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Review of OECD/OPPTS-Harmonized and OPPTS Ecotoxicity Test Guidelines for Their Applicability to Manufactured Nanomaterials

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Steve Diamond, Workgroup Chair, USEPA Office of Research and Development, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division
Dennis Utterback, Workgroup Organizer, USEPA Office of Science Policy
Christian P. Andersen, USEPA Office of Research and Development, National Health and Environmental Effects Research Laboratory, Western Ecology Division
Robert Burgess, USEPA Office of Research and Development, National Health and Environmental Effects Research Laboratory, Atlantic Ecology Division
Seishiro Hirano, National Institute for Environmental Studies, Japan
Kay Ho, USEPA Office of Research and Development, National Health and Environmental Effects Research Laboratory, Atlantic Ecology Division
Chris Ingersoll, Columbia Environmental Research Center, U.S. Geological Survey
Mark G. Johnson, USEPA Office of Research and Development, National Health and Environmental Effects Research Laboratory, Western Ecology Division
Alan J. Kennedy, U.S. Army, Engineer Research and Development Center Environmental Laboratory
David R. Mount, USEPA Office of Research and Development, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division
John Nichols, USEPA Office of Research and Development, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division
Pascal Pandard, INERIS, France
Paul Rygiewicz, USEPA Office of Research and Development, National Health and Environmental Effects Research Laboratory, Western Ecology Division
Janeck J. Scott-Fordsmand, National Environmental Research Institute, Denmark
Kath Stewart, AstraZeneca UK Limited, UK

U.S. Environmental Protection Agency
Office of Research and Development
National Health and Environmental Effects Research Laboratory
Mid-Continent Ecology Division
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EXECUTIVE SUMMARY

Assessing the environmental risk of manufactured nanomaterials (MNs) presents a significant and growing challenge for environmental regulators. These materials, defined as having at least one physical dimension between 1 and 100 nm are being developed, produced, and incorporated into a broad range of commercial, medical, environmental, and other products (USEPA 2007). The challenge for regulators derives in part from this rapid pace of MN development, but also because early evidence suggests that nanomaterials can be created in nearly unlimited variations in size, form, elemental composition, and the addition of functional groups (Colvin 2003, USEPA 2007). While the regulatory challenge of ever-growing numbers of substances is not unique relative to soluble chemicals, MNs also present several additional and novel challenges due to their particulate or fibrous properties. First, at sizes smaller than approximately 100 nm, MNs begin to exhibit behaviors and properties that are not apparent in their bulk forms, including electrical conductivity, elasticity, greater strength, different color, and greater reactivity (Parak et al. 2005). These novel properties are due to quantum effects that become dominant at the nanometer scale (most likely the lower range, approximately 10 to 20 nm). Smaller particle size enhances this phenomenon indirectly because total surface area for a given volume of material increases as a square function of decreasing particle size. Hence any quantum effects, assuming they are related to surface area, will have a higher probability of altering biological systems as surface area increases. It should also be noted that increased surface area will increase the probability of interactions that are related to bulk material properties as well, aside from any quantum effects.

A more immediate concern for regulators is what sort of guidance should be given to potential registrants on how nanomaterials should be tested. Generally, that guidance is provided by standard test guidelines within the USEPA, Office of Prevention, Pesticides and Toxic Substances (OPPTS), Series 850 Ecological Effects Test Guidelines (http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/850_Ecological_Effects_Test_Guidelines/). However, given the unique properties of nanomaterials, the applicability and adequacy of these test guidelines is questionable (Crane et al. 2008, Hansen 2009). To address that issue a workgroup was formed to evaluate the Series 850, as well as other, similar test guidelines, for their adequacy for testing nanomaterials. The workgroup was comprised of 14 international scientists with expertise in ecotoxicity testing and nanotechnology. This report summarizes the results of the review process, identifies specific areas where test guidelines are adequate or inadequate, and provides some recommendation for regulatory testing of nanomaterials.

The general conclusion of the workgroup is that none of the current ecotoxicological test guidelines reviewed are adequate for testing MNs. The breadth of the review, inclusion of selected, non-Series 850 guidelines, and the nature of the inadequacies, suggests that this is true of essentially all test guidelines (no colloid-specific toxicity test methods were identified). This is not to say that many aspects of the guidelines aren't adequate, but rather that any hazard testing undertaken with full adherence to the current guidance and without the addition of many critical nanomaterial-specific measurements and exposure approaches will yield data insufficient to reliably assess the hazard of nanomaterials. All of the inadequacies identified by the reviewers are directly related to the fact that MNs are generally particulate or fibrous and occur as colloidal suspensions in aqueous exposure media (including suspensions generated for wet application to, or mixing with, non-aqueous exposure media).

Methods and approaches for preparing exposure media, as well as measuring and characterizing materials both prior to testing and in prepared exposure media are absent in all test guidelines. Much of the terminology used in current test guidelines is specific to soluble chemicals and is either wholly inadequate for particulate and fibrous substances or not fully descriptive. An excellent example of this is the use of terms such as *dissolved*, *solution*, and *concentration*. The latter term, in particular, is used to describe exposure levels and exposure response; it is probable that endpoint responses will relate directly to additional factors such as surface area, particle size and count, and other nanomaterial properties. This also suggests that the terminology and metrology of exposure-effects relationships (e.g., LC50, EC50, NOEC, LOEC) are also not applicable (without some modification) for nanomaterials. The term *dissolved* is of specific concern because some nanomaterials are known to exist in media both as particles, and as ions that truly dissolve from the particles (e.g., nano-scale silver, [Benn and Westerhoff 2008]). However, the particles themselves are best described as being in suspension, so use of the term *dissolved* could lead to errors in interpretation of actual material exposure levels.

The review workgroup found that two aspects of current test guidelines are fully adequate for testing MNs. The first aspect is the toxicological principles inherent in all test guidelines, including use of healthy, viable organisms, incorporation of appropriate control treatments, selecting exposure levels, etc. The second aspect is the endpoints targeted in the test guidelines, and the species tested. In general, these endpoints, including survival, reproduction, growth, and others, are integrative of multiple mechanisms of toxicity, and should be as reflective of MNs toxicity as they are of soluble chemicals and formulations. It should be noted however that exploratory research may reveal nanomaterial-specific endpoints that, to be incorporated into regulatory testing, might require modification of existing, or drafting of new, test guidelines.

Specific, interim suggestions of the review group are:

1) Development of a nanomaterials-specific guideline document that would address the inadequacies common to all, or most guidelines. As discussed in the review, the Organization for Economic Co-operation and Development (OECD) guidance document on testing difficult substances (*Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures* [ENV/JM/MONO(2000)6]) provides an excellent framework for development of such a document.

2) Existing guidelines could be used to define required tests, but the inadequacies would need to be identified and approaches to addressing them stipulated to registrants. This represents an interim solution similar to generating the separate guidance document suggested above and would necessitate a case by case approach, but could be done immediately whereas generation of the new guidance document would require a significant period of development time.

3) It is likely that initial testing on MNs will require a more exploratory approach. For example, minor variation in test water chemistry and methods used to generate suspensions (including serial dilution) can cause significant variation in the as-tested properties of MNs. For these reasons, it is recommended that some investigation and quantitation of these effects be required.

4) Some consideration should also be given to material availability, which may be limited and preclude the use of flow-through or large-volume exposure approaches. Also, some brief discussion of the unique health and safety issues (e.g., their dustiness, potential ability to pass through commonly used laboratory gloves, ventilation from laboratory hoods, etc.) associated with nanomaterials should be included.

INTRODUCTION

Manufactured nanomaterials (MNs) present unique challenges for toxicity testing compared with most soluble chemicals and substances. NM are particles or fibers and when placed in wet media typically form colloidal suspensions (of varying stability). Despite the fact that nanomaterials (particles having at least one dimension between 1 and 100 nm) might pass through a 0.45 μm filter, and thus meet the widely-accepted definition of a *soluble substance*, their behavior is clearly distinct from a "truly" soluble substance, e.g., a metal ion or an organic molecule (Lead and Wilkinson 2006). Many suspended nanoparticles also have a strong tendency to agglomerate or aggregate, resulting in the formation of larger particles, or clusters of particles that rapidly settle out of suspension. In addition to the issue of exposure consistency during testing, particle-size variation may also alter the toxic potency of materials, in part because available surface area is rapidly reduced as agglomeration or aggregation occurs, but also because unique, quantum effects can predominate at sub-100 nm sizes (Parak et al. 2005). These characteristics are an inherent function of the material itself, but are also strongly affected by very small changes in ionic strength (perhaps to ionic composition as well), pH, dissolved organic matter (French et al. 2009, Domingos et al. 2009), and even the rate at which dilution media is added to more concentrated media to produce the concentration range necessary for exposure-response analysis (Fortner et al. 2006).

The purpose of this brief introduction is not to present an in-depth overview of nanomaterials and their toxicity. Rather it is intended to describe how the unique nature of nanomaterials presents problems for regulatory ecotoxicity testing. It should be clear from the brief discussion above, that test guidelines that limit the discussion of test media preparation to truly soluble substances cannot be expected to provide sufficient guidance for the preparation of stable colloidal suspensions. The common thinking among toxicologists, reflected in all of the reviewers' comments, is that nanomaterial toxicity is likely to be strongly related to particle size, surface area, possibly surface charge, bulk concentration, and additional factors that will likely be revealed in exploratory research (Handy et al. 2008, Klaine et al. 2008). Aside from bulk concentration, these factors are not considered in assessing risk for soluble substances, nor are they recognized in current test guideline language. In many cases, measurement techniques have yet to be developed (e.g., surface area in wet media) or the currently-used methods yield different results (e.g., electron microscopy and light scattering techniques often yield significantly different particle size values). Conversations among scientists working in this nascent field often begin with a discussion of what is meant by the term *soluble*: a sub-0.45 um particle, or an ion that actually dissolves from a larger particle; or whether an agglomerate is a particle, or is comprised of individual, discrete, as-produced particles.

In this summary we discuss these and other MN toxicity testing difficulties, and whether current test guidelines adequately address these issues. The reviews on which this summary is based were undertaken with the understanding that the specific goal was to address the issue of test guideline adequacy and to identify specifically where and how the guidelines might be inadequate. No effort was made to suggest specific alterations of guidelines to improve their adequacy.

TEST GUIDELINES REVIEWED

Two broadly overlapping sets of guidelines, listed in Tables 1 and 2, were reviewed in two distinct phases. The first set was comprised of 24 ecotoxicity test guidelines promulgated by the Organization for Economic Co-operation and Development (OECD) and closely harmonized with test guidelines within EPA's OPPTS Series 850. The OECD guidelines were reviewed by an international group of scientists at the request of the OECD's Working Party on Manufactured Nanomaterials (WPMN). The harmonization of OECD and OPPTS guidelines has resulted in nearly identical method descriptions; thus the OECD reviews were directly applicable to many OPPTS guidelines. In the second phase of the process, 25 OPPTS guidelines were reviewed by a group of U.S. scientists from EPA, U.S. Geological Survey, and the U.S. Army Corp of Engineers (Engineer Research and Development Center). The participation of several of the reviewers in both phases of the process contributed to the continuity and comparability of reviews and the summary process. The review of the OECD test guidelines was summarized in a final report delivered to the WPMN in March of 2008. That report comprises a large portion of the present document, with the addition of findings and observations unique to the OPPTS test guidelines.

In addition to the 24 OECD test guidelines, reviewers in the first phase also evaluated an OECD document on testing difficult substances (*Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures* [ENV/JM/MONO(2000)6]) and selected test guidelines from Environment Canada, The Ministry of the Environment, Japan, and the International Organization for Standardization (ISO). The documents were added to the review process to provide a survey of non-OECD guidelines that might provide a framework, terminology, or guidance more directly applicable to nanomaterials. The review of these additional documents is summarized near the end of this report.

Review Process

The OECD and OPPTS (as well as the additional documents) provide guidance 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, and 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. Subsequent to completion of the OECD reviews, the review group evaluated 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]). 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. The OECD review group also briefly reviewed five non-OECD test guidelines 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 document. The review of OPPTS test guidelines was completed using the same procedure.

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

Table 2. Reviewed OPPTS ecotoxicity test guidelines. Guidelines that were not reviewed by the OPPTS reviewers because of their comparability with previously-reviewed OECD guidelines are indicated with the comparable OECD identification. Those guidelines that were not reviewed are indicated with NR.

Guideline Identification	Description of Test	OECD Review	OPPTS Review
850.1000	Special Considerations For Conducting Aquatic Laboratory Studies	**	
	Group A—Aquatic Fauna Test Guidelines		
850.1010	Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids	202	
850.1020	Gammarid Acute Toxicity Test		X
850.1025	Oyster Acute Toxicity Test (Shell Deposition)		X
850.1035	Mysid Acute Toxicity Test (Shrimp, s.w.)		X
850.1045	Penaeid Acute Toxicity Test (Shrimp)		X
850.1055	Bivalve Acute Toxicity Test (Embryo Larval)		X
850.1075	Fish Acute Toxicity Test, Freshwater and Marine	203	
850.1085	Fish Acute Toxicity Mitigated By Humic Aid		X
850.1300	Daphnid Chronic Toxicity Test	211	
850.1350	Mysid Chronic Toxicity Test		X
850.1400	Fish Early-Life Stage Toxicity Test	210/212	
850.1500	Fish Life Cycle Toxicity	204/215	X
850.1710	Oyster BCF		X
850.1730	Fish BCF		X

Guideline Identification	Description of Test	OECD Review	OPPTS Review
850.1735	Whole Sediment Acute Toxicity Invertebrates, Freshwater	218/219	X
850.1740	Whole Sediment Acute Toxicity Invertebrates, Marine	218/219	X
850.1790	Chironomid Sediment Toxicity Test	218/219	X
850.1800	Tadpole/Sediment Subchronic Toxicity Test	218/219	X
850.1850	Aquatic Food Chain Transfer		X
850.1900	Generic Freshwater Microcosm Test, Laboratory		X
850.1925	Site-Specific Aquatic Microcosm Test, Laboratory		X
850.1950	Field Testing for Aquatic Organisms		X
	Group B—Terrestrial Wildlife Test Guidelines		
850.2100	Avian Acute Oral Toxicity Test	205	
850.2200	Avian Dietary Toxicity Test	205	
850.2300	Avian Reproduction Test	206	
850.2400	Wild Mammal Acute Toxicity		X
850.2450	Terrestrial (Soil-Core) Microcosm Test		X
850.2500	Field Testing for Terrestrial Wildlife		X
	Group C—Beneficial Insects and Invertebrates Test Guidelines		
850.3020	Honey bee Acute Contact Toxicity	213/214	
850.3030	Honey Bee Toxicity of Residues On Foliage	213/214	
850.3040	Field Testing For Pollinators		X
	Group D—Nontarget Plants Test Guidelines		
850.4000	Background—Nontarget Plant Testing	227	
850.4025	Target Area Phytotoxicity	227	
850.4100	Terrestrial Plant Toxicity, Tier I (Seedling Emergence)	208/227	
850.4150	Terrestrial Plant Toxicity, Tier I (Vegetative Vigor)	208/227	
850.4200	Seed Germination/Root Elongation Toxicity Test	227	
850.4225	Seedling Emergence, Tier II	208/227	
850.4230	Early Seedling Growth Toxicity Test	208/227	
850.4250	Vegetative Vigor, Tier II	208/227	
850.4300	Terrestrial Plants Field Study, Tier III		X
850.4400	Aquatic Plant Toxicity Test Using *Lemna* spp. Tiers I and II	221	
850.4450	Aquatic Plants Field Study, Tier III		X
850.4600	*Rhizobium*-Legume Toxicity		X
850.4800	Plant Uptake and Translocation Test		X
	Group E—Toxicity to Microorganisms Test Guidelines		
850.5100	Soil Microbial Community Toxicity Test	216/217	
850.5400	Algal Toxicity, Tiers I and II	201	
	Group F—Chemical-Specific Test Guidelines.		
850.6200	Earthworm Subchronic Toxicity Test	220/222/207	
850.6800	Modified Activated Sludge, Respiration Inhibition Test for Sparingly Soluble Chemicals	209/224	
	Group G—Field Test Data Reporting Guidelines.		
850.7100	Data Reporting for Environmental Chemistry Methods		NR
** Some similarity to OECD *Difficult Substances* document.			

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 1) *toxicological principles, 2) terminology, 3) test endpoints, 4) material*

characterization, and 5) *media preparation, delivery, exposure quantification,* and are discussed in that order in the following section.

ADEQUACY OF TEST GUIDELINES

Toxicological Principles

All reviewers agreed that 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 ecotoxicity 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.

Terminology

All of the current test guidelines reviewed use terminology that is primarily applicable to chemical substances. In many cases, the term *substance* is used rather than the term *chemical*; 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 will be used in estimation of effect levels, or dose-response relationships. It is the opinion of the reviewers that 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.

Chemicals

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 OECD test guidelines refer to the testing of preparations or formulations (e.g., OECD 213 and 214). This concept may be particularly applicable to some nanomaterials which may be dependent on surface treatments and coatings or specific solvents and emulsifiers to maintain their nano-scale characteristics.

Solution/solubility

Nanomaterials are generally in particulate or fibrous forms and their preparation and delivery is best described in terms of *preparations* or *suspensions*, rather than *solutions.* Some thought should also be given to the use of closely related terms such as *soluble*, *solvent*, or *dissolved*, which might be interpreted as precluding the testing of suspensions of nanomaterials. Terms such as *suspension agents* or *matrices* and *suspension* are more descriptive of nanomaterials. Such terminology might be interpreted as precluding the testing

of suspensions of nanomaterials. The importance of terminology in this case is exemplified by work with silver nanoparticle formulations, which typically consist of suspensions of elemental nano-sized cores, variably associated or bound silver ions, and free silver ions in solution. In this case, the particles are suspended (as opposed to *dissolved*) but may, to some extent, be soluble and release free silver ions into solution. An additional point is that the bulk concentration (see the next section) may remain the same yet the proportion of free ionic silver is very likely to be the major contributor to toxicity or potency of the mixture or formulation.

Concentration

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

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 dependent on concentration. Effect-level metrics may need to be developed to incorporate several properties specific to the biological activity of nanomaterials, including, but not limited to, particle size, surface area, or surface charge.

Test Endpoints

There is little evidence to suggest that the majority of 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 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. Early testing has suggested that some nanomaterials may have adverse effects that involve physical smothering of exterior surfaces, physical blockage of digestive processes, or physical inhibition of motility, e.g., coating of appendages in cladocerans. 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 be developed that are more predictive of adverse effects compared with the current test endpoints addressed by ecotoxicity 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 the reviewers from making specific recommendation for the development of such new test guidelines.

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. Current research suggests that particle count, size distribution, surface area, surface charge, and other surface characteristics might be better predictors of toxicity, and that their incorporation into exposure metrics will be necessary for accurate statistical determination of dose-response relationships. While 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 concentrations; 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.

An OPPTS work group has reviewed current physical-chemical test guidelines to assess their applicability to nanomaterials (Utterback et al 2008). As part of that process the workgroup made recommendations for characteristics that should be incorporated into new or existing physical-chemical test guidelines. We recommend 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 currently described should be removed if the test guideline is to be used 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 (see Utterback et al. 2008). It is also expected that new research on the ecotoxicity of nanomaterials will also guide the process of the revision of test guidelines.

The physical-chemical characteristics of nanomaterials have also been identified as being a primary concern relative to the other major test guideline components, discussed below.

Media Preparation, Delivery, Exposure Quantification

The test guidelines related to ecotoxicity involve several media, including soils, sediments, water, food, and direct application (Bee Test, OECD 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, ionic strength, and concentration of dissolved organic matter. Some test guidelines (e.g., OPPTS 1055) recommend the use of natural seawater, which could introduce considerable variability in exposures between laboratories, and even between tests due to small differences in water quality variables. In some test guidelines (e.g., OPPTS 1020), it is suggested that water quality factors vary month-to-month by no more than 10%. However, some factors, such as hardness or particulate concentrations might significantly alter agglomeration/aggregation behavior of nanomaterials even over this narrow range. 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 (Handy et al. 2008, Fortner et al. 2008). 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 significant exposure hazards for lab personnel; this issue should be addressed.

None of the test guidelines related to ecotoxicity provides information on how to measure, control for, or otherwise address these exposure preparation variables. SG4-2 recommends that such guidance be added to modified or newly-developed test guidelines to assure their applicability to nanomaterials.

Many existing test guidelines make specific recommendations about volumes of exposure media, organism loading rates, and the necessity of flow-through exposures. These recommendations may need to be reconsidered for nanomaterials that are particularly expensive or difficult to obtain in large quantities.

Some test guidelines (e.g., OPPTS 1020) recommend filtration of samples prior to their analyses for concentration of test substances. In the case of OPPTS 1020, the recommended pore size for filtration is 0.45 μm; this is a pore size that is very likely to remove particles from some nanomaterial preparations.

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, nor for documenting the consistency of materials obtained from different sources or production runs. 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 the OPPTS workgroup that reviewed physical-chemical guidelines (Loux et al. 2008) should also be incorporated into guidance on quantifying and characterizing exposure stability and consistency.

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 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, 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 the work of the OPPTS workgroup that

reviewed physical-chemical guidelines (Loux et al. 2008). It is the recommendation of the work group that these characteristics, as well as results of current toxicological research, be considered for the revision of existing, or the development of new, test guidelines.

ADDITIONAL REVIEWS

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

As described above, the reviews of ecotoxicology test guidelines indicated that their inadequacies for testing of nanomaterials are consistently related to material characterization and properties, and metrology. This finding suggests that, rather than extensive modification of all 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* (ENV/JM/MONO(2000)6). 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. Reviewers were also charged with making recommendations for modification of the *Difficult Substances* document, or a similar guidance document directed at nanomaterial testing.

A summary of the review findings are enumerated below:

a. 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 a 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;

b. 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 their stability, as well as media preparation and sampling. 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 mentioned. Specific nanomaterial properties to be measured or documented, and methods to do so will need to be described; and

c. 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; e.g., particle size, surface area, agglomeration potential or rate, as well as how to prepare and maintain stable suspensions or distribution of nanomaterials.

Recommendation of reviewers on the "Difficult Substances" document:

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 guidance document. Such guidance could provide a means to rapidly advance toxicity testing of nanomaterials using existing test guidelines; an outcome that would also provide critical information necessary for development of nanomaterial-specific test guidelines. To serve these purposes, the guidance would need to address the issues just discussed. Key considerations would include the physical-chemical properties of nanomaterials identified by the OPPTS P/Chem workgroup.

Non-OECD/OPPTS 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 for the workgroup 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/OPPTS test guidelines.

Table 3. Reviewed non-OECD/OPPTS 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 by the workgroup in the OECD/OPPTS 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 U.S. EPA/OPPTS Test Guidelines, the harmonization process has led to identical language in most cases.

Observations on Specific OPPTS Guidelines

(850.5400) **Algal toxicity, Tiers I and II**: Methods described for estimating algal population growth might be confounded by the presence of nanoparticles, especially where

agglomeration or aggregation results in particle sizes that overlap the size of tested lifestages. In addition, some nanomaterials may agglomerate with algal cells or cause cell-to-cell clumping. The methods discussed include optical particle counting, gravimetry, and spectroscopy. The test guideline also includes the requirement that the accuracy of these approaches be confirmed using some form of microscopy. These measurement approaches, including confirmatory microscopy, will need to be evaluated, and possibly modified for use in nanomaterial testing.

(850.1055) **Bivalve acute toxicity test (embryo-larval)**: Endpoints should be evaluated with the recognition that early lifestages of some mollusks may have size ranges that overlap the size range of the particles being tested, particularly where agglomeration/aggregation are expected or observed. Adverse effects of such an overlap in size might include direct physical interference with movement, respiration, feeding, etc., and could make quantification of effects on the end-point stages (veliger larvae) difficult. It is also notable that this is the only reviewed test guideline that specifically suggests that ultrasonic dispersion is an acceptable method for dispersing test substances. No specific guidance is provided on how ultrasound should be employed, e.g., energy intensity, duration of sonication, or whether probe or bath systems are acceptable.

(850.1085) **Fish acute toxicity mitigated by humic acid**: This guideline describes an approach for examining the effect that humic substances might have on the toxicity of traditional chemicals, a question that is equally important for assessing the hazard and risk of nanomaterials. The guideline provides an excellent framework for examining these effects, but will require considerable modification to address the terminology, metrology, and other issues associated with the particulate or fibrous nature of nanomaterials that are described here for all other test guidelines.

(850.1850) **Aquatic food chain transfer**: This is a very brief guideline that provides limited and very general guidance on examining food chain transfer of soluble chemicals. The guideline could serve as a framework for nanomaterial testing (albeit limited and very general), but would need a few key modifications. Most notable among these is that, as currently written, the need for this testing is based on water solubility and log Kow values. The former of these would essentially exclude nanomaterials (due to their colloidal character), whereas the applicability of the latter has yet to be determined for nanomaterials. Issues associated with Kow approaches are discussed relative to OPPTS 850.1730, below.

(850.1730) **Fish BCF**: This guideline describes methods for determining bioconcentration factors and the rates of uptake and depuration for contaminants. All of the issues associated with defining and using the term *concentration* apply to this guideline, with the added complication that the concept of proportionate concentrations, e.g., octonol/water, as a surrogate for lipophylicity, are not yet defined or well-investigated for most particulate or fibrous materials. The critical unknowns are how particle size and level of aggregation or agglomeration should be incorporated into these concentration metrics.

(850.4800) **Plant uptake and translocation test,** and (850.4600) ***Rhizobium*-legume toxicity**: These guidelines describe methods for collecting data on rates of uptake and translocation of chemical substances, and toxicity to *rhizobium*-legumes, respectively. There are three unique concerns relative to applying these guidelines to nanomaterials testing. The first is the requirement for nutrient addition to growth media, either dry or wet, that is likely to strongly influence rates and levels of aggregation and agglomeration. The second concern

is the assumption that substances can be added to stable solutions that will infiltrate sand-based media. As well as the previously-mentioned concern with suspension issues, it is also very likely that nanomaterials will interact with sand surfaces, and the interactions will vary significantly depending on the specific nanomaterial being tested. The third concern is the requirement for illumination that optimizes plant growth and vigor. The photo reactivity of some nanomaterials, e.g., the anatase form of TiO_2, has been shown to increase their toxicity. This reactivity is likely to be wavelength dependent suggesting that additional guidance on how to incorporate or address this factor in testing will need to be developed.

OPPTS (850.1000) **Special considerations for conducting aquatic laboratory studies**: This guideline discusses many of the basic principles of aquatic toxicity testing and could provide a framework for incorporation of guidance specific to nanomaterials testing, or development of a similar document focused on nanomaterials. Many of the principles discussed are as applicable to nanomaterials as to soluble substances. There is some discussion of the presence of colloids in test preparations; however, the focus is on removing them by centrifugation, a procedure that would certainly remove nanoparticles from suspension (with rates dependent upon particle size, surface charge, and a variety of media characteristics). Of particular interest is the following section:

> *(3) Current policy allows chemicals that are poorly soluble (solubility <100 ppm) or dispersible in water to be tested up to the maximum water solubility or dispersibility limit obtainable for the given test conditions employed, provided that certain prerequisites apply:*
>
> *(i) Concentrations of test chemical in test media are measured at appropriate intervals and from appropriate test chambers of all test levels are determined from centrifuged supernatant or other appropriate separation (e.g., filtrate). Self-dispersing industrial chemicals (e.g., surfactants, detergents, or charged polymers) should be sampled directly.*

Given some modification, in particular discussion of recommended particle characterization, this section could be adapted to address nanomaterials specifically.

OPPTS 850.1950, 850.2400, 850.2500, 850.3040, 850.4300, 850.4450 **(generally, field-testing guidelines)**: These test guidelines describe approaches to testing for effects of pesticides in natural settings or media. They may be generally more adequate for nanomaterials due to the lack of detailed methods, and thus lack of terminology that precludes testing of suspensions or colloids. In some cases, the intent is to test formulations, rather than pure compounds, a goal that is likely to apply equally to nanomaterials used as field pesticides, which would also be likely to involve formulations of emulsifiers, stabilizers, solvents, etc.

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