for nanomaterials. Additionally, some consideration should be given to how representative test media are of nanomaterial-specific fate processes that might occur in natural systems. The suggestions made by Section 1 relative to nanomaterial physical/chemical properties should also be incorporated into guidance on quantifying and characterizing exposure stability and consistency.

1.4.4 Metrics and measurement

As mentioned above, the particulate or fibrous nature of nanomaterials will require new approaches to estimating and predicting levels of effects based on biota exposure. Current test guidelines recommend dose-response metrics based on substance concentration (EC50, EC50, NOEC, LOEC, etc.). While (mass)concentration may remain as a major component in expression of exposure for nanomaterials, it is likely that other metrics including (amongst others) particle size, surface area, number concentration and surface charge may be essential for the development of predictive exposure metrics. Specific nanomaterial properties that might be critical for development of these metrics are identified in Section 1. It is recommended that these characteristic, as well as results of current toxicological research be considered for the revision of existing, or the development of new, test guidelines.

2. ADDITIONAL REVIEWS

2.1 OECD Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures

As described above, the review of OECD's ecotoxicology test guidelines indicated that their inadequacies for testing of nanomaterials are consistently related to material characterization, properties and metrology. This finding suggests that, rather than extensive modification of all OECD test guidelines, these nanomaterial-specific issues might be addressed in a single document that would provide guidance on how existing test guidelines could be used in testing nanomaterials. This approach has been applied to other substances that are deemed, "difficult substances" in OECD's Guidance Document: *Aquatic Toxicity Testing of Difficult Substances and Mixtures*. The goal of this document is to describe the preparation, delivery, and measurement of substances that would not be adequately tested if existing test guidelines were used. This document was reviewed to determine if the guidance provided would be adequate for nanomaterials, and might address some of the issues identified in the test guideline reviews. The review also includes making recommendations for modification of the *Difficult Substances* document, or a similar guidance document directed at nanomaterial testing.

A summary of finding are enumerated below:

1. The document provides a good framework for developing guidance for the aquatic toxicity testing of nanomaterials. Such specific guidance could be incorporated into the existing document or developed as similar, but separate document. It should be noted, however, that the guidance is specifically for testing in aquatic systems. Similar guidance may be necessary for terrestrial testing as well;

2. As with the review comments above for the OECD Ecotoxicity Test Guidelines, the "*Difficult Substances*" document lacks sufficient guidance for the characterization of nanomaterials. The guidance does describe procedures for characterizing traditional test substances including (amongst others) their stability, media preparation and sampling of test substances. However, many of the properties defined are unlikely to be applicable to nanomaterials (e.g. volatility), and many that are presumptively critical for nanomaterials (e.g. agglomeration and aggregation), are not. Specific nanomaterial properties to be measured or documented, and methods to do so will need to be described; and

3. Many physical and chemical properties that make substances difficult to test are described, as are approaches for overcoming these difficulties in toxicity testing and some of this guidance might be applicable to nanomaterials. However, many properties specific to nanomaterials will also need to be addressed, for example, particle size, surface area, agglomeration potential or agglomeration rate, as well as how to prepare and maintain stable suspensions or distribution of nanomaterials.

Recommendation

Guidance very similar to that provided by the *Difficult Substances* document, but specific to nanomaterials toxicity testing, should be either added to the existing document or developed as a new comparisons guidance document. Such guidance could provide a means to rapidly advance the toxicity testing of nanomaterials using existing test guidelines: to rewriting or devising new test guidelines. To do so, the guidance would need to address the issues presented here, and in the OECD ecotoxicity test guidelines reviews. It is strongly recommended that the WPMN develop a framework for such a guidance document. Key considerations would include the physical/chemical properties of nanomaterials identified by Section 1, the inadequacies identified by Section 2 in current test guidelines, and the biotic effects

testing devised by the testing of manufactured nanomaterials under the OECD's sponsorship programme, and would be best addressed by representatives of these steering groups.

2.2 Non-OECD Test Guidelines for Ecotoxicity

Five non-OECD biological testing guidelines (Table 2) were briefly reviewed. This was an effort to identify sources of guidance that might be directly applicable to nanomaterial testing, and thus inform the revision or rewriting of OECD test guidelines. As it was not possible to review a large number of additional test guidelines, a small putatively representative sample was selected based on environmental media (water, sediment or soil) and compartment (pelagic or sediment). These abbreviated reviews involved scanning the guidelines to identify descriptions or terminology adequate for nanomaterials. These were not intensive, section-by-section reviews as was undertaken with the OECD test guidelines.

Table 2. Reviewed non-OECD Ecotoxicity Test Guidelines.

Guideline Identification	**Description of test**
Environment Canada EPS1/RM/45E	Test for Measuring Emergence and Growth of Terrestrial Plants Exposed to Contaminants in Soil
Environment Canada EPS1/RM/11E	Acute Lethality Test Using Daphnia spp.
Japan, Ministry of the Environment	Algal Growth Inhibition Test, Daphnia Acute Immobilization Test, and Fish Acute Toxicity Test
International Standard ISO 11267:1999	Soil quality -- Inhibition of reproduction of Collembola (*Folsomia candida*) by soil pollutants
International Standard ISO 6341:1996	Water quality -- Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) -- Acute toxicity test

None of the non-OECD test guidelines provided guidance that addressed the inadequacies identified in the OECD test guidelines. This is not surprising given the unique nature of nanomaterials and the fact that new test guidelines are typically based on existing, well-vetted guidelines. In the case of OECD, EU Testing Methods, and US EPA/OPPTS Test Guidelines, the harmonization process has led to identical language in most cases.

SECTION 3
DEGRADATION AND ACCUMULATION

INTRODUCTION

Information on the fate of manufactured nanomaterials in the environment is necessary to understand their exposure to humans and other organisms. Research on the transport and potential transformation of nanomaterials in soil, subsurface, surface waters, wastewater, drinking water, and the atmosphere is essential. To support these investigations, existing testing methods should be evaluated and if necessary, they should be modified or new methods should be developed to take into account the unique characteristics of nanomaterials.

Due to the limited amount of testing data the considerations are often theoretical, and a tiered approach could be more appropriate than starting the testing with all test-guidelines in parallel. Most important test-guidelines should be evaluated for prioritization and some kind of integrated testing strategy could be developed for assessing the environmental fate of nanomaterials.

1. CONSIDERATIONS

The following specific questions are those needs to be addressed when examining existing test guidelines to determine if they are adequate.

1.1 Test Guidelines relevant to Transport and Deposition in the Environment

These test guidelines include both the methods for physical and chemical properties of the nanomaterials and methods for testing abiotic fate of nanomaterials.

- What are the physicochemical factors that influence the transport and deposition of manufactured nanomaterials in the environment?
- Do nanomaterials move through environmental media differently from their bulk counterparts? Can existing information on colloidal fate and transport, atmospheric ultrafine particulate fate and transport inform our thinking?
- How are nanomaterials transported in the atmosphere? Which properties and atmospheric conditions control the atmospheric fate of nanomaterials?
- To what extent are nanomaterials mobile in soils and in groundwater? What is the potential for these materials, if released to soil or landfills, to migrate to groundwater and within aquifers, with potential exposure to general populations via groundwater ingestion?
- What is the potential for these materials to be transported bound to particulate matter, sediments, or sludge in surface and ground waters?
- How do the aggregation, sorption and agglomeration of nanoparticles affect their transport?
- Do nanoparticles react differently in the environment from their larger scale counterparts?
- In what quantity and in what forms may nanoparticles be released from materials that contain them, as a result of environmental forces (rain, sunlight, heat, solution chemistry variables – pH, salinity, ionic strength, temperature etc.) or through use, re-use, and disposal?

- What is the potential for nanomaterials to interact with the environment, and in particular catalytic nanomaterials?
- Are current harmonized test methods capable of determining the environmental transport and deposition of nanomaterials? *(Note: current OECD guidelines do not address e.g. potential for air transport)*
- Are new methods needed to understand fate and transport of nanomaterials (for example, is the octanol-water partition relevant for nanomaterials?)

1.2 Test Guidelines related to Degradation (biotic and abiotic)

These test guidelines include both abiotic and biotic degradation (including simulation methods) of the nanomaterials.

- Do particular nanomaterials persist in the environment in nano- form, or undergo degradation via biotic or abiotic processes? Is the degradation of a nanomaterial different from chemically similar macro scale material? If they degrade, what are the transformation products and their characteristics? Are the degradation products the same as those of the bulk material? Is the nanomaterial likely to be in the environment, and thus be available for bioaccumulation/biomagnification?
- What are the physicochemical factors that affect the persistence of intentionally produced nanomaterials in the environment? What data are available on the physicochemical factors that affect the persistence of unintentionally produced nanomaterials (e.g., carbon-based combustion products) that may provide information regarding intentionally produced nanomaterials?
- How are the physicochemical and biological properties of nanomaterials altered in complex environmental media such as air, water, and soil? How do redox processes influence environmental transformation of nanomaterials? To what extent are nanomaterials photoreactive in the atmosphere, in water, or in environmental surfaces?
- How does the aggregation, sorption and agglomeration of nanoparticles affect transformation?
- Are current test methods capable of determining the abiotic (e.g. phototransformation, hydrolysis) and biotic degradation and transformation of nanomaterials?

1.3 Test Guidelines related to Bioaccumulation

The methods here should be able to detect bioconcentration, bioaccumulation and possibly biomagnification.

- Are nanomaterials bioavailable for aquatic organisms? For bulk material it is assumed that the concentration in the organism is not increased if the concentration in the environment exceeds the water solubility, since the undissolved portion is not bioavailable. Is this true for nanomaterials as well?
- In the case that dispersed nanomaterials are bioavailable: What is the mechanism/pathway of uptake and depuration? Is the kinetic of uptake and depuration the same as for other test materials? Do unique characteristics of the nanomaterials affect their bioavailability?
- How do nanomaterials bioaccumulate? Do their unique characteristics affect their bioavailability? Do nanomaterials bioaccumulate to a greater or lesser extent than macro-scale or bulk materials?
- Are current test methods capable of determining the bioaccumulation (and biomagnification) of nanomaterials?

- What is the appropriate physical form of a nanomaterial to use for bioaccumulation assessment (e.g. dispersed, agglomerated, aggregated)? Based on knowledge of the physical chemical behaviour of a nanomaterial in environmental media, can 'worst case exposure scenario' conditions be defined and used to design relevant bioaccumulation tests?
- Are new methods needed to understand bioaccumulation of nanomaterials?

2. INFORMATION REQUIREMENTS AND POSSIBLE OECD METHODS

2.1 Endpoints identified the WPMN ; Fate and Corresponding OECD Test Guidelines

The WPMN identified endpoints[8] for the phase one of the OECD's Sponsorship Programme on the Testing of Manufactured Nanomaterials[9]. The following endpoints were selected to address environmental fate. These endpoints will be investigated in the testing of manufactured nanomaterials within the Sponsorship Programme. The test guidelines below, identified with an asterisk (*) have been reviewed by Section 1 (Physical-chemical Properties) in this document.

Physical-chemical properties

- Agglomeration/aggregation
- Water solubility OECD105*
- Octanol-water partition coefficient OECD 107*, 117,* 123*
- Dispersion stability in water

Biotic degradability:

- Ready biodegradability OECD 301, 310
- Surface water simulation testing OECD 309
- Soil simulation testing OECD 307
- Sediment simulation testing OECD 308
- Sewage treatment simulation testing OECD 303
- Identification of degradation product(s)
- Testing of degradation product(s) as required-

Abiotic degradability and fate:

- Hydrolysis (surface modified nanomaterials) OECD 111*
- Adsorption- desorption OECD 106*
- Adsorption to soil or sediment OECD 121*
- Leaching in soil OECD 312

Bioaccumulation potential:

- Bioconcentration from water to fish OECD 305
- Bioaccumulation in sediment worms OECD draft 2007

8. ENV/JM/MONO(2008)13/REV.

9. OECD's Sponsorship Programme on the Testing of Manufactured Nanomaterials, http://www.oecd.org/document/47/0,3343,en_2649_37015404_41197295_1_1_1_1,00.html

Other relevant information (when available)

2.2 Issues on the applicability and use of testing methods

2.2.1 Transport and deposition in the environment

General

If a specific nanomaterial is soluble in water to the degree that the dissolved concentration is high enough to elicit organism or ecosystem effects, then many of the current protocols and guidelines developed for existing hazardous contaminants are probably applicable. However, for materials that do not readily dissolve, it should be determined whether the nanomaterial will form stable aerosols in air or stable suspensions in aqueous media (in both fresh and sea waters). To understand the fate of these nanomaterial dispersions in the environment it will be necessary to characterize the nanomaterial aerosol properties and the aqueous phase physical-chemical properties to a greater degree than is necessary for gas phase or dissolved contaminants. For example, moderate changes in the ionic strength will have little effect on the solubility of many organic contaminants (ex: PAHs, most pesticides) but can have major effects on the suspension stability of fullerene nanoparticles.

Determining the aggregation and sorption characteristics of the nanomaterials can provide valuable information when developing new testing guidelines, or interpreting the results from existing test guidelines. Nanomaterials that are highly sorbed to soil and sediment surfaces are not likely to partition into the water column as dissolved solutes. For nanomaterials that can form stable aggregates in water, detailed information on particle size and surface characteristics are needed to evaluate nanomaterial transport and deposition potential.

The data requirements identified for the phase one of the OECD's Sponsorship Programme on the Testing of Manufactured Nanomaterials will represent the core endpoints which should be addressed at an early stage in order for projects of the WPMN to proceed. However, many other endpoints might also be relevant for nanomaterial fate studies e.g. fat solubility (OECD 116), and agglomeration.

Method applicability

Physical-chemical properties

In principle the OECD test methods for a number of endpoints are applicable, but as with most of the test methods, the detection and analysis (and quantification) of the nanomaterials is the most challenging issue.

Some of the physical-chemical methods in relation to environmental transport and fate are already evaluated in Section 1 "Physical chemical Properties".

Nanoparticle aggregation/ Dispersion stability in water – it is possible that a standard method for measuring particle zeta potential could be used to estimate the degree of particle aggregation and hence the stability of nanomaterial aqueous dispersions. Colloid stability is an important measure in many industrial applications – perhaps an SOP from industry or some standards group would be useful here.

Water solubility OECD TG 105 may be useful for some nanomaterials, but many of the organic based materials (e.g. fullerenes) are so insoluble that specialized methods will need to be employed to measure or

estimate solubility. For example, the solubility of fullerenes are usually estimated by measuring solubility in alcohols and extrapolating to a zero carbon alcohol, i.e. water.

Octanol-water partition coefficient (OECD 107, 117,123) - as for water solubility, many organic nanomaterials have such low water solubility that measuring their concentration in the aqueous phase is problematic.

2.2.2 Degradation (biotic and abiotic)

General

Based on the detailed evaluation of the different test guidelines and the current knowledge, the test protocols seem to be applicable to the same extent for nanomaterials as for the comparable bulk material. Strictly inorganic nanomaterials will not require testing in any of the biotic degradation tests. It should be first determined, therefore, whether the nanomaterial contains carbon which will serve as an energy and nutrient source for microorganisms.

Secondly the material's physical-chemical and compartmentalization properties can provide insight into whether some of the simulation tests are unnecessary. For example, if the material is unlikely to reside in the water column, then simulation testing in surface water may be unnecessary.

In addition, if several conclusive aerobic degradation test results indicate very low or negligible degradation, then it is likely that other aerobic degradation tests will also be negative and a decision can be made whether to proceed with additional tests. For example, if the *ready biodegradation test* is below 10%, then the simulation test in surface water will also likely be very low and therefore it may be concluded that the more elaborate test is not required.

Likewise for hydrolysis testing, the necessity of undertaking this test will be dictated by the chemical structure of the material and whether it contains groups which would be subject to hydrolysis.

Many carbon nanomaterials, such as fullerenes and nanotubes are not water soluble. Guidance for biodegradation testing of poorly soluble materials is given in ISO 15462.

The international test methods (OECD, ISO, OPPTS) for assessing biodegradability are listed and evaluated in Annex 1. Guidance on the ISO biodegradation methods is also given in ISO TR 15462.

It is important to remember that to some extent re-defining the understanding and meaning of degradation is needed. It would depend on if the interest lies on only the "nano-related property" of the nanomaterial (NM). Firstly, if a NM is made of a readily biodegradable substance, e.g., a polymer, it is quite simple to demonstrate that the nanoformulated substance behaves similarly to the "non-nanomaterial", but even though a nanomaterial can be shown to be e.g., readily biodegradable, it may act differently in the environment due to aggregation/agglomeration of other nanomaterials, natural nano-particles or other substances adhering to the surface. It is in fact changes in these differences in behavior that testing should consider.

Secondly, if a nanomaterial seems to be persistant, it is still important to consider whether its unwanted "nano-properties" can be changed. Nanomaterial can act as a carrier for other pollutants, or this ability may be neutralised by aggregation with other substances. Such issues should be considered when designing especially simulation degradation tests. It could for example be done by testing binary mixtures (consisting of a NM and a well known chemical) just to see if the behavior of the well know chemical in the presence of the NM is changed over time.

Method applicability

The detailed evaluation of existing biodegradation methods is presented in Annex 1. Most of carbon based nanomaterials e.g. fullerenes and nanotubes have a very limited solubility in water. This means that the biodegradation screening methods e.g. for ready biodegradability measuring dissolved carbon are not applicable. In principle the methods measuring carbon dioxide production or oxygen uptake are applicable, but these methods involve a rather high amount of test material. The most promising candidate of the OECD screening methods could be OECD 310, which measures carbon dioxide production. A limited amount of test material is needed and the test material does not have to be soluble.

It could be also questioned whether it is possible that the carbon based nanomaterials e.g. fullerenes are biologically degraded at all. Limited data has indicated that fullerenes could be taken up by wood decay fungi, suggesting that the carbon from fullerene could be metabolized (Filley et al., 2005).

Simulation tests for biological degradation in various environmental compartments are also applicable in principle, but again the detection of the presence of nanomaterials is the challenge. The possible degradation to carbon dioxide (mineralization) integration into biomass or other partitions could be followed by radio labeled test material. The advantage to use simulation test with labeled substances would also allow low test material concentration, provide degradation kinetics and mass balance on the fate of the carbon from the tested material.

However, radiolabelled nanomaterials can only be used with great caution - it is quite obvious that the optimal thing would be to place the label uniformly on the nanomaterial, but here we are dealing with a complicated issue that will need specialized input from experts on radio-chemistry.

2.2.3 Bioaccumulation

General

For simple organic chemicals, there is an established relationship between octanol water partition coefficient (Kow) and bioaccumulation or bioconcentration factor (BCF). However, this relationship may not hold true for many nanomaterials but more data is needed to judge this.

The data requirements for the phase one of the OECD's Sponsorship Programme on the Testing of Manufactured Nanomaterials will represent the core endpoints for bioaccumulation potential. Applicable methods should be evaluated rather soon in order for projects of the WPMN to proceed. As there exist today only one OECD TG for bioconcentration test (TG 305) from water into fish, and another draft for bioaccumulation into sediment worms, also the promising methods published in scientific literature should be considered. One of these methods could be the fish dietary bioaccumulation method (Fisk et al. 1998, Stapleton et al. 2004, Parkerton et al. 2007).

Standard BCF testing protocol may have critical limitations in sole testing of bioaccumulation of nanoparticles. It is likely that in most cases the relative big size (1-100 nm) of nanoparticles compared to macromolecules limits the uptake of these particles to fish compared to standard molecular chemical substances. Therefore it is unlikely that OECD 305 method is suitable enough for these particles since it has been observed that large molecular size effectively limit direct uptake of large molecules (> 0.5 nm cross diameter).

Fish dietary BAF testing is not yet a standard OECD testing protocol. This spiked food method is suitable for testing of poorly soluble large molecules and might be very suitable in testing of several classes of nanoparticles as well (entirely or in combination with the standard 305. However, more data using a harmonized OECD dietary protocol especially for testing nanomaterials are needed.

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

Method applicability

Overall the two OECD methods TG 305 and a new OECD TG on sediment worms are a valuable starting point for assessing the bioaccumulation potential of nanomaterials. The detailed evaluation of the OECD methods is presented in Annex 2. Some parts of them should be modified as described further on, and the method should be continually revised as the relevant factors regarding the biological uptake and depuration of nanomaterials are determined. Hence, at this point it is impossible to give a precise answer which kind of guidance is needed if they are applied for testing nanomaterials.

Still many questions remain without answers. One of the questions again is the detection and quantification of the nanoparticles and their detection and characterization in tissues and body fluids. The use of C-14-labelled materials seems promising and the labeling techniques of carbon nanomaterials are developing. One novel possibility could be neutron activation of metal and metal oxide nanoparticles (Oughton *et al.* 2008). It enables both localization and quantification within tissues or organisms. Also more traditional chemistry e.g. ICP-MS analysis for metals could provide valuable information on the toatal amounts of material accumulated to the organism.

3. CONCLUSIONS

- As with testing biotic effects, the detection and analysis methods for various nanomaterials is a perquisite to use most fate related test guidelines.
- The biodegradation methods are of course only applicable to organic nanomaterials. However, it is not very probable that pure carbon materials e.g. fullerenes or carbon nanotubes would be biodegradable in normal test conditions.
- If the fate of carbon nanomaterials (e.g., carbon nanotubes) is to be assessed the carbon-14 labeling technique enables the detection of carbon originated from labeled material.
- In the bioaccumulation studies, a possibility could be neutron activation of metal and metal oxide nanoparticles. This enables both localization and quantification within tissues or organisms.
- Traditional chemistry e.g. ICP-MS analysis for metals could provide valuable information on the total amounts of material accumulated to the organism.

4. RECOMMENDATIONS

Several OECD Test Guidelines are applicable also for testing the environmental fate of nanomaterials. However, for testing and especially for the detection of the nanomaterials the analytical methods has to be developed before the OECD test guidelines are applicable to testing the environmental fate of nanomaterials. As stated above for testing the carbon based nanomaterials could possibly rely on radio-labelling techniques.

As summary conclusion and recommendation, a general guidance documents has to be developed for testing the fate and degradation of nanomaterials with existing OECD Test Guidelines and those under development. It is also especially important to record and documentate the dosage, exposure and the final

detection and characterisation in order to evaluate the results in a retrospective way after novel scientific developments.

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ANNEX III-1: INTERNATIONAL GUIDELINES (OECD, ISO, OPPTS) FOR ASSESSING BIODEGRADABILITY - APPLICABILITY FOR NANOMATERIALS

Method	Test duration	Inoculum	Test conditions	Measurements (additional analysis)	Limitations	Applicability for nanomaterials
Ready biodegradability tests						
OECD 301A DOC die away (ISO 7827)	Up to 28 days	Micro-organisms (~10^7 – 10^8 cells/L) from surface waters, un-chlorinated sewage treatment works effluents or activated sludge. Not pre-adapted inoculum	Agitation in the dark or diffuse light under aerobic conditions at 20-24°C Test material conc. (10 - 40 mg DOC/L)	DOC removal	Test substance has to be soluble, non-volatile, not sorbed to vessel or sludge and non-toxic at test conc.	In principle not applicable as the nanomaterial has to be soluble.
OECD 301B CO_2 evolution test (ISO 9439, OPPTS 835.3120)	Up to 28 days	Micro-organisms (~10^7 – 10^8 cells/L) from surface waters, un-chlorinated sewage treatment works effluents or activated sludge. Not pre-adapted inoculum	Agitation in the dark or diffuse light under aerobic conditions at 20-24°C Test material conc. (10 - 20 mg DOC/L)	CO_2 production (Phys.chem characterization of remaining material)	Test substance must be non-volatile and non-toxic at test concentration.	Applicable, but higher test material concentration needed e.g. compared to OECD 310 (2-40 mg C/L). Measures mineralization
OECD 301C Modified MITI Test	Up to 28 days	Micro-organisms (~10^7 – 10^8 cells/L) in surface waters, un-chlorinated sewage treatment works or industrial effluents or activated sludge. Not pre-adapted inoculum	Agitation in the dark under aerobic conditions at 24-26°C Test material conc. (100 mg/L)	O_2 uptake (Phys.chem characterization of remaining material)	Test substance has to be non-toxic at test concentration, subject to interference from nitrification.	In principle applicable, but high conc. needed

Method	Test duration	Inoculum	Test conditions	Measurements (additional analysis)	Limitations	Applicability for nanomaterials
OECD 301D Closed bottle test (ISO 10707)	Up to 28 days	Micro-organisms (10^4 - 10^6 cells/L) in surface waters or un-chlorinated sewage treatment works effluents Not pre-adapted inoculum	Agitation in the dark under aerobic conditions at 20-24°C Test material conc. (2 - 10 mg/L)	O_2 uptake (Phys.chem characterization of remaining material)	Test substance has to be non-toxic at test concentration, subject to interference from nitrification.	In principle applicable.
OECD 301E Modified OECD screening test (ISO 7827)	Up to 28 days	Micro-organisms (~10^5 cells/L) in unchlorinated sewage treatment works effluents Not pre-adapted inoculum	Agitation in the dark or diffuse light under aerobic conditions at 20-24°C Test material conc. (10 - 40 mg DOC/L)	DOC removal	Test substance has to be soluble, non-volatile, not sorbed to vessel or sludge and non-toxic at test conc.	In principle not applicable as the nanomaterial has to be soluble.
OECD 301F Manometric respirometry test (ISO 9408)	Up to 28 days	Micro-organisms (~10^7 – 10^8 cells/L) in surface waters, un-chlorinated sewage treatment works effluents or activated sludge Not pre-adapted inoculum	Agitation in the dark or diffuse light under aerobic conditions at 20-24°C Test material conc. (100 mg/L)	O_2 uptake (Phys.chem characterization of remaining material)	Test substance has to be non-toxic at test concentration, subject to interference from nitrification.	In principle applicable, high conc.
OECD 310 (Headspace test) ISO 14593	Up to 28 days	Micro-organisms (~10^5 – 10^8 CFU/L) in surface waters, un-chlorinated sewage treatment works effluents or activated sludge Not pre-adapted inoculum	Closed batch culture, agitation in the dark or diffuse light under aerobic conditions at 20-25°C Test material conc. (2 - 40 mgC/L)	CO_2 production in sealed vessels giving % degradation	Test substance must be non-toxic at test concentration. (pH 2 for analysis of $CO_{2)}$	Applicable, test material need not to be soluble, carriers can be used. Measures mineralization.

Method	Test duration	Inoculum	Test conditions	Measurements (additional analysis)	Limitations	Applicability for nanomaterials
Simulation Tests for Freshwater (Marine) and Sediment Systems						
OECD 308 Aerobic and anaerobic transformation in aquatic sediment systems	Less than 100 days	Microorganisms in sediment (not pre-adapted)	Static test with natural water and sediment, with non-volatile ^{14}C labelled compounds at natural levels.	Chemical analysis of transformation products or $^{14}CO_2$ analysis where labelling used.	Simulates suspended sediment only. Test substance has to be non-toxic, non-volatile and soluble. Site specific with respect to sediment. Sorption to sediment may be misleading if ^{14}C not used.	Applicable, but the bioavailability may limit degradation Measures mineralization from labeled particles.
OECD 309 Aerobic mineralisation in surface water	Up to 90 days for the batch test	Microorganisms in surface water (fresh-water and marine) Not pre-adapted May include suspended sediment and/ or semi-continuous operation	Agitation in the dark or diffuse light under aerobic conditions at field temperature or 20-25^{o}C Test material conc. (1 – 100 µg/L) preferred 10 µg/L For radio-labeled substances, approx. 50 µCi/mg	Chemical analysis of transformation products or $^{14}CO_2$ analysis where labelling used.		Applicable Measures mineralization from labeled particles.

Method	Test duration	Inoculum	Test conditions	Measurements (additional analysis)	Limitations	Applicability for nanomaterials
ISO 14592-1 (OPPTS 835.3170)	No fixed duration	Micro-organisms in surface water samples filtered through 100 um filter for a 'pelagic test' which may be amended with an aerobic sediment slurry from the study site for a 'suspended sediment test'.	Agitation in the dark or diffuse light under aerobic conditions at field temperature or 20-25^{o}C Test material conc. (1 – 100 µg/L) For radio-labeled substances, 15 – 30 Bq/ml	Specific chemical or radio-chemical analysis (and DOC or TOC if possible) giving 1st order rate const.	Test substance has to be non-toxic, non-volatile and soluble. Site specific with respect to sediment. Sorption to sediment may be misleading if ^{14}C not used.	In principle applicable. Labeled substances needed Measures mineralization from labeled particles.
ISO 14592-2	No fixed duration but <60 days	Micro-organisms in surface water.	Natural diffuse daylight or constant illumination of artificial white light (400-700 nm) with an energy of 50 uE/m^2/s at the water surface. Flow through system	Specific chemical or radio-chemical analysis giving 1st order rate const.	Test substance has to be non-toxic, non-volatile and soluble. Site specific with respect to sediment if used – glass beads may not be representative of sediment. Sorption to sediment may be misleading if ^{14}C not used.	In principle applicable. Labeled substances needed Measures mineralization from labeled particles. High amounts of test material needed.

Method	Test duration	Inoculum	Test conditions	Measurements (additional analysis)	Limitations	Applicability for nanomaterials
OPPTS 835.3180 Sediment/ water microcosm	Less than 60 days	Natural microbial assemblage.	Sediment microcosms using intact cores with (semi) continuous water replacement. ^{14}C labelling at environmentally realistic levels recommended.	Chemical analysis of transformation products or $^{14}CO_2$ analysis where labelling used.	Test substance has to be non-toxic, non-volatile and soluble. Site specific with respect to sediment. Sorption to sediment may be misleading if ^{14}C not used.	In principle applicable. Labeled substances needed Measures mineralization from labeled particles. High amounts of test material needed.
Sewage Treatment Simulation Test						

Method	Test duration	Inoculum	Test conditions	Measurements (additional analysis)	Limitations	Applicability for nanomaterials
OECD 303A Aerobic sewage treatment: coupled unit test The guideline can be performed with two type of vessels: activated sludge plant model (Hussman unit) and porous pot unit (ISO 11733)	Up to 12 weeks	Aerobic sewage	Elimination of test chemicals (20 mg l^{-1} DOC) from continuously fed laboratory scale coupled sewage treatment units. In annex the use of labeled material is explained	DOC or COD giving % degradation. For nanomaterial only $^{14}CO_2$ analysis and activity analysis of residuals possible	Test substance must be water soluble and non-volatile.	Applicable only with radio-labeled material
Primary Biodegradability Test						
OPPTS 835.3220 Porous Pot Method,	At least 21 days	Activated sludge mixed liquor from a domestic plant.	Test and control pots filled with inoculum and 10-20 mgC/l test substance.	Primary biodegradation determined by test chemical removal, DOC analysis provides measure of ultimate biodegradation.	Test substance has to be soluble, non-volatile, not sorbed to vessel or sludge and non-toxic at test conc.	Not applicable

Method	Test duration	Inoculum	Test conditions	Measurements (additional analysis)	Limitations	Applicability for nanomaterials
Simulation Tests for Marine Waters						
OECD 306 (ISO 7827 and 10707, OPPTS 835.3160)	Up to 60 days: Shake flask method : 60 days Closed bottle method : 28 days	Micro-organisms[2] in test seawater Not pre-adapted inoculum	Agitation in the dark or diffuse light under aerobic conditions at 15-20°C. Test material conc. - 5 – 40 mg DOC/l (Shake Flask Method), - 2 – 10 mg test substance /l (Closed bottle test).	DOC; O_2 uptake	Test chemical must be non-toxic at test concentrations, soluble and not sorbed by vessel. Closed bottle test subject to interference from nitrification. High nutrient concentrations with respect to seawater	Two methods are presented : a seawater variant of the modified OECD Screening Test (Shake Flask Method) and a seawater variant of the Closed Bottle Test. The latter can be applicable to nanomaterials. As the closed bottle test is more appropriate an indication of differences in limitations could be useful e.g. in closed bottle method test substance can be less soluble.
Simulation Tests for Soil						
OECD 307 Aerobic and anaerobic transformation on soil	Up to 120 days, longer under some circumstances	Indigenous soil microbes	The degradation of labeled substances are followed in a soil sample	$^{14}CO_2$	- phys-chem analysis of nanomaterials could be impossible	Mineralisation by $^{14}CO_2$ measurement possible

Method	Test duration	Inoculum	Test conditions	Measurements (additional analysis)	Limitations	Applicability for nanomaterials
Inherent Biodegradation Tests - Water						
OECD 302A Modified SCAS test (OPPTS 835.3210)	Months (often up to 120 days).	Settled domestic sewage and activated sludge. Inoculum to be sourced from a domestic treatment plant	Test chemical (20 mg DOC l^{-1}) aerated with settled domestic sewage and activated sludge (ca. 2500 mg l^{-1} TSS) for 23h at 20-25°C. Aeration stopped, sludge settled and supernatant removed. Fresh sewage and test chemical are added and the cycle repeated. ^{14}C-radiolabelled chemicals can be used for increased sensitivity.	DOC CO_2 production in sealed vessels giving % degradation. Potential to measure $^{14}CO_2$ Only DOC in test guideline 302A	Test substance must be non-volatile, not lost by foaming and non-toxic at test conc. Sorption potential needs to be determined.	Applicable for mineralisation
OPPTS 835.5045 Modified SCAS for insoluble and volatile chemicals	Months (often up to 120 days).	Settled domestic sewage and activated sludge.		CO_2 production in sealed vessels giving % degradation Potential to measure $^{14}CO_2$		Applicable for mineralisation
OECD 302B Zahn Wellens (ISO 9888) (OPPTS 835.3200)	28 days	Inoculum of 200 - 1000 mg l^{-1} (TSS) of activated sludge. Unadapted or pre-adapted inoculum	Aerated batch culture, using the test chemical as the sole carbon source (50 – 100 mg l^{-1} DOC) and with the inoculum at 20-25°C. Assesses ultimate biodegradation.	DOC or COD or Specific analysis for primary transformations	Test substance must be non-volatile and soluble not lost by foaming and non-toxic at test conc. Sorption potential needs to be determined	Limited use as DOC needed

Method	Test duration	Inoculum	Test conditions	Measurements (additional analysis)	Limitations	Applicability for nanomaterials
OECD 302C MITI (II)	14-28 days	Aerobic mixed, specially grown, unadapted micro-organisms at 100 mg l^{-1} (TSS, or approx. 3×10^7 - 3×10^8).	Agitated batch culture, using the test chemical as the sole carbon source (30 mg ThOD/l) with inoculum. Assesses ultimate biodegradation.	O_2 demand and possibly specific chemical analysis	Test substance must be non-volatile, not lost by foaming and non-toxic at test concentration.	Not applicable as very high amount of carbon needed.
OPPTS 835.3100 Aerobic aquatic biodeg	28 days after pre-adaptation	Pre-adapted inoculum	Agitated aerated aquatic test using test chemical (10 mg l^{-1} DOC) pre-adapted inoculum from a medium concentration of aerobic mixed micro-organisms at 20-25°C. ^{14}C labelled compounds may be used	DOC removal and CO_2 evolution ^{14}C provides mass balance phase distribution data	Test substances must be soluble and non-volatile.	Limited use as DOC needed

Method	Test duration	Inoculum	Test conditions	Measurements (additional analysis)	Limitations	Applicability for nanomaterials
OPPTS 835.5045 Modified SCAS test for insoluble and volatile chemicals	40 to 120 days	Settled domestic sewage and activated sludge Unadapted or pre-adapted inoculum	Same principle as for OECD 302A but with a volatiles trap on the aeration unit and additional analytical requirements for trapped volatiles and sludge solids. 20 mg l^{-1} DOC test concentration at 20-25^{o}C. ^{14}C labelled compounds may be used.	DOC. Specific analysis can provide primary transformation data. Kinetic data and half-life determination available. >20% removal of DOC =inherent biodegradation, >70% =ultimate biodegradation.	Additional analytical requirements.	Limited use as DOC needed
Inherent Biodegradation - Soil						
OECD 304A (ISO 14239 – biometer system) OPPTS 835.3300	Up to 64 days	Disturbed soil – alfisol, spodosol, entisol. In special cases can use soil with high silt fraction content or soil with high clay content (30%).	Incubation in the dark at 22^{o}C ± 2^{o}C	CO_2 evolution giving % degradation	Labeled substances needed	Labeled substances needed
Anaerobic Degradation Test Methods						
OECD 311 ISO 11734	Up to 60 days	Washed digester sludge at 1-3 /l in nutrient amended anaerobic medium, containing a redox indicator in sealed vessels.	Batch culture with test concentration of 20-100 mg l^{-1} as OC, at 35^{o}C. Assesses ultimate biodegradation	Total gas production (CH_4+CO_2) using a pressure transducer and DIC	Test substance must be non-toxic at test concentration.	Very high amount of test material needed. May not be applicable.

Method	Test duration	Inoculum	Test conditions	Measurements (additional analysis)	Limitations	Applicability for nanomaterials
OPPTS 835.3400 Anaerobic biodegradability of organic chemicals	Up to 56 days.	Sludge from an anaerobic sludge digestor. Recommendations are for a well-mixed primary sludge from a digester with a retention time of 15 to 25 days.	Test sample concentrations at around 50 mg l^{-1} with tests carried out at 35°C.	CO_2 and CH_4 production.	Not applicable to toxic chemicals, reproducibility not yet fully defined. Uses high concentrations of test substances.	Might be applicable with labeled substances

ANNEX III- 2. DETAILED REVIEW OF OECD BIOACCUMULATION METHODS

Detailed review of OECD Guideline 305 Bioconcentration in Fish

Paragraph 3 – In introduction it seems like this test was designed for assessing the uptake of hydrophobic organic contaminants given the focus on K_{ow} and empirical relationships based thereon. It is highly unlikely that this coefficient will be important for inorganic nanoparticles. The extent to which octanol-water partitioning relationships have relevance for carbon-based nanomaterials such as nanotubes and fullerenes is also unknown. Hence it is no use to refer to the follow tests: octanol-water partition coefficient, vapor pressure, hydrolysis (especially for inorganic nanomaterials), and surface tension. For biodegradability and phototransformation, it is unlikely these processes would destroy most nanomaterials especially inorganic ones although changes in the surface coatings of the nanomaterials is a distinct possibility that could influence their fate.

Paragraph 5 – A method to measure metal concentrations in the fish such as inductively coupled plasma mass spectroscopy (ICP-MS) could be included here to determine concentrations of inorganic nanomaterials.

Paragraph 7 – There are several differences between the biouptake of nanomaterials and typical hydrophobic organic chemicals that may be important factors in long-term bioaccumulation experiments. First, one concern is the stability of the nanoparticle suspension in the aqueous phase; if the nanoparticles settle, the concentration in the aqueous phase could change with time. Second, biotransformation of the surface coatings on the nanomaterials or enhanced aggregation of the nanoparticles during passage through the fish may cause the aqueous phase concentration to decrease during the course of the experiment. Third, it is possible that the size of the nanoparticles could change during the course of the experiment if they aggregate, and this change in the size of the nanoparticles could influence biouptake.

Paragraph 10 – The text starting after "Since, for many organic substances, …", may be irrelevant because this may not hold true for organic or inorganic nanoparticles. It is also unknown whether the lipid content of the organisms would influence biological uptake of various nanoparticles.

Paragraph 12 – On validity criteria: as described above in comment on paragraph 7, it may be difficult to keep the chemical concentration within 20% of the mean unless new nanoparticles are added to the water throughout the experiment given that many nanoparticles aggregate and settle with time.More relevant would be to achieve homogenuous dispersion of the material rather than dissolved concentration.

Paragraph 15 – The water characteristics are particularly important since they could influence the aggregation and settling behaviors of the nanomaterials. Perhaps having a test conducted first to assess the stability of the nanoparticles in water with predetermined characteristics would be a valuable step before the accumulation experiment.

Paragraph 18 – This discussion of stock solutions may not be relevant because many nanomaterials are not stable in solutions at elevated concentrations unlike hydrophobic organic chemicals. The discussion of what dispersants or solvents is very applicable for many nanomaterials though.

Paragraph 50 –Given that degradation of carbon nanomaterials has not yet been shownand significant degradation is highly unlikely given their inert structure, this section could be irrelevant. If degradation is shown, this part could be re-inserted.

Paragraph 52 – Because the relationship between lipid content and nanomaterial uptake is unknown and unlikely at least for inorganic nanomaterials, This paragraph should be revised and more specific

Detailed review on Bioaccumulation in sediment worms (OECD draft 2007)

Paragraph 7 – These relationships may not hold true for nanoparticles and this paragraph could be irrelevant for testing nanoparticle accumulation.

Paragraph 8 – The references to K_{ow} might not be relevant for nanoparticles.

Paragraph 9 – Most of these typical physicochemical data will not apply to nanomaterials e.g. the references to K_{ow}, vapor pressure, Henry's Law, critical micelles concentration, and surface tension. For biodegradability and phototransformation, it is unlikely these processes would destroy most nanomaterials especially inorganic ones although changes in the surface coatings of the nanomaterials is a distinct possibility that could influence their fate.

Paragraph 20 - It is currently unknown whether the lipid content of the organisms would influence biological uptake of various nanoparticles.

Paragraphs 33, 34 and 35 – The most appropriate way to spike nanomaterials to sediments is currently unclear and the effects of different spiking approaches on bioaccumulation are unknown. For a discussion of this topic (although the author focuses on spiking to soils), please review the attached learned discourse by Dr. Scott-Fordsmand et al. 2008 These are the four principal methods for spiking the nanoparticles (NP): "1) direct addition of the NP to the soil, 2) stirring of the NP in water for a prolonged time (to disperse the nanoparticles), 3) sonication of NP into a water solution, and 4) dissolving in water with the use of a carrier (e.g., tetrahydrofuran or alcohol)."

Paragraph 60 – The sentence: "Compounds with log K_{ow} above 5 are not expected to be eliminated significantly during any gut-purging period in water only, while chemicals with log K_{ow} lower than 4 may be lost in notable amounts."is not relevant for nanomaterials

Paragraph 63 – How quickly the samples are measured may not be as important for nanomaterials since biodegradation and volatilization is unlikely. However, there could potentially be aggregation of the nanomaterials which could potentially affect certain analytical methods for nanomaterial quantification.

Paragraph 69 – The reference to the lipid concentration in this paragraph might not be needed given the uncertain relationship between lipid content and bioaccumulation for nanomaterials.

Paragraph 75 – A different set of properties should be measured for the nanomaterials other than K_{ow} and solubility. Some important properties for nanomaterials include size, surface area, number concentration or surface charge, impurities, chemical composition, and the presence of surface coatings.

SECTION 4

HEALTH EFFECTS

INTRODUCTION

The purpose of this document is to review the applicability for testing manufactured nanomaterials of the OECD Test Guidelines for investigating health effects of chemicals.

There are currently 52 different OECD Test Guidelines for investigating the health effects of chemicals. These cover a range of endpoints and duration of exposure and allow the comprehensive investigation of the health effects of chemicals. Some guidelines have been introduced, or updated, relatively recently, whilst others have been identified as priorities for updating. Some however are only very rarely used in the general chemicals area, for example several of the *in-vitro* methods for investigating genotoxicity of chemicals or the in-vivo germ cell assays since there is no clear evidence for germ cell specific mutagens. It is unlikely that these would be needed for investigating the health effects of nanomaterials (Further explanation is given in the Annex).

Consideration of the individual OECD guidelines, with comments on their applicability and relevance to nanomaterials, is given in the Annex to this report. The position regarding those guidelines considered most relevant for investigating the health effects of nanomaterials is summarised below, after an important generic point relevant to all the health effects guidelines.

1. GENERIC COMMENT REGARDING ALL TEST GUIDELINES

Although the current OECD guidelines generally are likely to be applicable for investigating the health effects of nanomaterials there are some important considerations that need to be taken into account which particularly relate to their physicochemical characteristics, including in the dosing vehicle. In all cases the test guidelines need to be modified to ensure that appropriate consideration is given to adequate characterisation of the nanomaterial tested and also to the actual exposure of the test system, allowing for possible agglomeration/ disagglomeration. Consideration should be given to the most appropriate dose metric if known. If this is not the case a number of measurements need to be made including mass, particle number concentration and surface area. Furthermore it is important to build upon current knowledge and practical solutions in relation to approaches to test-sample preparation and the avoidance of artefact formation in approaches using in-vitro tests (including the impact on aggregation/agglomeration vs disaggregation/disagglomeration of the nanomaterial being tested , and on the test parameters selected as endpoints)

2. GUIDELINES CONSIDERED MOST RELEVANT FOR INVESTIGATING HEALTH EFFECTS OF NANOMATERIALS

2.1 Acute Toxicity

For oral exposure the Test Guidelines 420, 423 or 425 (adopted in 2001, 2001 or 2006 respectively) would be appropriate for initial investigation. It should be recognised that the extent of pathology at autopsy is limited.

The current guideline for exposure by inhalation, namely Test Guideline 403 (adopted 1981) includes only very limited histological examination at autopsy. In studies investigating the acute toxicity of nanomaterials by inhalation, detailed examination of the respiratory tract would be appropriate with consideration of the addition of BAL (broncho-alveolar lavage) and possibly pulmonary cell proliferation endpoints. The OECD guideline is currently being updated, but this does not take into account nanomaterial assessment . Further revision should be planned after adoption or a specific guideline should be developed.

The current guideline for dermal exposure, 402 (adopted 1987), only requires minimal pathology; it would be desirable to have enhanced pathology when investigating nanomaterials.

2.2 Skin and Eye Irritation/Corrosion and Skin Sensitization

The recently adopted *in-vitro* methods for investigating skin corrosion, Test Guidelines 430, 431, and 435 may be used but noting that measurement of cell viability using MTT (or other metabolically converted vital dye) may not be appropriate due to marker inactivation.

The investigation of skin and eye irritation using Test Guidelines 404 and 405 respectively would be applicable for investigating the irritancy of nanomaterials.

The local lymph node assay (LLNA), Test Guideline 429 would appear to be the most appropriate method for investigating skin sensitization potential of nanomaterial.It has advantages with respect to animal welfare considerations, objectivity of end-point and uses fewer animals than the guinea-pig method described in test guideline 406; it also uses less compound which may be a distinct advantage when investigating well characterised nanomaterial. In addition the test permits an estimation of the potency of the sensitization reaction.

2.3 Repeated dose toxicity studies (28 and 90 days) in rodents

The studies via the oral route, Test Guidelines 407 (28 day) and 409 (90 day) have relatively recently been updated (2008 and 1998 respectively) to enhance their ability to detect neurotoxic and immunotoxic effects and also effects on the reproductive system. Test guideline 407 has recently been updated to give enhanced ability to detect effects on the endocrine system. These guidelines are applicable for investigating the repeated dose toxicity of nanomaterials by the oral route. It should be noted however that consideration needs to be given to enhancing the ability of these methods to detect adverse effects that are a particular concern with some nanoparticles. For example cardiovascular effects are believed to be a concern with ultrafines (nanoparticles) in air pollution.

The situation is different regarding the repeated dose inhalation test guidelines, 412 (14-28 day) and 413 (90 day), both of which were adopted in 1981. (Draft proposals for updating are currently being considered). The current test guidelines have not been enhanced to detect neurotoxic or immunotoxic effects. The 14-28 day Test Guideline (412) also has very limited pathology. Detailed histological examination of the entire respiratory tract would be expected when investigating the effect of

nanomaterials following repeated exposure by inhalation, with consideration of the addition of BAL (broncho-alveolar lavage) and possibly pulmonary cell proliferation endpoints. Both guidelines need to be enhanced as in the updated oral guideline, particularly with respect to neurotoxicity and immunotoxicity when investigating nanomaterials. Similar comments also apply as to those made for the oral study regarding the need to consider investigating adverse effects that are a particular concern with some nanomaterials.

2.4 Genotoxicity

It is possible to investigate the mutagenic potential of chemicals using in-vitro assays. It is felt that the following 3 assays, all well validated for chemicals and based on mutation as endpoint (rather than an indicator of DNA damage), will be applicable for an initial investigation of the mutagenic potential of nanomaterials ; these guidelines have been updated relatively recently (1997).

- Test Guideline 471 Bacterial reverse mutation assay (adopted 1997)
- Test guideline 473 In-vitro mammalian cell gene mutation test (adopted 1997)
- Test guideline 476 *In-vitro* mammalian cell gene mutation assay (adopted 1997), with the mouse lymphoma assay being the preferred assay.

There have been some questions raised about the appropriateness of the OECD Test Guidelines for testing nanomaterials, particularly the bacterial assays, based on concerns as to whether the mechanisms of genotoxicity differs from other chemicals, although the evidence for this is limited. It is believed that these concerns would be mitigated by testing in both bacterial and mammalian cell assays, preferably all 3 of the above. In addition, it has been recognised that treatment of mammalian cells in vitro with insoluble particles may lead to misleading results. This has been taken into consideration by current OECD and ICH guidelines for genetic toxicity testing. It is suggested that the current test guidelines be used and that the situation be reviewed in the light of the results obtained from the program of testing selected.

Positive results in vitro would need to be followed up in vivo using the OECD guidelines 474, 475, or 486 if the bone marrow or liver were appropriate target organs, and this would be dependent on systemic availability. It is possible that the initial sites of contact, such as the respiratory tract following inhalation, would need to be investigated for genotoxicity. There are currently no OECD guidelines available for such studies.

2.5 Reproductive toxicity

The OECD guidelines to investigate reproductive toxicity (421, 422, 415, and 416) and developmental toxicity (414) appear, in principle, applicable for investigating the reproductive toxicity of nanomaterials. The extent of testing in this area would need to be carefully justified. All the guidelines relate to use of the oral route. They would need to be modified if exposure was by inhalation, and this route presents additional difficulties for reproductive toxicity studies, and would need careful consideration.

2.6 Toxicokinetics

There is general agreement that studies on the absorption, distribution, metabolism and excretion (ADME) of nanomaterials will be of fundamental importance in assessing their potential health effects. The current OECD guideline on toxicokinetics is the original version, adopted in 1984, and gives only very general guidance. Although this is currently being updated it is questionable whether modifications would be sufficient for investigating nanomaterials. It is likely that specific studies on the absorption and

distribution of nanomaterials will need to be designed on a case-by-case basis. In particular, due to the likely property of nanoparticles to translocate whatever the exposure conditions, studies tracking the distribution of nanomaterials in-vivo at realistic exposure scenarios will be necessary, but will also be technically challenging. Labelled nanomaterials will be needed for such studies. The main issues associated with ADME studies with nanomaterials are the following:

- Ensuring that the label remains with the nanoparticles following route of entry into the body; and

- Ensuring that the label does not alter the biological activity of the nanoparticle particularly since the changes in surface chemistry of the nanoparticle can significantly influence the physicochemical properties of the nanoparticle and, as a consequence, the toxicity of the nanoparticle.

Therefore preliminary studies should be undertaken to certify that the above 2 criteria are met before undertaking a toxicokinetic study.

There are specific OECD guidelines for one area of absorption studies, namely skin absorption using either an in-vivo method (test guideline 427) or an in-vitro method (test guideline 428); these were both adopted in 2004. The use of the in-vitro method has been questioned for nanomaterials since it has been claimed that mechanical aspects such as flexing may be important and some further development of this assay may be needed for nanomaterials (This issue may be considered by the other project of the WPMN concerned with the role of alternative methods in nanotoxicology).

3. CONCLUSIONS

In general the OECD guidelines are applicable for investigating the health effects of nanomaterials with the important proviso that additional consideration needs to be given to the physicochemical characteristics of the material tested, including such characteristics in the actual dosing solution. In some cases there will be a need for further modification to the OECD guideline. This applies particularly to studies using the inhalation route and to toxicokinetic (ADME) studies. Finally it is important to build upon current knowledge and practical solutions in relation to in-vitro test approaches.

ANNEX IV
REVIEW OF CURRENT OECD HEALTH EFFECTS TEST GUIDELINES

The OECD test guidelines are considered below. Information is given on when the current version was adopted, with brief comments on the method and its applicability to manufactured nanomaterials.

A. ACUTE TOXICITY STUDIES

A.1 Oral Route

The earlier OECD guideline to investigate acute toxicity by the oral route has been subject to updating, with the emphasis on avoiding the need to calculate LD_{50} values with confidence limits and other animal welfare issues, resulting in a range of new test guidelines which may be used (with the original guideline being deleted) .

TG 420 Acute Oral Toxicity ; Fixed dose Procedure.(Adopted 2001)

Animals observed for signs of toxicity. Limited pathology at autopsy, namely gross lesions with microscopic examination of organs showing evidence of gross pathology.

TG 423 Acute Oral Toxicity; Acute Toxic Class Method. (Adopted 2001)

Observations essentially as above.

TG 425 Acute Oral Toxicity; Up and Down Method (Adopted 2006)

Allows calculation of an approximate LC_{50} value. Observations essentially as above.

A. 2 Inhalation route

TG 403 Acute Inhalation Toxicity. (Adopted 1981)

Based on observing signs of toxicity and estimating LC_{50} Minimal pathology namely; 'Consideration given to gross necropsy when indicated by signs of toxicity observed. Microscopic examination of any target organs should be considered.'

Discussions are ongoing in the test guidelines programme regarding updating this guideline.

A.3 Dermal Route

TG 402 Acute Dermal Toxicity (Adopted 1987)

Based on observing signs of toxicity and estimating $LC_{50.}$ Minimal pathology, namely. 'All gross lesions recorded with microscopic examination of organs showing evidence of gross pathology in animals surviving 24 hours or more should also be considered'.

Comment regarding use of acute toxicity guidelines for testing nanomaterials

The extent of histological examination, particularly of the initial sites of contact eg respiratory tract after inhalation, appears to be less than would be needed for an appropriate examination of the acute toxic effects of nanoparticles. In addition consideration needs to be given to broncho-alveolar lavage (BAL) and possibly pulmonary cell proliferation endpoints.

B. SKIN AND EYE IRRITATION/ CORROSION AND SKIN SENSITIZATION

B.1 Skin Corrosion

There are OECD test guidelines for 3 validated in-vitro methods.

- TG 430 *In-vitro* Skin Corrosion Test. Transcutaneous Electrical Resistance Test (TER). (Adopted 2004).
- TG 431 *In-vitro* Skin Corrosion Test. Human Skin Model Test. (Adopted 2004).
- TG 435 *In- vitro* Membrane Barrier Test for Skin Corrosion. (Adopted 2006).

Comment regarding testing nanomaterials

TG 431quantifies cell viability using MTT (or other metabolically converted vital dye). The ILSE report (and others) have noted that this is an unpredictable measure of cytotoxicity with nanoparticles due to marker inactivation.

B.2 Skin irritation

TG 404. Acute Dermal Irritation (Adopted 2002)

Prior to testing in animals corrosive substances should have been identified for example by consideration of physicochemical properties such as pH or by use of one of the in-vitro methods listed above. The animal test (rabbit) is used to confirm absence of irritation or to grade mild/moderate irritation.

Note: ECVAM now consider that EPISkin has been validated as an in-vitro method for assessing skin irritation. However it uses MTT reduction (and IL-1 α release) which may present problems when testing nanomaterials.

B.3 Eye Irritation

TG 405 Acute Eye Irritation/Corrosion (Adopted 2002)

There is no need for testing if the compound is a severe irritant to skin or if results from validated in-vitro tests for severe eye irritation (e.g. isolated eye models) are positive(there are currently no OECD test guidelines for in –vitro models)

Comment regarding testing of nanomaterials

The OECD test guidelines for investigating skin and eye irritancy would appear to be appropriate for investigating the irritancy of nanomaterials

B.4 Skin sensitization

There are 2 OECD guidelines available:-

- TG 429 Skin Sensitization; Local Lymph Node Assay. (Adopted 2002)
- TG 406 Skin Sensitization. (Guinea pig models) (Adopted 1992)

Comment regarding testing nanomaterials

The local lymph node assay in mice has significant advantages over TG 406 (based on the guinea pig maximization test or the Buehler assay) with respect to animal welfare considerations (including use of fewer animals), objectivity of end-point and estimation of potency of sensitizing agents. Furthermore less compound is needed, a distinct advantage when testing well characterised nanomaterials. It is felt that this should be the preferred method for testing nanomaterials, and that TG 406 need not be considered in this context.

B.5 Phototoxicity

There is an OECD test guideline for a phototoxicity test, namely TG 432 In vitro 3T3 NRU Photoxicity Test. This is an in-vitro assay to measure the phototoxic potential of a test substance induced by the 'excited' chemical after exposure to light. It is based on Balb/3T3 cells and measuring cytotoxicity by neutral red uptake.

Comment regarding testing nanomaterials

This assay is mainly used for cosmetics, specifically testing UV filters in sunscreens for phototoxicity.

C. REPEATED DOSE TOXICITY STUDIES

C.1 Oral route

TG 407 Repeated dose 28-day Oral toxicity study in rodents (Revised version Adopted 2008)

The update in 1995 put more emphasis on examination of signs of neurotoxicity and enhancing histopathology at autopsy to detect effects on the gonads and on the immune system. Histology is now nearly as extensive as in the 90 days study, recognising that this may be the basic repeated dose study for compounds where a 90 day study was not warranted. To increase the ability to detect signs of neurotoxicity a Functional Observation Battery (FOB) of tests is included (measuring response to a range of sensory stimuli, assessment of grip strength and motor activity).

This guideline has been updated to enhance its ability to detect effects of the endocrine system.

TG 408 Repeated dose 90 day oral toxicity study in rodents (Adopted 1998)

The ability to detect signs of toxicity was enhanced in the 1998 revision by inclusion of a FOB as above, and the histopathology enhanced further with regard to neurotoxic and immunotoxic effects and also effects on the reproductive organs (including accessory sex organs).

This study should allow the identification of compounds with the potential to cause neurotoxic or immunological effects or effects in reproductive organs. Further studies may be needed to characterise such effects.

Comment regarding nanomaterials

The oral 28 and 90 day oral repeated dose toxicity studies in rodents are applicable for investigating the effects of nanomaterials. However in view of presence of specific concerns about adverse effects, consideration may need to be given to enhancement of the ability to detect such effects in both the 28 day and 90 day study.

TG 409 Repeated dose 90 day oral toxicity study in non-rodents (Revised version adopted 1998)

This guideline is mainly concerned with studies in the dog although other species could be used (e.g. mini-pigs). Primates are not recommended and their use needs to be justified. The extent of histopathology was enhanced in 1998 and is now very comprehensive and comparable to the rodent 90 day study.

Comment regarding nanomaterials

In view of the amount of compound needed and other considerations, it is unlikely that studies on nanomaterials will be needed in the dog except in exceptional circumstances. They are only rarely used in the general chemicals area, but are required for approval of pesticides (and medicines)

C.2 Inhalation route

TG 412 Repeated dose inhalation toxicity 28 day or 14 day study (Adopted 1981)

This current guideline has not been updated since first adopted in 1981, but a draft revised guideline is currently under consideration.

The current guideline does not include the additions made relatively recently to the repeated dose oral guidelines to enhance their ability to detect compounds with neurotoxic potential (such as the FOB) and also contains very limited pathology compared to what is required in the 28 days oral study.

Comment regarding investigating nanomaterials

As a minimium more detailed histological examination of the entire respiratory tract would be expected at autopsy when investigating the effects of nanomaterials by inhalation. Consideration also needs to be given to enhancing ability to detect neurotoxic effects and any specific concerns about adverse effects, (as in the oral guidelines).

TG 413 Sub-chronic Inhalation Toxicity 90 day study (Adopted 1981)

This guideline has also not been updated since originally adopted in 1981. There is a requirement for fairly comprehensive histological examination at autopsy, but somewhat less than in the 90 day oral study in rodents (especially regarding peripheral nerves and accessory sex organs). Also there is no requirement for a FOB to enhance the ability to detect compounds with neurotoxic potential.

Comment regarding investigating nanomaterials

Consideration needs to be given to enhancing the ability of this guideline to detect neurotoxic and other specific adverse effects, if appropriate.

Note. Both TG 412 and 413 are currently being updated by the OECD; USA leads on 413. An expert meeting was held in the Netherlands in June 2007 and it is anticipated that revised draft guidelines will be circulated in 2008 for comment

C.3 Dermal route

TG 410 Repeated Dose Dermal toxicity 21/28 day study (Adopted 1981)

This guideline also has not been updated since originally adopted in 1981. It is a very limited study compared to the current oral repeated dose study guidelines. There is the requirement for only very limited pathology at autopsy, only skin, liver, kidney and organs showing gross lesions.

TG 411 Sub-chronic Dermal toxicity 90 day (Adopted 1981)

More comprehensive pathology than above, but less than the 90 day oral study.

Comment regarding testing of nanomaterials for repeated dose toxicity by the dermal route

The repeated dose dermal studies appear to be little used for chemicals in general. They have low priority for updating by OECD. Most chemical risk assessments for dermal exposure are based on information from repeated dose studies using the oral route together with information on skin absorption. It is likely that any testing of nanomaterials by the dermal route would be limited to acute toxicity and investigation of the extent of absorption through skin.

D. GENOTOXICITY STUDIES

There are currently 15 OECD test guidelines for investigating the genotoxicity of chemicals. However in practice only a limited number are routinely used for regulatory purposes. These comprise assays considered below

D.1 In-vitro methods

Many regulatory guidelines require chemicals to be initially investigated using in-vitro studies to assess mutagenic potential using three (or two if there is limited exposure) mutagenicity assays to cover both gene mutation and chromosome aberrations.

The methods recommended are:

- TG 471 Bacterial Reverse Mutation Assay (Adopted 1997): This measures gene mutations in Salmonella typhimurium and Escherichia col.
- TG 473 In-vitro mammalian chromosome aberration test (Adopted 1997): This measures chromosome aberrations by metaphase analysis in cultured mammalian cells.
- TG 476 In-vitro mammalian cell gene mutation test (Adopted1997): This detects gene mutations in mammalian cells. A range of cell systems may be used but that using L5178Y mouse lymphoma cells and measuring mutations at the thymidine locus is preferred by some because of its ability to detect both gene mutations and chromosome aberrations, with the ability to distinguish these by colony sizing.

All these in-vitro methods are well validated and measure mutation as an endpoint, rather than an indicator of genetic damage. The other *in-vitro* assays, together with the one assay using insects (Drosophila melanogaster) are now not commonly used when investigating the mutagenic properties of chemicals and will not be described here.

D.2 *In–vivo* methods in somatic cells

For compounds that are positive in any one of the above in- vitro assays there will be a need to investigate whether their mutagenic potential can be expressed in somatic cells in animals. There are 3 relevant OECD test guidelines which cover bone marrow and liver as target organs.

- TG 475 Mammalian Bone Marrow Chromosomal Aberration Test (Adopted 1997): This measures structural chromosome aberrations by metaphase analyses in bone marrow cells of rodents.
- TG 474 Mammalian Erythrocyte Micronucleus Test (Adopted 1997): Micronuclei are an indirect measure of damage to chromosomes or the mitotic apparatus of erythroblasts, and thus are an indicator of structural or numerical chromosome aberrations. Samples are obtained from bone marrow or peripheral blood cells of rodents.
- TG 486 Unscheduled DNA synthesis (UDS) Test with Mammalians Liver Cells In Vivo.(Adopted 1997): UDS is an indirect indicator of DNA damage. This method thus detects DNA damage in the liver of treated animals.

D.3 *In–vivo* methods using germ cells

There are a number of OECD test guidelines to investigate mutagenicity in germ cells in vivo. These are not commonly used when investigating the mutagenic properties of chemicals. There is currently no clear evidence for germ cell specific mutagens. All established germ cell mutagens have also been shown to produce positive results in somatic cells (bone marrow) assays in animals (e.g. Shelby MD Selecting Chemicals and Assays for assessing mammalian germ cell mutagenicity, Mutation Research 352 156-69 (1996) [The reverse is not true and all somatic cell mutagens are not also germ cell mutagens]. Thus it is possible to screen for germ cell effects in vivo using the in vivo assays in somatic cells noted above.Thus germ cell assays will not be described here.

Comment regarding testing nanomaterials

The following OECD guidelines covering in-vitro methods should be considered when investigating the mutagenic potential of the nanomaterial:

- OECD TG 471 Bacterial reverse mutation test.
- OECD TG 473 *In vitro* mammalian chromosome aberration test.
- OECD TG 476 *In vitro* mammalian cell gene mutation test.

It is recognised that questions have been raised about the appropriateness of these test methods for investigating the mutagenicity of nanomaterials , particularly the bacterial assays , based on concerns that the mechanisms of genotoxicity of nanomaterials differs from that of other chemicals. However the evidence for this is limited, and, provided that both bacterial and mammalian cell assays are used, and preferably all 3, this should provide adequate information on mutagenic potential of the nanomaterials. In addition it has been recognised that treatment of mammalian cells in vitro with insoluble particles may lead to misleading results. This has been taken into consideration by current OECD and ICH Guidelines on genetic toxicity testing. The situation should be reviewed in the light of the results obtained from the testing program.

Positive results would need to be followed up by investigations to assess whether the activity seen in vitro can be expressed in vivo. The available OECD test guidelines listed below cover the bone marrow and liver, and may be appropriate depending on the systemic availability of the nanomaterial:

- OECD TG 474 Mammalian erythrocyte micronucleus test

- OECD TG 475 Mammalian bone marrow chromosome aberration test
- OECD TG 486 Unscheduled DNA synthesis (UDS) test with mammalian liver cells in vivo.

However it may be that the main concern would be in investigating activity at sites of first contact such as the respiratory tract following inhalation. In such cases specially designed studies would be necessary.

The remaining genotoxicity test (TGs 477,478,479,480,481,482,483,484,and 485) are unlikely to be used when investigating the mutagenicity of nanomaterials.

E. REPRODUCTIVE TOXICITY

TG 421 Reproductive Toxicity Screening Test.(Adopted 1995)

This screening test guideline can be used to provide initial information on effects of a chemical on reproduction and development. Male and female rodents are dosed for two weeks prior to mating and during the mating period. The males are dosed for a further two weeks and then subject to autopsy with testes and accessory sex organs examined histologically. Females are dosed throughout pregnancy and for four days lactation. The adult animals are then subject to autopsy with particular attention given to identifying the number of implantation sites and corporea lutea and histological examination of ovaries and accessory sex organs. Offspring are observed for any visible abnormalities and weight gain. The method generates information on reproductive performance, but this is limited particularly regarding post-natal manifestations of pre-natal exposure or effects that may be induced during post natal exposure.

The guideline assumes oral administration. Modifications maybe required if other routes are used.

TG 422 Combined Repeated Dose Toxicity Study with Reproduction Developmental Toxicity Screening Test (Adopted 1995)

This method is intended for use in those circumstances where initial data are needed from both a reproductive toxicity screening test and a 28 day repeated dose toxicity study. Observations are similar to those in the oral 28 day study (TG 407) and the reproductive toxicity screening test.

The guideline assumes oral administration. Modifications may be required if other routes are used.

Comment regarding testing nanomaterials using TG422

This method is recommended as a possible tier 2 study for nanoparticles with inhalation exposure by the ILSI Research Foundation/Risk Science Institute expert group.

TG 415 One-Generation Reproductive Toxicity Study (Adopted 1983)

This study provides general information on the reproductive performance of rodents over one generation. Male animals are dosed for at least one complete spermatogenesis cycle (about 56 days in mouse, 70 in rat) and females for at least two complete oestrous cycles before mating. Both sexes are dosed throughout the mating period and the females also throughout pregnancy and the nursing period. Growth and development of the offspring is noted from birth to weaning.

The guideline recommends that the oral route (diet or drinking water) be used, but notes that other routes are also acceptable.

TG 416 Two-Generation Reproduction Toxicity Study.(Adopted 2001)

This is similar to the above guideline, but dosing of the F_1 generation (both sexes) continues during their growth into adulthood, mating the production of an F_2 generation and until these are weaned. In addition, when this guideline was updated in 2001 its ability to detect endocrine disrupter effects relating to sex hormones was enhanced by much more detailed examination of sperm parameters and effects on the oestrus cycle.

The guideline notes that the oral route is the preferred route. If another route is used justification should be provided and appropriate modifications may be necessary.

TG 414 Pre-natal Developmental Toxicity Studies (Adopted 2001)

This study provides information on the effects of exposure during pregnancy on the developing offspring (e.g. malformations, variations, delayed growth etc.) When this guideline was updated in 2001 the exposure period was extended to cover the entire period from implantation to the day before caesarean section near full term, when the uteri and contents are examined. (Previously the exposure had been only during the period of organogenesis). The preferred species are the rat or rabbit.

Test substance is usually administered orally; if another route is used justification must be given and appropriate modifications may be necessary.

Comment regarding testing nanomaterials

The guidelines described above would appear to be applicable in principle, for investigating the reproductive toxicity (both fertility and effects on developing offspring) of nanomaterials. The extent of testing in this area would need to be carefully considered in view of the complexities of the studies. The Combined Repeated Dose Toxicity Study with Reproduction Developmental Toxicity Screening Test., TG 422 may be appropriate in certain circumstances. For all studies the OECD guideline relates to the use of the oral route and modifications would need to be made for use of the inhalation route. Such studies present additional difficulties with regard to reproductive toxicity and the use of this route would need to be carefully justified.

F. TOXICOKINETICS

The OECD toxicokinetics guideline, 417 (adopted 1984) is a very general guideline covering absorption, distribution, metabolism, excretion studies. The need for flexibility is noted with actual studies being designed to suit the particular substance in question (and the specific aspect being investigated).

This guideline is currently being updated (USA lead). A draft text is being considered by a small expert group. It is anticipated that a first draft text for an updated TG 417 will be formally circulated for comment in 2008

In addition to this very general guideline there are two specific OECD guidelines covering skin absorption using either an in-vitro or an in-vivo method.

- TG 427 Skin Absorption: In vivo method (Adopted 2004)
- TG 428 Skin Absorption: In vitro method (Adopted 2004)

There is an OECD guidance document (no. 28) on the conduct of skin absorption studies which gives further information particularly relating to the in-vitro method.

Comments considering investigating nanomaterials

There is general agreement that absorption/distribution studies are of key importance with regard to investigating the likely toxicity of nanomaterials. However, studies will probably need to be designed on a case by case basis rather than using a specific guideline. In addition to the need for well characterised material and measurement of actual exposures, there will be the added analytical difficulty of tracking distribution in vivo at realistic exposure scenarios. Labelled nanoparticles are likely to be needed for such studies.

This is also a specific concern regarding the in-vitro TG for skin absorption (TG 428). Knowledge of skin absorption is a key factor for nanomaterials applied to the skin. This assay has the potential to provide such information using an in-vitro method. The recent opinion of the EC's Scientific Committee on Consumer Products on nanomaterials in cosmetics question the adequacy of this method arguing that mechanical aspects (presumably such as flexing) are important with nanoparticles. Further consideration of this is needed before recommendations can be made in this area.

G. NEUROTOXICITY

The oral 28 day and 90 day repeated dose toxicity studies in rodents (TG 407 & 408) and also test guideline 422 (Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test) provide general information on a range of potential toxic effects including neurotoxicity. Indeed TG 407 and 408 have been enhanced to give better coverage of neurotoxicity, and similar studies are required by TG 422. Such studies should normally identify compounds with neurotoxic effects, but that more detailed studies, as specified in the OECD neurotoxicity guidelines may be warranted to characterise neurotoxic effects when justified.

TG 424 Neurotoxicity Study in Rodents

The test guideline is used when detailed information is needed on the neurotoxicity of a chemical. This may be because information from TG 407 or 408 indicates that the compound has neurotoxic effects that warrant characterisation or sometimes because of other concerns.

The guideline is written for oral exposure. It is flexible about duration of exposure and as to what approaches to use. Regarding duration this may be 28 days, 90 days or one year or more; it also states that the procedures described maybe used in an acute neurotoxicity study.

The aim is to detect major neurobehavioural and neuropathological effects in adult rodents. A subgroup is used for perfusion fixation and detailed examination of the CNS and PNS at autopsy. There is the usual requirement for detailed clinical observations including a FOB. A range of more specialised tests of memory and learning, and for specific neurobehavioural neuropathological, neurochemical and electrophysiological effects are listed as possibilities depending on what aspect of neurotoxicity warrants specific consideration with a given compound. Literature references to appropriate techniques are given, rather than being described in the guideline itself. Further information is provided in the OECD guidance document series No. 20 "Guidance Document on Neurotoxicity Testing (2004).

Comments regarding investigation of nanomaterials

The repeated dose oral toxicity guidelines should be able to detect compounds with neurotoxic potential and it is unlikely that the OECD neurotoxicity guideline will be needed, at least in the initial stages of testing. With regard to studies by the inhalation route modification of the repeated dose inhalation guidelines will be needed as noted earlier, and indeed also of the neurotoxicity guideline, if used, since this is based on the oral route.

TG 426 Development Neurotoxicity Assay. (Adopted 2007)

This is a complex study designed to investigate functional and pathological changes in the developing nervous system of the offspring that may arise from exposure in-utero and during early life. Test substance is administered during gestation and lactation. Dams are tested to assess neurotoxic effects both during pregnany and lactation. The offspring are subject to a range of evaluations to assess gross neurologic and behavioural abnormalities including the assessment of physical development, behavioural ontogeny, motor activity, motor and sensory function and learning and memory. Animals are killed at several time intervals during postnatal development and adulthood and brain weights recorded, together with detailed neuropathological examination (perfusion fixation).

The preferred species is the rat. It is noted that the route of exposure will generally be oral, but other routes may be used depending on the characteristics and likely human exposure. It is only the oral route that is described.

Comments regarding investigating nanomaterials

This is a particularly complex study and its use has been very limited with chemicals (mainly pesticides with concerns regarding developmental neurotoxicity). Also few, if any, such studies have been carried out using the inhalation route. The added value of results from this assay with pesticides (in addition to data from a 90 day repeated dose toxicity study and from appropriate reproductive toxicity studies) to date is unclear. It is unlikely that this test guideline wikl be used for investigating nanomaterials.

Other OECD test guidelines relating to neurotoxicity

There are 2 OECD guidelines that specifically cover delayed neurotoxicity in organphosphates, namely:

- TG 418 Delayed neurotoxicity of organophosphorus substances following acute exposure. Adopted 1995
- TG 419 Delayed neurotoxicity of organophosphorus substances; 28 days repeated dose study. Adopted 1995.

Comments regarding investigation of nanomaterials

These guidelines are specific for organophosphates and are unlikely to be needed for testing nanomaterials.

H. CHRONIC TOXICITY AND CARCINOGENICITY

There are currently 3 OECD test guidelines, all in the original form as adopted in 1981. These are:

- TG 451 Carcinogenicity studies
- TG 452 Chronic Toxicity Studies
- TG 453 Combined Chronic Toxicity/Carcinogenicity Studies

Work has started on the updating of these guidelines with an expert group meeting in February 2008. The major issues identified for discussion at this meeting are outlined in the following 2 paragraphs.

The current chronic toxicity guideline is less detailed with regard to investigating neurotoxicity and other aspects of pathology than the 90 day oral study. This aspect needs updating. The current guideline also covers both rodents and non-rodents (e.g. dog). It is being suggested that any update covers only

rodents, the need for a chronic guideline in the non-rodent (e.g. dog) being questioned. There is also support for the view that chronic toxicity in rodents should always be investigated as part of the carcinogenicity bioassay, by use of appropriate satellite groups. It is possible that any future update will concentrate on the combined chronic toxicity carcinogenicity study in rodents.

Regarding the carcinogenicity study little change is expected regarding number of animals or duration of study or the already comprehensive examination of autopsy for tumours. Some consideration will be given to advice in dose levels (especially the top dose) and whether it will be practical to include information on mechanisms.

Any revision is likely to concentrate on the oral route of exposure, as do the current guidelines.

Comments regarding investigating nanomaterials

In view of the high cost of the studies in terms of time (minimum two years exposure in the rat), number of animals (50 per sex per dose level plus 100 in the controls) and amount of compound needed these studies are unlikely to be practical for nanomaterials except in very exceptional circumstances. This is particularly true for the studies using the inhalation route. The number of facilities available to carry out such studies are extremely limited.