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Nanomaterials in the Environment

Critical Review

ECOTOXICITY TEST METHODS FOR ENGINEERED NANOMATERIALS: PRACTICAL EXPERIENCES AND RECOMMENDATIONS FROM THE BENCH

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Abstract—Ecotoxicology research is using many methods for engineered nanomaterials (ENMs), but the collective experience from researchers has not been documented. This paper reports the practical issues for working with ENMs and suggests nano-specific modifications to protocols. The review considers generic practical issues, as well as specific issues for aquatic tests, marine grazers, soil organisms, and bioaccumulation studies. Current procedures for cleaning glassware are adequate, but electrodes are problematic. The maintenance of exposure concentration is challenging, but can be achieved with some ENMs. The need to characterize the media *during* experiments is identified, but rapid analytical methods are not available to do this. The use of sonication and natural/synthetic dispersants are discussed. Nano-specific biological endpoints may be developed for a tiered monitoring scheme to diagnose ENM exposure or effect. A case study of the algal growth test highlights many small deviations in current regulatory test protocols that are allowed (shaking, lighting, mixing methods), but these should be standardized for ENMs. Invertebrate (*Daphnia*) tests should account for mechanical toxicity of ENMs. Fish tests should consider semistatic exposure to minimize wastewater and animal husbandry. The inclusion of a benthic test is recommended for the base set of ecotoxicity tests with ENMs. The sensitivity of soil tests needs to be increased for ENMs and shortened for logistics reasons; improvements include using *Caenorhabditis elegans*, aquatic media, and metabolism endpoints in the plant growth tests. The existing bioaccumulation tests are conceptually flawed and require considerable modification, or a new test, to work for ENMs. Overall, most methodologies need some amendments, and recommendations are made to assist researchers. Environ. Toxicol. Chem. 2012;31:15–31. © 2011 SETAC

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INTRODUCTION

The ecotoxicity of engineered nanomaterials (ENMs) has been the subject of several extensive reviews [1–4]. These reviews highlight the importance of understanding particle chemistry in the context of the bioavailability and ecotoxicity of ENMs. Some environmental factors that may alter the toxicity of ENMs, such as pH, salinity, divalent ions, and the presence of natural organic matter, also have been discussed [1,2]. Much of the research has focused on fundamental aspects of ecotoxicity, such as estimates of lethal concentrations, documenting sublethal effects on organisms, identifying potential mechanisms of toxicity, as well as describing the fate and behavior of ENMs. Inevitably, researchers have customized exposure protocols and selecting biological endpoints to match their research objectives; therefore, they have used a variety of methods.

In addition to the scientific community's use of nonstandard, customized, methods in fundamental research, a plethora of standardized methods exists for the regulatory ecotoxicity testing of chemical substances. These standardized tests have aspects of international harmonization (the Organisation for Economic Co-operation and Development [OECD] tests, [5]; International Organization for Standardization, *Daphnia* immobilization test [6]), and many countries and institutions also have their own protocols. For example, the American Society of Testing and Materials Committee on Biological Effects and Environmental Fate has written protocols for testing substances on a range of aquatic invertebrates and fish [7]. Similarly, the U.S. Environmental Protection Agency (U.S. EPA) has protocols for testing aquatic, terrestrial, and microbial organisms [8].

All of these tests, however, were established with traditional chemicals in mind, not ENMs. Consequently, some debate has

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ensued about the utility of existing regulatory tests for ENMS. The consensus is that the existing methods and framework for hazard assessment (standard test organisms, mortality, growth and reproduction endpoints) are generally fit for this purpose, but the details within each group of tests may require modification or optimization to work well with ENMs [5]. Recently, the OECD made some preliminary recommendations on how to dose toxicity test systems with ENMs [9], and the long process of validating standard regulatory tests for new ENMs has begun (the OECD sponsorship program, [10]). In addition, academic researchers are constantly refining methodologies as the scientific community gains experience in working with ENMs.

Collective experience from researchers working at the bench has not been summarized, however, and the practical details of conducting ecotoxicity experiments with ENMs have been given less attention in the peer-reviewed literature. The Society of Environmental Toxicology and Chemistry (SETAC) Nano Advisory Group hosted a technical workshop in the summer of 2010, gathering a group of researchers with considerable hands-on experience with ENMs at the bench to document the practical issues and options for conducting ecotoxicity experiments with ENMs. The present paper reports the technical content of those discussions and aims to move the field forward by identifying what practical aspects of methodology need to be modified specifically for ENMs, but also to highlight what parts of protocols do not need to be changed. Clearly, the scientific community is using numerous variations of exposure methods and endpoints for research and many standardized protocols for regulatory testing. Going through the details of every single test method here is not possible, nor is it our purpose. Instead, our approach is to address practical considerations at the bench for groups of tests with similar methodologies, including tests using soil and sediments, aquatic organisms, long-term exposures for chronic toxicity and bioaccumulation, as well as some microbial tests that are relevant to the environmental regulation of ENMs. The considerations include generic issues such as how to maintain the exposure during the test, but also very specific practical problems within individual groups of tests.

GENERIC ISSUES IN TEST METHODOLOGY

Some generic methodological issues are common to different types of ecotoxicity tests and ENMs. These generic issues can present practical problems, such as how to clean and prepare test vessels, but they also can represent issues of fundamental scientific importance, such as experimental design and the use of controls. The scientific issue around experimental design, including the use of reference ENMs, particle size controls, whether to use dispersing agents, and so forth, have been discussed extensively [5,11,12]. The present study focuses on the practical aspects of deciding what to do and how to do it.

Setting up at the bench

The first practical consideration at the bench is cleaning and preparing the test vessels. Regulatory test methods often have some guidance on selecting equipment and cleaning test chambers. Recommendations usually include the use of good quality glass such as grade A glassware rather than low-grade soda glass. If plastic ware must be used, one must assess its suitability and recognize that this may be harder to clean. Consequently, plastic containers must be disposable. Experiences at the bench so far suggest that in the case of glass vessels, routine cleaning methods such as acid washing in dilute nitric acid or aqua regia [8] are adequate for preparing test vessels for most ENMs, including metallic nanoparticles (NPs) [13] and carbon-based materials such as single-walled carbon nanotubes (SWCNT) [14].

Most ecotoxicity experiments require some monitoring of the test environment to ensure the health of the test organisms, such as dissolved oxygen, pH, salinity, or nitrogenous compounds (ammonia, nitrite, or nitrate). Concerns about ENM interference with colorimetric assays and dyes have already been raised [15], and each assay should be checked before beginning each experiment. Less attention has been given to the effect of ENMs on the performance of ion selective electrodes, or similar probes that work by potentiometry, such as pH probes and dissolved oxygen probes. Practical experience at the bench (R. Handy, unpublished observations) showed that ENMs do adsorb onto the surfaces of the probes, which can block the sintered plug in combination electrodes or coat the sensitive glass surface, so that the electrode is less responsive. This is a problem for all ENMs that adsorb to glass, such as TiO_2 NPs and carbon-based ENMs such as SWCNT. In addition to the manufacturer's recommended cleaning procedure, short (seconds–minutes) periods of etching the surface with strong nitric acid may help restore the probe as a last resort. In any event, the responsiveness of probes should be monitored carefully. For combination pH electrodes, this can be done by following the voltage predicted by the Nernst equation; typically 59.2 mV/pH unit at 25°C). Many combination ion-selective electrodes are filled with 3 M KCl and have an internal $Ag/AgCl_2$ reference. Anecdotal concerns about interferences, from silver nanoparticles (AgNPs) especially, seem unlikely, because the sensitive glass bulbs are an ion-exchange surface to create voltage [16], not directly porous to the materials. This may not be the case for gas-sensing electrodes, however, in which the pore sizes in the membranes used can be into the micron range, depending on the gas to be detected [17]. Even if AgNPs penetrated inside the electrode, for most probes (gas and ion-selective), the high ionic strength inside the probe would precipitate the AgNPs directly, or as relatively insoluble silver chloride. Concerns that ENMs will generate spurious voltages can be checked with a mixed calibration procedure for ion-selective electrodes (Handy [18]).

Confirming exposure; maintaining test concentration

Confirming that the test organisms have been exposed to the test substance is fundamental to any ecotoxicology experiment, but it is particularly challenging in the case of ENMs. Measurements should be taken in addition to simply monitoring the mass concentration. For example, particle size distribution and particle number in the test media may need to be monitored to aid data interpretation or simply to give the researcher the option of plotting the data against a dose metric other than concentration [19–21]. Concerns have been expressed about when and how to change test media during a test. Of course, the prime purpose of changing the media is to ensure that the exposure is maintained and that the general quality of the media meets the test organism's requirements. Most aquatic and terrestrial experiments with ENMs have so far been conducted using either a static (i.e., no change of the test media) or semi-static exposure regimen, in which the medium is replaced periodically during the experiment and for good, practical reasons (Fig. 1). For example, aquatic flow-through tests, especially for fish, would require large quantities of potentially expensive ENM and generate a wastewater disposal problem. For intact marine sediments (Fig. 1), dosing the overlying water is environmentally relevant and may achieve an even spread of the ENM over the surface of the sediment, but this method of dosing may limit

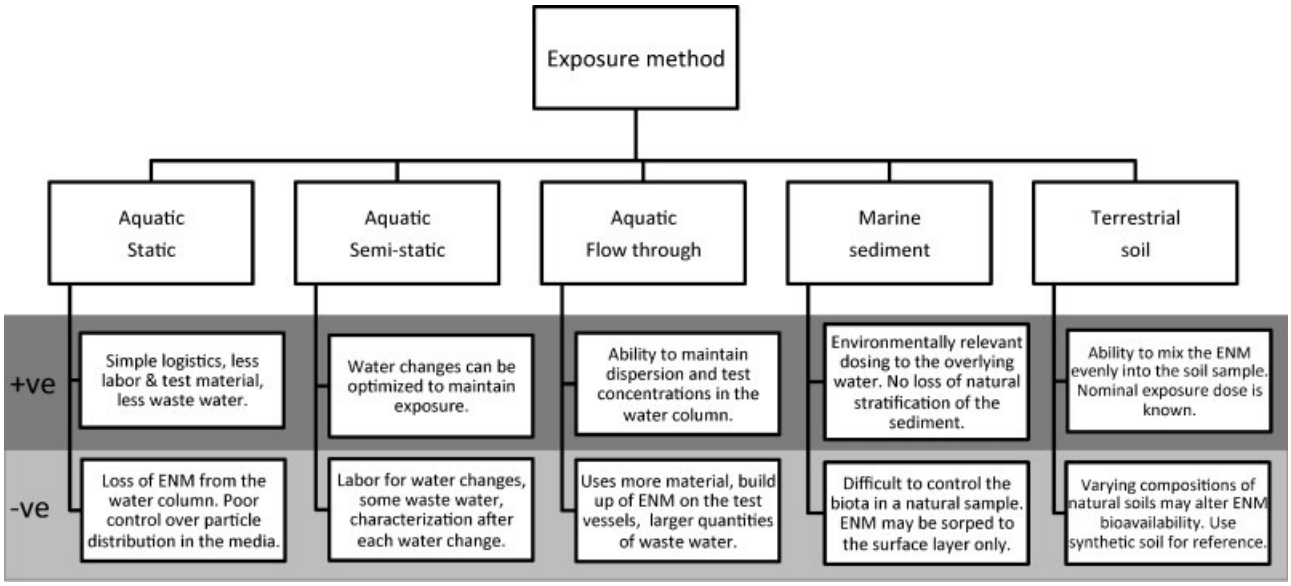


Fig. 1. Some advantages (dark gray) and disadvantages (light gray) of exposure methods used in ecotoxicology for aquatic tests, protocols using marine sediments, and terrestrial soil tests. See text for details. ENM = engineered nanomaterial.

ENM penetration into the sediment layers. For terrestrial soils, dry or wet mixing of ENMs into the soil is possible, and this may ensure a reasonably even spread of the ENM in the test container. With huge variability in the composition of natural soils that may alter bioavailability, however, a reference (synthetic) soil should be included for benchmarking (Fig. 1).

Currently, regulatory aquatic tests are often required to maintain at least 80% of the nominal test concentration during the exposure. This is an important validation criterion, and the data may be rejected if this is not achieved. This criterion likely will not be achievable for many ENMs or even relevant if particle number or some other dose metric turns out to be more important. The experimenter also may be faced with the technical challenge of maintaining several parameters in colloid chemistry simultaneously, including primary particle size/shape, dispersion state, and therefore energy additions to the test system, just to achieve a notional 80% of the target concentration.

In short acute tests, replacing the test media is an option, but even this would need to be done more frequently compared with traditional chemicals. For example, milligram levels of TiO_2 can be maintained at a mass concentration about the nominal 80% in freshwater, but only for a few hours. Consequently, water changes may be needed every 8 h [13]. This frequency of water changes, however, may not be a financially viable or practical proposition for a commercial contract research laboratory. The problem is compounded by the behavior of mucous secretions from the animals (see Handy and Maunder [22] for a review on mucus). For example, in rainbow trout, SWCNT are attached rapidly to the mucus secretions of the fish [14], which then sink to the bottom of the tank, rapidly removing SWCNT from the test media along the way. A key point learned from these experiences is that the decision on exactly when, or if, to change the water should be driven by the characteristics of the test system and should not be some arbitrary decision (e.g., water changes every 24 h just because it is logistically convenient). Validating the exposure will often require pilot experiments to establish when the water changes need to be done.

For soil and sediment tests, replacing the test media to maintain the exposure is often impractical. For example, earthworms hidden in the soil may be damaged inadvertently or stressed, and it may be scientifically desirable to leave the soil alone once the test has started so that the partitioning of the test substance in the pore water, soil matrix, or test organism can be measured. Chronic exposure studies, or any test lasting more than a few days, may suffer from one of the many possible ageing and transformation reactions of ENMs that have been reported [23]. It also may be desirable to monitor this in the test system. Exactly how such transformations will affect the practical ability of the experimenter to maintain the test concentration is unknown.

One should also recognize that the priorities for maintaining the exposure in a regulatory ecotoxicity test may be very different from those needed for fundamental academic research. For example, academic laboratories do not have an 80% of the nominal concentration rule with which to comply, but instead might be much more focused on the problem of achieving an environmentally relevant exposure. This might even include deliberately letting the test material aggregate so that the natural fate and behavior of the ENM can be followed. Clearly, imposing the concepts and validation criterion used in regulatory methods on the scientific community for ENMs generally is not desirable. Some commonly agreed-upon aspects will emerge, such as the use of suitable controls [5], but at this early stage, the opportunity exists to incorporate environmentally relevant conceptual models for ENM in the testing strategy [24].

Characterization of the test media during experiments

The minimum characterization requirements for stock solutions of ENMs have been discussed at length, with agreement that stock solutions can be verified [5,11], but less consideration has been given to practical measurement during an ecotoxicity test. A consensus view from the scientific community on what dose metric should be used routinely in ecotoxicity testing is still forming, and collecting a sufficient weight of evidence for or against using a particular dose metric (such as particle size, particle number, or mass concentration) in a regulatory test will take some years, and it may be specific for some types of ENMs. While the debate continues, ensuring that ecotoxicity tests minimally include measurements of mass concentration

(e.g., mg/L) and some measurement of particle size distribution in the test media (see von der Kammer et al. [25], this issue) would be prudent. The latter would at least allow retrospective correction for size and estimates of surface area for hard, nonporous spherical particles. For metal-based ENMs that gradually dissolve, such as ZnO and AgNPs, the dissolved versus particulate metal also should be measured, or at least benchmarked against a metal salt control. The measurement issues, such as detection limits, and dealing with background levels of similar substances, such as the problem of measuring carbon-based nanomaterials in carbon-rich natural water, are discussed at length in von der Kammer et al. [25]. For ecotoxicity tests, however, the speed at which the chemistry data can be returned to the experimenter can be crucial to decision making during the experiment. For example, if the exposure has evidently not been maintained, then the test can be terminated to save wasting time and money on data that would be discarded in any event. For short acute tests, the researcher is often faced with not getting the analytical chemistry on the fate and behavior of the ENM in the test system until after the test has finished. Consequently, the entire experiment may be questioned if the chemistry shows that the exposure was not maintained. Conducting pilot studies on the fate and behavior of the ENM in the test system can mitigate this risk, but this inevitably requires more time. Thus, rapid detection methods (same-day results), in which the worker at the bench can monitor exposures while the experiment is running, are urgently needed. For ethical reasons, this is also especially important for fish tests.

A second problem is that environmentally relevant ENM mass concentrations are in the low microgram per liter range [26]. Some fairly rapid and sensitive techniques are available to confirm mass concentration. For metal ENMs, current inductively coupled plasma mass spectrometry and inductively coupled plasma atomic emission spectroscopy methods are adequate for detecting low microgram per liter concentrations in water and in some tissues. Currently, however, no rapid, reliable, practical, routine methods exist to detect carbon-based ENMs in natural water or soil matrices, although this area of research is developing rapidly [25]. In addition, most routine instrumentation for particle size distribution becomes less reliable below approximately 1 mg/L, and with predicted environmental concentrations of ENMs in the low microgram range or less [26], highly sensitive techniques are required. Such techniques are under development [25], but often they are not used routinely or they are not available or rapid enough for the biologist at the bench. For example, for a typical triplicate aquatic test with five concentrations, the researcher would have at least 15 samples per time point. With semistatic exposure in which the new media needs to be verified, one can easily expect to generate 50+ water samples every day. A typical particle distribution measurement may take approximately 20 min to obtain careful triplicate measurements from each water sample. Clearly, running 50 water samples for particle size distribution alone would take several days. One potential way forward would be to develop instruments with autosamplers, so that dynamic light scattering or similar measurements could be completed overnight. Equally, this could create a new set of problems such as particle aggregation in peristaltic pumps and settling of ENMs in the test tubes waiting on the autosampler.

Dispersing agents and solvent controls

Whether ENMs should be administered as well-dispersed suspensions is an ongoing debate [5], and some of the practical considerations of dispersion methods for test media are summarized in Figure 2. In ENM experiments, dispersing the test material may simply be needed to dispense the dose accurately into the test media. Researchers also must be accurate with terminology in written protocols, especially regulatory protocols that may constitute a formal part of a legislative document. For example, the term *aqueous* may be inappropriate. One is not dealing with aqueous solution chemistry, but instead, with a colloidal *dispersion* [1]. The term *solvent control* as currently used in testing protocols is also therefore inaccurate; indeed, perhaps a better technical term would be *dispersing agent control*.

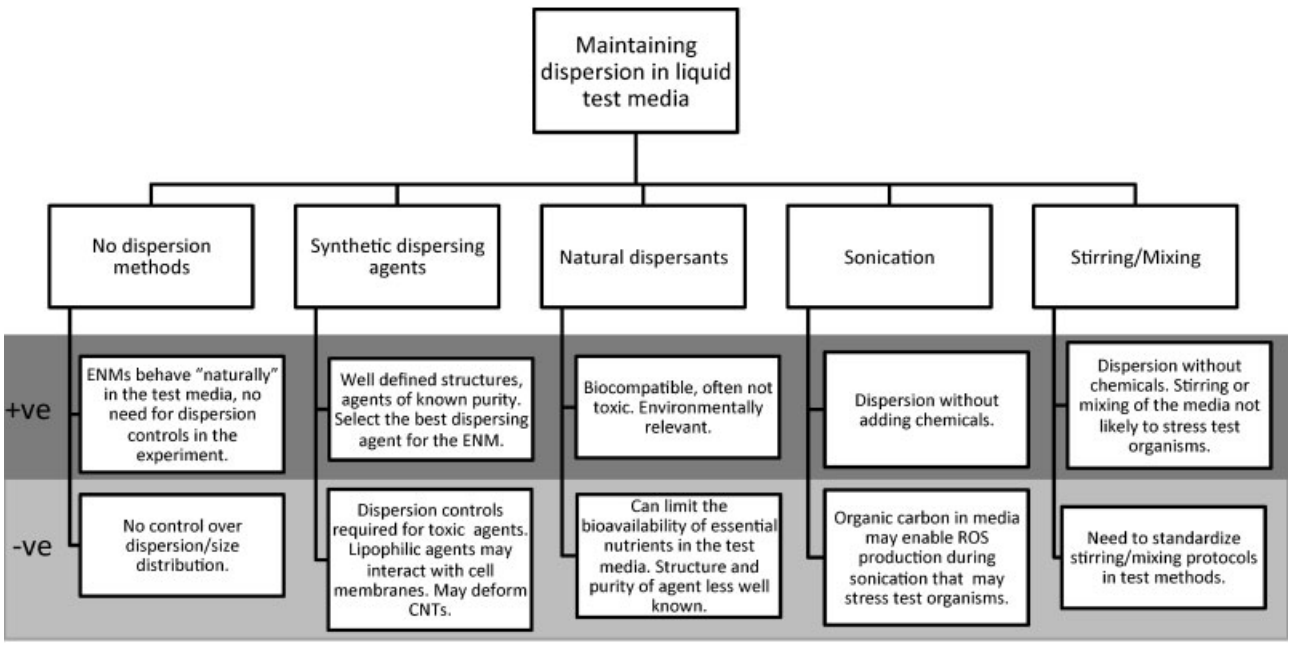


Fig. 2. Some advantages (dark gray) and disadvantages (light gray) of dispersion methods used in ecotoxicology. See text for details. ENM = engineered nanomaterial; CNT = carbon nanotube; ROS = reactive oxygen species.

The use of dispersing agents and sonication methods [5] has identified, with the benefit of hindsight, some substances that are excellent at dispersing ENMs such as tetrahydrofuran, but such agents are often incompatible with whole organism biology [12]. Compromises have also been achieved in which dispersant concentrations have been selected carefully to minimize the toxicity of the solvent control on the animals, while maintaining a reasonable dispersion of the test materials (see Smith et al. [14] for discussion on sodium dodecyl sulfate with SWCNT). Figure 2 provides some practical guidance to the experimenter on the use of dispersing agents. The dispersants fall into two broad categories from the perspective of biology. These are natural materials such as humic acids, proteins, and gum arabic. Alternatively, synthetic dispersants such as pluronic solution, sodium dodecyl sulfate, or other non-ionic surfactants can be used.

The benefits of using natural dispersants (Fig. 2) include less likelihood of acute toxic effects on the test organism as well as environmental relevance, but disadvantages are possible also. Natural dispersants can create analytical problems for the chemist. For example, adding more carbon from natural organic dispersants to the test system can only compound the problem of analyzing SWCNT. One should also remember that these natural substances, although often not readily biodegradable, are not biologically inert. For example, dissolved organic matter can be critical in controlling metal bioavailability to aquatic organisms [27]. Conversely, humic and fulvic acids are also known to reduce the toxicity of ENMs [28,29] and slow the dissolution of metal-based ENMs [30]. Moreover, natural dispersants are usually not well characterized, and the properties of natural organic matter often used in ENM experiments, such as Suwannee River humic acid, may not be representative of the wide range of organic matter in different environments. Some types of aquatic natural organic matter promote aggregation of particles [31].

Alternatively, synthetic dispersants may be used to circumvent the disadvantages of natural dispersants (Fig. 2). Synthetic materials at least have a well-defined chemical structure and composition and have no intended biological functions that would make them bioreactive. However, one cannot exclude incidental biological effects of synthetic dispersant. For example, theoretically, a lipid-soluble synthetic dispersant could interact with the cell membrane (also lipophilic) to deliver more ENM to the test organism inadvertently, thus increasing the apparent toxicity of the test. In such circumstances, this additional effect on toxicity would not be evident in the dispersion agent control [32,33].

Some practical concerns are worth noting about routine procedures for including solvent controls (dispersion controls for ENMs) in the experimental design of regulatory toxicity tests. Most protocols include only one such control at the highest solvent concentration used in the test. In the case of traditional chemicals, one would normally spike all the test vessels with an appropriate amount of solvent so that the solvent concentration was the same in all containers. For ENMs, this approach would also normalize the dispersing agent concentration across all the test containers. Spiking with some extra dispersant, however, would also change the ratio of dispersant to ENM in each test container, which may result in each test concentration having very different ENM dispersion states. Performing some pilot studies to determine whether the extra dispersant appreciably changes the dispersion state or deforms the structure of the particular ENM being investigated would be important. For some materials, keeping the ratio of dispersant to ENM the same is important. For example, SWCNTs are particularly prone to deformity with excess dispersant (see discussion in Smith et al. [14]), but it also creates additional work, because each ENM concentration will require an equivalent dispersion agent control. The workload can be reduced by using only the highest dispersant:ENM ratio with the hope that no effect will be observed. If this is not the case, however, then the entire experiment will need to be repeated with a full series of dispersing agent controls.

After considering all of these technical details, the experimenter also must be mindful of why dispersing agents were used (or not) in each test and reflect on how this may alter the interpretation of the data. Although natural dispersants may be regarded as more ecologically relevant than synthetic dispersants, fundamentally, any dispersing agent has the potential to change the dynamics of ENM behavior. Furthermore, any surface coating of the ENM with the dispersant may alter how the ENM interacts with the cell surfaces of the test organism and therefore alter bioavailability. Clearly, an ecological argument could be made that dispersants should not be used at all, but this must be offset by the experimental errors generated when solution handling is poor and the exposure is heterogeneous. The alternatives of using stirring or sonication to create dispersions (see below) may be criticized equally for a lack of environmental realism because laboratory methods, for example, cannot reproduce the complex mixing that might occur in a river bed. Ultrasonication is certainly not a natural phenomenon, and yet the shear forces at water interfaces can have a profound effect on aggregation rates [1].

Sonication

Sonication is often used to produce well-dispersed ENM suspensions as an alternative to, or in combination with, dispersing agents. Advantages and disadvantages of using sonication for ecotoxicity tests (Fig. 2) exist, with the main advantage being the ability to disperse the ENM without adding extra chemicals. Presently, however, no standard sonication protocols are available in terms of time, temperature, sonication power, volume of the solution that is sonicated, type (batch vs probe), and properties (micro vs macroprobe) of the sonication device. Sonication time affects the aggregation properties of ENMs, with prolonged sonication leading to increased aggregation [34]. The optimal sonication time depends on the suspended ENM concentration, but it is usually less than 1 h [35]. In addition, sonication can introduce artefacts in toxicity studies because it fragments multiwalled carbon nanotubes [36] and possibly alters the coatings on ENMs [37] or increases reactive oxygen species (ROS) production (see later discussion). Sonication settings and procedures should therefore be reported carefully at all times. Similar to using dispersants, using sonication is not environmentally realistic and may lead to overestimation of toxicity. Some authors therefore suggest adding a control with nonsonicated (e.g., stirred) ENM to improve extrapolation of ENM toxicity results toward risk assessment [38].

Nano-specific endpoints in ecotoxicity tests

The current view is that most of the generic biological endpoints critical to risk assessment at the population level (mortality, growth, reproduction) remain appropriate for ENMs [5]. Numerous sublethal endpoints are also used in fundamental research, including physiological endpoints, histopathology, biochemistry, molecular, and -omic responses (see Handy et al. [39] for review). Many of these approaches are now being

used for research on the biological effects of ENMs [1–4], and the issue of whether nano-specific endpoints are also needed in ecotoxicity tests is worth discussing. Developing nano-specific biological measurements seems unlikely at this stage. Engineered nanomaterials exhibit many of the fundamental toxic responses (oxidative stress, enzyme inhibition, ionoregulatory disturbances, genotoxicity, and so forth [14,15,40]), which are well known for other chemicals. To establish a nano-specific biological endpoint, a unique nano-specific biological response that could form the basis of an assay is a prerequisite. This would be analogous to developing the vitellogenin assay [41], which is now widely used as a specific diagnostic tool for endocrine disruption. To date, such an assay has proved elusive for ENMs, and seeking such an assay for ENMs may be inappropriate. Given the numerous chemical and physical forms of ENMs, the idea that one biomarker will be diagnostic of all is unlikely. At best, eventually identifying some biological responses associated with major types of ENMs (i.e., a biomarker for nanotubes or a biomarker for particular types of metal NPs) may be possible.

More likely, however, is that a suite of responses could be developed to indicate a possible ENM exposure or effect (Fig. 3). The idea of using response syndromes as a tool for identifying groups of contaminants is not new to ecotoxicology. For example, respiratory and cardiovascular response syndromes in fish were used 20 years ago to identify narcotic compounds [42]. The scientific community also uses tiered approaches with suites of biomarkers to identify contaminants in environmental monitoring schemes [43]. One approach could simply involve modifying an existing tiered monitoring scheme to include the responses to ENMs (Fig. 3). Environmental samples, such as tissue homogenates or cells from biota, water samples, soil extracts, can be screened initially for toxicity using well-established rapid screening assays for existing chemicals, such as neutral red retention assays or the Microtox assay. If the sample is toxic, further investigation can include solvent extraction to isolate the fraction containing organic pollutants and hydrophobic ENMs. The presence of ENMs in the solvent-extracted phase can then be identified by nano-specific assays (Fig. 3). Nano-specific assays have yet to be developed for routine environmental monitoring. However, using physical effects of ENMs on cells, such as the frustrated phagocytosis measurement [44], may be possible, or one may design an assay around the notion of toxicity after endocytosis of the ENM. For example, in such an endocytosis assay, ENM toxicity to cells should decline in the presence of endocytosis inhibitors. For metals, and ENMs that release metals by dissolution, the addition of metal chelators such as ethylenediaminetetraacetic acid will identify a decrease in toxicity attributable to bioavailable metal ions (Fig. 3). The presence of dissolved metals can be confirmed using well-known assays for metals such as the metallothionein assay or ATPase inhibition. Inorganic ENMs that remain in the aqueous phase but do not release appreciable free metal ions may then be assessed by the nano-specific assays outlined previously (Fig. 3). The assays also could be used in biomonitoring by repeating the program with fresh samples

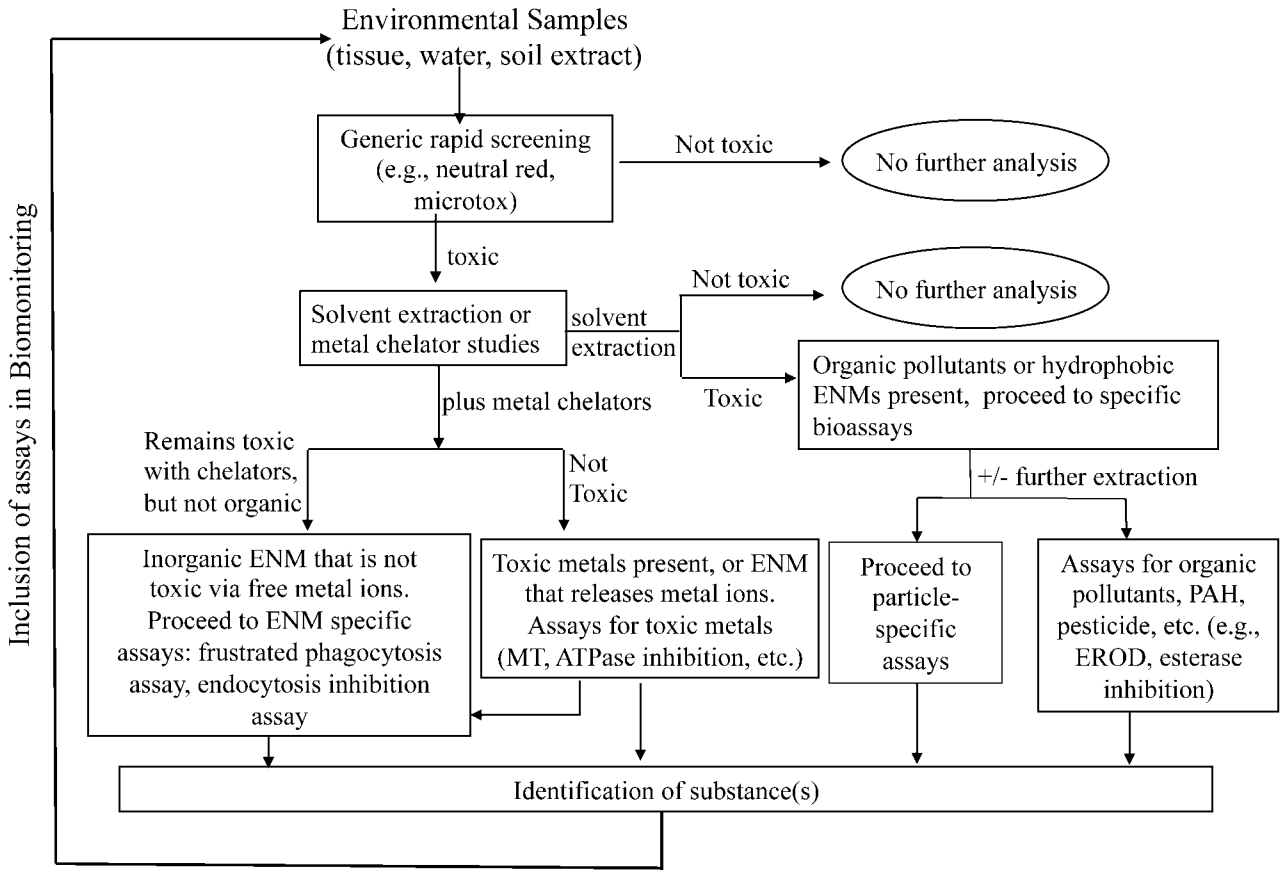


Fig. 3. A suggested program for ecotoxicological screening and diagnosis of environmental samples for engineering nanomaterials (ENMs) modified from Handy et al. [43]. Program involves initial screening using traditional rapid assays, then extracting hydrophobic and hydrophilic phases, followed by nano-specific assays. The release of metal ions from ENMs can be assessed using chelators and traditional metal toxicity assays. See text for details. MT = metallothionein; PAH = polyaromatic hydrocarbon; EROD = ethoxyresorufin-*O*-deethylase activity.

collected at different times. The weight of evidence approach [43] for a nano-effect also could be enhanced by looking for more unusual histopathology seen with ENMs (e.g., hyperplasia in fish gill [13]), along with genomic profiling [45] and biochemical assays around phagocytosis or endocytosis.

TESTS USING AQUATIC ORGANISMS IN THE WATER COLUMN

Many of the generic issues discussed apply to organisms in the water column. These tests include work on algae, invertebrates, and fish. Of particular interest are the acute tests, such as the algal growth test, *Daphnia* immobilization test, and 96-h fish toxicity test, which are often the first suite in the hazard assessment strategy for normal chemicals. The tendency of ENMs to aggregate and precipitate, however, provides an ecological argument for including a sediment or benthic test within this first tier of the ecotoxicity testing strategy for ENMs.

Like other regulatory tests, the acute tests have standardized protocols [5], but of special concern for ENMs is that the small variations allowed in methodology between the different agencies may also be the aspects that affect ENM behavior or toxicity the most. Thus, methodological details that are not as important for traditional chemicals become critical to testing ENMs. Here, particular attention is drawn to this dilemma by referencing the algal growth test, as a case study, in which several variations in the fine detail of methodology are currently allowed in regulatory testing.

Critical variations in protocols for the microalgae test

The algal growth test is based on the notion of following the multiplication of unicellular algae in water over a period of usually 72 h. The test methods include OECD 201, EPA 797.1050, and OPPTS 850.5400 (Table 1). Some differences in the protocols, such as the choice of algae species, do not appear to have any nano-specific ramifications. Differences in the recipes of test media, shaking the test vessels, and lighting (Table 1), however, may have profound effects on test results when using ENMs. The algal test has been examined for reproducibility across different methods and between laboratories for traditional chemicals [46], but this has not been done for ENMs.

First consider the effects of shaking on ENMs. From a biological perspective, shaking benefits aeration and gas exchange in the media and therefore growth of the culture. Indeed, keeping cultures at optimal growth [46] is essential. The mixing of medium induces orthokinetic (shear) aggregation [1], however, and depending on the precise particle energy, shear forces and particle size ranges in the dispersion may decrease or increase aggregation. The accurate and detailed reporting of shaking techniques in experiments is therefore important for tests with ENMs. Unfortunately, most published studies assessing the effects of ENMs indicate that shaking, stirring, or mixing took place, but the level of information reported is frequently sparse and highly variable (Table 1). The units used to report shaking are often different, making any useful comparison between tests difficult. Similar arguments apply to reporting lighting (discussed later) and sonication (discussed previously).

Finally, and perhaps most important, the amount of shaking will also alter the nature and frequency of ENM collisions with the cell wall of the test organism itself. Engineered nanomaterials can readily sorb onto the exterior surface of the algae, including the exudates and the cell wall [47]. At milligram per liter concentrations, this may lead to physical restraint of the test organism, but the accumulation of the ENM on the surface of the cell wall also may inhibit photosynthetic activity because of shading effects (decreased light availability in the microenvironment at the surface of the organism). This may be in addition to the general shading effect caused by the turbidity or color of the ENM in the test media. The former will be particularly important for positively charged ENMs that are attracted to the polyanionic matrix of the cell surface (see Handy et al. [48] for discussion of ENMs in unstirred layers). Darkly colored materials such as SWCNT may be particularly important to shading effects in the bulk solution.

Lighting is also a special concern for certain photo-reactive ENMs. Lighting is essential for algal growth, but too much light may promote ROS generation from ENMs. For example, the energy from ultraviolet light especially can promote free radical reactions on the surface crystal structure of nano titania [49] and nanoceria [50]. Notably, the rates of ROS production can be substantial (μmol/h levels for the final CO_2 product [49]) and are strongly dependent on the presence of organic matter to facilitate the movement of electrons on the surface of the ENM. This effect is exacerbated if the media is also sonicated. Thus, the combination of shaking and the wavelength and intensity of the light determine photoactivation in the test media. Sonication of the test media should be avoided because this generates ROS, although this should not be a problem for pure stock solutions that do not contain carbon sources for moving electrons (stocks made in ultrapure water).

Given the variability in methods used, photoactivation experiments with algae are, not surprisingly, producing contrasting results. One of the earlier studies using the green algae *Desmodesmus subspicatus* found no statistically significant differences associated with irradiation before TiO_2 NP (25 and 100 nm) exposures, nor an effect of shading [51]. Similarly, Van Hoecke et al. [52] found no effects of shading on *Pseudokirchneriella subcapitata* during experiments with CeO_2 NPs. Aruoja et al. [53] reported a similar view for CuO and ZnO NP. In other studies, however, differences have been found [54–56], but comparisons are very difficult because of variations in lighting intensity, lamp type, temperature, and shaking conditions used. Clearly, standardizing both lighting and shaking in the algal tests for ENMs is vitally important, and especially for materials with photoreactive properties or materials that absorb light.

Photosynthesis is a well-known rate-limiting step in algal growth, and these effects will greatly influence the main endpoint of growth in the test. Mayer et al. [57] examined a gradient of eight light intensity levels (6–250 $\mu E/m^2/s$) and suggested that light intensity could be standardized in the algal toxicity test by increasing the light level to achieve light-saturated photosynthesis (that is, so that it is no longer rate limiting on growth). This approach may reduce variability in the test results, but it also has the risk of promoting photoactivation of ENMs and therefore overestimating the hazard.

Another potential source of error that may be specific to ENMs is interfering with techniques for quantifying growth. Each test method gives the option of using different methods, such as automated cell counting (flow cytometry), traditional microscopic sterology, or spectrophotometric methods. The concerns about ENM interference with dyes and probes apply equally here. Counting methods should be checked and validated against traditional microscopy, in which such errors are less likely. These checks will need to be done for each type of ENM tested.

Table 1. Key differences in the test conditions for standardized algal growth tests

Organization (test number)[a]	OECD (201)	U.S. EPA (797.1050)	U.S. EPA (Guideline OPPTS 850.5400)
Test species	*Pseudokirchneriella subcapitata* *Desmodesmus subspicatus* *Navicula pelliculosa* *Anabaena flos-aquae*	*P. subcapitata* *Skeletonema costatum* (marine)	Written specifically for *P. subcapitata* and *S. costatum.* Use of *A. flos-aquae* or *N. pelliculosa* may require some specific modifications in test procedures.
Test seeding density	*P subcapitata*: 5×10^3–10^4 cells/ml *Scenedesmus subspicatus:* 2–5×10^3 cells/ml *N. pelliculosa*: 10^4 cells/ml *A. flos-aquae:* 10^4 cells/ml *Synechococcus leopoldensis*: 5×10^4–10^5 cells/ml	*P. subcapitata:* approximately 1×10^4 cells/ml *S. costatum:* 7.7×10^4 cells/ml	*P. subcapitata*: approximately 1×10^4 cells/ml *S. costatum:* 7.7×10^4 cells/mL *N. pelliculosa:* approximately 1×10^4 cells/ml *A. flos-aquae:* approximately 1×10^4 cells/ml
Test medium	OECD growth medium (or AAP[b]—U.S. EPA)	AAP (U.S. EPA) growth medium	AAP (U.S. EPA) growth medium
Dosing method	Dosed into medium. Solvents can be used.	Dosed into medium. Solvents can be used.	Dosed into medium. Solvents can be used.
Test duration	72 h	96 h	96 h
Endpoint	Growth inhibition or stimulation, EC10, EC20 and EC50, LOEC, NOEC[c] values.	Growth inhibition or stimulation, EC10, EC50, and EC90 values.	Growth inhibition or stimulation, EC10, EC50, and EC90 values.
Techniques permitted for measuring growth.	Measurement of biomass by manual cell counting by microscope or an electronic particle counter (cell counts and/or biovolume; the biovolume correlates directly with biomass) are preferred. Alternative techniques, for example in vitro chlorophyll fluorescence or optical density can be used providing a satisfactory correlation with biomass (dry wt mg/L) can be demonstrated over the range occurring in the test.	Number (or wt of algal cells/ml) in all test containers and controls shall be determined by an indirect (spectrophotometry, electronic cell counters, dry wt, etc.) or a direct (actual microscopic cell count) method.	Electronic cell counter, light microscope sterology, spectrophotometric methods-fluorimetry or colorimetry. For sterology, at least 400 cells per flask should be counted.
Recording procedure	Cell counts or surrogate measure that correlates with biomass (biovolume, in vitro chlorophyll fluorescence, or optical density).	Determine whether the altered growth response between controls and test algae was due to a change in relative cell numbers, cell sizes, or both.	Determine whether the altered growth response between controls and test algae was due to a change in relative cell numbers, cell sizes, or both.
Shaking	Constant shaking or stirring should be used.	Test containers shall be placed on a rotary shaking apparatus. *P. subcapitata:* 100 cycles/min *Skeletonema:* 60 cycles/min The rate of oscillation should be determined at least once daily.	Test containers shall be placed on a rotary shaking apparatus. *P. subcapitata:* 100 cycles/min *Skeletonema:* 60 cycles/min The rate of oscillation should be determined at the beginning of the test or at least once daily if the shaking rate is changed.
Light conditions[d]	Continuous, uniform fluorescent illumination of cool-white or daylight type. Light intensity at the level of the test solutions: 60–120 $\mu E/m^2/s$ and in the range of 400–700 nm. The light intensity should be selected within this range to suit the test organism. For light-measuring instruments calibrated in lux (range: 6,000–10,000 lux).	*P. subcapitata:* continuous illumination *Skeletonema:* 14:10 light:dark photoperiod with a 30 min transition. Fluorescent lamps providing 300 ± 25 $\mu Ein/m^2.sec$ (~400 ft-c) measured adjacent to the test chambers at the level of test solution.	*P. subcapitata*, *Navicula*, and *Anabaena:* continuous illumination Skeletonema: 14:10 light:dark photoperiod. Fluorescent lights providing 4.3 K 1× (4,306 lm/m^2 or $400 \pm 10\%$ fc) for *P. subcapitata*, *Skeletonema*, and *Navicula*, and 2.2 K 1× for *Anabaena*. Lamps should have a photosynthetically active radiation of approximately $66.5 \pm 10\%$ $uEin/m^2.sec$

Table 1. (*Continued*)

Organization (test number)[a]	OECD (201)	U.S. EPA (797.1050)	U.S. EPA (Guideline OPPTS 850.5400)
pH	pH in the control cultures shall not increase more than 1.5 unit.	*P. subcapitata:* 7.5 ± 0.1 *Skeletonema:* 8.1 ± 0.1 pH may be adjusted prior to test chemical addition.	*P. subcapitata:* 7.5 ± 0.1 *Skeletonema:* 8.1 ± 0.1 *Navicula* and *Anabaena:* 7.5 ± 0.1 pH is not adjusted after the addition of the algae.
Temperature	Temperature in the range of 21–24°C maintained at ± 2°C.	*P. subcapitata:* 24°C *Skeletonema:* 20°C Deviations, no greater than ± 2°C.	*P. subcapitata, Navicula*, and *Anabaena:* 24°C *Skeletonema:* 20°C Deviations, no greater than ± 2°C.

[a] The precise protocols described are taken from the following versions of the published protocols: OECD (201) [114]; U.S. EPA (Guideline OPPTS 850.5400) [115]; U.S. EPA (797.1050),[116]; OECD = Organisation for Economic Co-operation and Development; U.S. EPA = U.S. Environmental Protection Agency.

[b] AAP medium is a type of algal and plant cell culture solution.

[c] EC10, EC20, EC50, EC90 are effective concentrations for 10, 20, 50, and 90% responses, respectively. LOEC = lowest-observed-effect concentration; NOEC = no-observed-effect concentration.

[d] Units for light measurements are written exactly as reported in the relevant document, some of the abbreviations are ambiguous, and documents use a mixture of system international (SI) and imperial units. Illuminance is measured in lux (SI units, lm/m^2, or lx) or foot candles (the imperial unit is fc = lm/ft^2, also less common to use the unit abbreviation ft-c). K lx is presumably kilo lux (lux x 10^3). Photon irradiance, the correct imperial unit is Einstein m^{-2} s^{-1}, and agencies have abbreviated this to Ein/m^2/sec, Ein/m^2.sec or E/m^2/s. The Einstein unit should not be abbreviated to E (as this is the abbreviation for energy).

Daphnia *immobilization test*

The principle of this acute test is to demonstrate the toxicity of the chemical by measuring immobilization of the invertebrate. For traditional chemicals, the test design usually involves static beakers without a water change or aeration, with the endpoint being measured over 48 h. The test beakers are replicated, normally with up to four replicates of each test concentration.

Clearly, the absence of water changes is likely to compromise the maintenance of ENM dispersion in the water column (aggregation over 48 h), but replacing the test media during the experiment may increase stress and lead to additional mortalities. Aeration is required for many aquatic tests and would aid mixing of the ENMs in the test beakers. This, however, would need to be done carefully so that the animals are not damaged and shear aggregation of ENMs is avoided as much as possible. Redesigning the test vessels so that an airlift is created on one wall of a rectangular test vessel to help circulate the media could accomplish this. A simple mesh could be used to keep the animals separate from the stream of small air bubbles. Such approaches have been used with semistatic exposures with fish to successfully mix water that contains ENMs [13].

One concern for the immobilization endpoint is the physical effect of ENMs on the mobility of the test organism, especially at high mg/L concentrations of the ENM. Like the algal test, concerns arise about the precipitation of the test material onto the surface of the *Daphnia*; in addition, the ENMs may prevent respiration. Daphnids ventilate under the carapace by moving their appendages to create water flow over the respiratory surfaces. The ENMs could stick to these appendages, and eventually cause death by preventing ventilation. For example, milligram per liter levels of lysophosphatidylcholine-coated single-walled carbon nanotubes do stick to the surfaces of *Daphnia magna* in sufficient quantities to prevent swimming, causing the animals to sink to the bottom of the test vessels [58]. This in itself is not a problem for the test method—it is simply a nonchemical method of producing mortality—but it should be measured and distinguished from traditional chemical toxicity. This could be done by including controls of ENMs with a known size that have been standardized and are chemically inert (e.g., polystyrene beads) but produce the desired mechanical effect. The disadvantage of such a control is that although a particle could be of the right primary particle size and surface charge, it would not behave precisely the same as the test material. Thus, the results of such a control could only be used as a guide to indicate whether the toxicity is mainly mechanical or chemical in origin.

Acute fish test

The generic issues about cleaning and preparing test vessels and measuring water quality are especially important for any toxicity test with fish. Salmonid fish, in particular, are very sensitive to ammonia and declining levels of dissolved oxygen. For these reasons, flow-through test methods are often preferred with fish, but the volumes of water required may use large quantities of potentially expensive ENMs. In addition, waste regulations currently prevent discharging ENM effluents from the laboratory. Consequently, the test water is treated as hazardous waste. The flow-through method is therefore not as practical for ENMs, and the semistatic method has been used successfully for exposures lasting up to 14 d in trout [13,14].

Some practical considerations must be noted for fish tests that are specific to ENMs. Fish produce mucus that will cause

the ENM to agglomerate or form aggregates with the mucus (e.g., with SWCNT [14]). These mucous aggregates of ENMs will deposit in the bottom of the tank, and during water changes one should gently siphon off the old test media from the bottom of the tank to ensure that this debris is captured. Fish also require routine health observations as part of ethical approval for running any toxicity test; therefore, the ENM should not discolor or cloud the water to such an extent that these husbandry observations cannot be made.

Changes in schooling behavior and increased aggression have been noted in trout during ENM experiments (TiO_2 [13]; SWCNT [14]). Trout, especially, also become aggressive when they are hungry, and this has implications for the test design. In the 96-h acute test, this may not be a problem, because 5 or 6 d may pass before aggression appears [14], but it would be important for any test lasting a week or more. A single feeding (maintenance ration, 2% or less of body mass) just after the water change can ameliorate this aggression without subsequent fecal contamination of the water [14]. Do not underestimate the importance of behavioral change or aggression. The latter alone can cause fish mortality and needs to be monitored, especially in experiments with SWCNT [14]. One also should avoid using air stones for aeration, because these can release particulates into the water and can become a focus for the aggressive behavior.

Finally, the replication of the test needs to be considered. Pseudo replication is used in the regulatory acute test methods for fish (one tank/treatment). This is partly to reduce the use of animals, but given the difficulty of maintaining ENM dispersions, perhaps a triplicated design should be used instead. Evidence from triplicate semistatic tests shows that the replication is good [13,14] and allows data to be pooled for statistical analysis. This suggests that the current approach of pseudoreplication may be adequate for regulatory testing, although it is accompanied by all the usual concerns about statistical analysis of data from pseudoreplicated designs. A risk remains, however. Experience at the bench shows that when one tank of fish appears to be different for ENMs, it is usually very different in terms of the response of the fish or the water chemistry; this would add substantial error to the dose–response curve plotted from data obtained by pseudoreplication. The risk of a substantial experimental error needs to be balanced against the animal welfare.

MARINE SEDIMENT FEEDING TESTS

One concern for the hazard assessment strategy is that some benthic tests need to be included in the initial tier or base set of tests to account for the aggregation and settling behavior of ENMs. These concerns are well founded in the microenvironment chemistry and biology of the sediment surface. Benthic test methods involve freshwater and marine taxa, and this would have the advantage of adding a marine species to an early step in the testing strategy where the colloid behavior and bioavailability of ENM is likely to differ because of the higher ionic strengths of marine environments. Sedimentary environments typically contain high concentrations of organic detritus and microorganisms (e.g., bacteria, diatoms), which together form an abundant food source for sediment grazing animals. Here, most microbial cells occur as attached *biofilms* in which cells are surrounded by a sticky matrix of extracellular polymers [59]. Biofilms coat the surfaces of most sediment and clumps of detritus and offer efficient sites for the sorptive accumulation and concentration of ENMs [60,61]. For this reason, the sediment surface represents an environment of potentially high bioavailability of ENMs to grazing organisms and an efficient vehicle for trophic transfer.

The present test methods have been used to measure bioaccumulation using ^{14}C-radiolabeled SWCNTs [62] that are based on earlier bioaccumulation studies [63]. A typical experimental design involves mixing nanotubes with processed sediment (natural sediment processed to remove any animals) for several days under continuous mixing to ensure a homogenous distribution of the nanotubes. The sediments are then added to microcosms and allowed to equilibrate with overlying water. Then, test animals such as polychaetes or harpacticoid copepods are added and allowed to feed naturally for approximately 14 d. The animals are then removed, allowed to depurate while feeding on non-test sediments, and then measured for ^{14}C activity. Sediments are also characterized to determine levels of SWNT as the experiments commence and conclude.

ENM coatings and sediment feeding experiments

In marine and freshwater grazing experiments, the type of ENMs and the types of coatings on ENMs will strongly affect their partitioning and persistence within sediments, which in turn influence bioaccumulation and toxicity to the grazing animals. Most types of ENMs will also behave differently under marine conditions compared with freshwater. For example, sparingly soluble ENMs such as inorganic NPs (CeO_2, TiO_2) and pristine SWCNTs should remain quite stable in marine sediments, whereas more soluble forms such as AgNPs will dissolve rapidly under marine conditions to form chloride ($AgCl_4^{3-}$) complexes. All of these forms will flocculate into sediments on addition, because of the high ionic strength of the marine environment.

To enhance dispersion and solution handling during dosing, ENMs can be dispersed with various organics such as humic or fulvic acids, gum arabic, polyvinylpyrrolidone, sodium dodecyl sulfate, or citrate (see earlier discussion). These coatings differentially affect the persistence and dispersion of ENMs. Importantly, however, certain coatings such as polyvinylpyrrolidone and sodium dodecyl sulfate may present significant toxicity to grazing animals [64]. Some coatings may be labile (easily digested and removed by the animal) or relatively refractory (not easily digested and removed). Humic and fulvic acids are derived from natural organics and are relatively refractory. When used as coatings, humic or fulvic acids may enhance the persistence (reduce dissolution) and potentially reduce the potential toxicity of ENMs. Finally, any coating added to the ENMs could ultimately be replaced by the natural organic matter present within sediments.

Adding ENMs to the food of grazing organisms

In grazing studies, the food is the biofilm and detritus on the sediment surface, and one should provide a relatively homogeneous dispersion of the test material over this surface. The ENMs can be added to the overlying water above the sediments and will flocculate along with other organic matter to eventually arrive at the sediment. If the ENM is evenly mixed in the medium, then it should give a relatively homogeneous coverage of the exposed sediment surface. This would also be an ecologically relevant way introduce contaminants to the sediment without disturbing the delicate structure of the biofilm. Alternatively, ENMs may be homogeneously mixed with the sediment before setting up the test. This would destroy the in situ biofilm structure in natural sediments, and only a fraction of the dose would be in the surface layer. A variation on the latter method would be to try to spike the surface sediment directly

(not via the water) with the ENM, but this would be problematic in terms of maintaining an even surface covering of the test material. Of course, the test organisms themselves may mix the sediment surface as they graze and burrow, and so any conditions carefully imposed by the experimenter at the start may be undone rapidly by the animals' behavior. The general composition of the sediment (e.g., organic matter, clay content) may also alter the bioavailability of the food, and the detailed composition of the sediment should be reported.

Feeding behavior of different test species

A wide range of animals can be used for grazing experiments, including polychaetes, oligochaetes, amphipods, copepods, nematodes, snails, and bivalves. These animals represent different feeding behaviors. Some examples include suspension feeders (*Streblospio* sp., polychaetes), and filter-feeding bivalves, surface grazers (snails), epibenthic (harpacticoid) copepods and amphipods, and selective and nonselective deposit feeders [65]. One must understand the type of feeding a test animal uses to ensure that added ENMs are accessible to the animal during feeding. Failure to do so can result in false negative results, such as no uptake or toxicity of the ENM simply because it was not ingested. Harpacticoid copepods [66] and nematodes may be particularly useful as test organisms for ENMs because of their efficient feeding and rapid generation times. As molecular studies of ENM progress, use of animals whose genome has been sequenced (e.g., nematodes such as *Caenorhabditis elegans*; see later discussion) will be important.

Uptake, digestion, and assimilation in grazing experiments

In sediment tests with grazing invertebrates, inevitably both dermal and dietary exposure will occur. The relative contributions of each uptake route to accumulation/toxicity are presently unknown for ENMs and, like traditional chemicals, will also vary with the type of grazer and sediment. Measuring dermal versus dietary uptake is very challenging, and methods to confirm ENMs in the tissues of the test organisms are needed to inform data interpretation. One founding assumption of feeding tests, however, is that the toxicity is due to the assimilation of the toxic substance by the organism, not merely the presence of the substance in the gut lumen. Whether this assumption is true for ENMs is unclear. Nonetheless, several important parameters must be measured to quantify the ingestion and retention of ENMs by animals, including gut transit time, adsorption versus true assimilation in the tissues, depuration, and excretion [67].

A key fundamental research need for grazing and food chain studies is to understand the biology of the animals over long time scales with ENMs to interpret test results properly. Uptake, distribution, retention, and depuration studies should be conducted with labeled ENMs to understand the ENMs' dynamics in the test organisms. Techniques such as using uncontaminated food to purge the gut of ingested ENMs need to be validated. As a general rule, feeding on clean food should be conducted for approximately three times the gut transit time to adequately clear the gut. We should also recognize that the particle biology of invertebrates is very different from that of mammals. Many invertebrates have a well-known high capacity to manufacture inert storage granules in the tissues. Also filter-feeding bivalves and snail grazers have two pathways for the digestion of particulates: a rapid digestion extracellular pathway (using intestinal passage of food) and a slower, more efficient, intracellular form of digestion (using a hepatopancreas or digestive gland). Interestingly, small particulates, and possibly ENMs, are processed through the slow pathway, a process that may enhance retaining and incorporating ingested ENMs into tissues [68,69]. These differences in ENM handling should be taken into account. Finally, future studies involving feeding and sublethal and multigenerational effects of toxicity can benefit from using test animals having relatively short generation times. These include harpacticoid copepods (~14 d) and nematodes such as *C. elegans* (24 h).

ECOTOXICITY TESTS WITH SOIL ORGANISMS

Several regulatory test methods use soil as the test matrix [5], including the 21-d plant growth test (OECD 208), the acute (14-d) earthworm toxicity test (OECD 207), and the earthworm 56-d reproduction test (OECD 222 [70]). Tests also are under development for the nematode, *C. elegans* [71], one of the most abundant soil organisms [72] widely used as an environmental indicator for both aquatic and soil toxicity tests [73,74].

Behavior of ENMs in the soil matrix

Many researchers use their preferred natural soil for ecotoxicity testing, or several different soils, and an artificial standard soil as a reference. The type of soil matrix may have profound effects on the fate, behavior, and bioavailability of the test material. Although this is also well known for traditional chemicals, some challenges relate specifically to ENMs. Natural soils represent a large and reactive sink, and recent studies have shown that natural soil will filter ENMs during transport through the soil matrix, particularly if the clay content or ionic strength is elevated [75–77]. Likely the bioavailability of ENMs to soil organisms, as for aquatic sedimentary tests, will differ for natural and artificial soils, and the method of dosing the soil will alter this bioavailability. Simple spiked doses in artificial soil are likely to overestimate toxicity [2], because the bioavailability is artificially high with the ENMs not properly incorporated into the soil structure.

Similar to methods with sediment grazers, soil tests thus need some fundamental research to strengthen our understanding of the regulatory tests with soil organisms. The mechanisms that determine the bioavailability of ENMs to soil organisms are largely unknown. A method to determine ENM retention and dissolution in bulk soils can be used to estimate the bioavailable fraction of ENMs to plants [77], but whether root exudates may improve dispersion and diffusion of ENM in the rhizosphere, thus enhancing their bioavailability relative to the bulk soil, as is well-known for traditional organic–metals complexes [78], is unclear. Nonetheless, test methods clearly should take into account the diversity of natural soils such as their pH, clay content, cation exchange capacity, texture, amount/type of organic matter, and mineralogy, as well as include a standard soil in the test.

Confirming ENM exposure via the soil

For measuring concentration (mg/kg soil), the problems are similar to traditional chemicals. For example, techniques are needed that can measure the concentration of the ENM against a high background of the substances with the same chemistry, such as measuring C_{60} against a large carbon background in the soil. Methods are also required to confirm the compartmentalization of the ENM within the soil matrix and pore water. Techniques are being developed to overcome these technical difficulties (Von der Kammer et al. [25], this issue). Although these techniques have great utility in the research laboratory, they often require sensitive equipment, high levels of technical

skill, and lengthy sample preparation procedures (e.g., field-flow fractionation [25]). Few of these methods represent inexpensive and rapid tools for quantifying exposure during experiments in the routine regulatory testing laboratory.

ENM characterization in soil and dose metric

Measuring particle size distributions of ENMs in soils is complicated by the high background of particulate material already present in the soil [25]. Such information may be vital, however, because, for plants at least, a need exists for alternative dose metrics. Particle number or surface area appears to be more relevant than mass-based toxicity to plants for ENMs [79]. Because of these current technical difficulties, ecotoxicity testing on soil organisms can be supported by a strategy that uses liquid media instead of soil, such as aquatic nematode tests or hydroponic plant tests. Such an exposure scenario may induce false positives, for example, because of damage to the otherwise impermeable Casparian strip of plants in hydroponic tests [80]. Engineered nanomaterials may be more bioavailable in liquid media/hydroponics compared with a natural soil, but likely particle distribution will be easier to measure. This can always be supported by a reduced tier of work later on in the testing strategy using natural soils, which are still the preferred medium for environmentally relevant conditions. In the nematode aqueous tests, the medium (K-Medium: 32 mM KCl, 51 mM NaCl) has significant ionic strength and some 83 mM of chloride. The high ionic strength is likely to create a similar problem with particle distributions (aggregation/agglomeration) as that in seawater [2] or salt solutions in electrodes (discussed previously). For some ENMs, this may be a particular problem. For example, the previous arguments about the precipitation of insoluble silver chloride from AgNPs apply equally here. Liquid media can be characterized more conveniently using existing techniques (ultrafiltration to characterize dissolution, dynamic light scattering, zeta potential measurements), however, and tests with *C. elegans* could be conducted with a lower ionic strength medium, such as the standardized U.S. EPA moderately hard, reconstituted water [81].

Aging and modifying ENMs during soil tests

Soil organism tests tend to take several weeks or months to conduct, and researchers are concerned that the test material may age or be modified in the soil. This problem is also found with traditional chemicals, but some nano-specific issues exist. For example, for a 14- or 21-d plant growth test, very unstable NPs may completely dissolve during the test. This may be the case for AgNPs in liquid media [29], but it may not be a realistic representation of the fate of AgNPs in soil. For example, Shoults-Wilson et al. [82] demonstrated little or no oxidative dissolution of AgNPs during a 56-d earthworm reproduction assay. To minimize aging effects, one may adopt shorter tests in the soil testing strategy. Here, *C. elegans* may be useful. Nematode tests have been used for metals and organic contaminants in soils [83–86], and although they have not yet been used extensively for testing of ENMs, data show that the tests do work with ENMs [87]. The tests are much shorter than the equivalent earthworm method (28 d for mortality, 56 d for reproduction in earthworms). The nematode mortality test is 24 h and the reproduction test 96 h [71,88].

Measuring uptake in soil organisms

Given the difficulties of measuring ENMs in soil matrices, an alternative approach to confirming exposure and understanding uptake will be to measure ENMs in the soil organisms directly. In the research laboratory, this can be done with labeling methods. For example, ^{14}C-radiolabeled SWCNT has been used in an uptake and depuration study in *Eisenia fetida* [89]. Several studies have attempted to address uptake into tissues of nematodes (AgNP [90]) and earthworms (Au NP and Cu NP [91,92]), but these generally use sophisticated methods that are unlikely to find routine application in regulatory testing. For example, Meyer et al. [90] used a visible and near-infrared hyperspectral imaging system to demonstrate that AgNP can be detected in the embryos of *C. elegans*.

Similar to the sediment grazer tests, soil organisms also can be exposed simultaneously via the dermal and dietary routes. For the earthworm, allowing the animals to void the gut lumen contents and washing the animals (e.g., in ethylenediaminetetra-acetic acid solution) to remove ENMs adsorbed to the cuticle is possible. This enables internal tissue levels to be differentiated from any surface-bound material. Short-term studies designed to look only at dermal exposures, for example with dauers (a stage of *C. elegans* that does not feed) or with earthworms with their mouths sealed with surgical glue would be helpful to evaluate which of the routes, dermal or gastrointestinal, is more important for ENM uptake. Like the sediment grazer tests, however, the relative proportions of dermal or dietary uptakes are largely unknown.

Sensitive endpoints for soil organism

One specific concern for ENMs is that the endpoints used for traditional chemicals (survival, growth) may not be sensitive enough to detect the effects of ENMs in some soil organisms. For example, Scott-Fordsmand et al. [93] showed that the most sensitive toxicological parameter for earthworms was reproduction (cocoon production) during exposures up to 495 mg/kg of double-walled CNTs and 1,000 mg/kg C_{60}, with no effect on hatchability or survival. Unfortunately, the one sensitive endpoint of reproduction used in regulatory tests often involves long experiments (e.g., earthworm tests lasting weeks) and therefore would require considerable effort to maintain the exposure. Clearly, both scientific and practical considerations are the impetus for finding new endpoints for soil tests with ENMs. These arguments also might apply to some traditional chemicals, but the nano problem presents a timely opportunity to develop some new endpoints. For instance, behavior is regarded as a very sensitive endpoint. Avoidance behavior in earthworms (*E. fetida*) has been demonstrated for AgNPs [82]. The locomotor movements or activity of *C. elegans* also may have some utility as a sensitive endpoint, and behavioral measurements can be quantitative. A computer tracking method has been developed to simultaneously assess behavior of nematodes in real time [94], and this could be applied to studies with ENMs.

Changes in behavior also may be important for interpreting false-negative results in soil toxicity tests. Earthworms, in particular, may stop feeding and moving around in contaminated soil. This defense mechanism prevents exposure and in an acute mortality tests could lead to a false negative. Body mass and body burden measurements of the earthworms may aid data interpretation, because a worm that is not feeding may show a decrease in body mass or growth. Another alternative, instead of finding more sensitive endpoints in existing regulatory test organisms, is to find more sensitive test species. For example, springtails have been used in metal toxicity studies [95,96], and may be sensitive to metal ENMs.

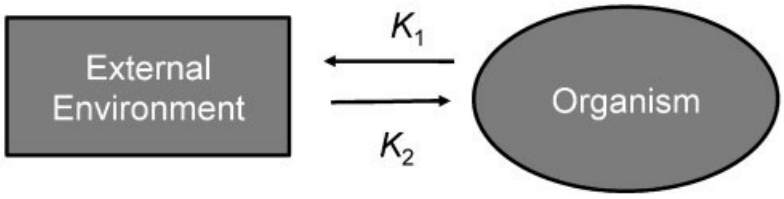
Sensitive endpoints are particularly needed for plants. The type of test media may help. Toxicity tests using hydroponics to

expose terrestrial plants do show some toxic effects [97]. In comparison, artificial or natural soils (OECD 208, terrestrial plant test) usually result in little or no phytotoxcity [98,99]. Commonly used endpoints such as germination and root growth have so far shown limited sensitivity to ENMs, even in hydroponic settings [100]. The way forward could include selecting plant species that are more sensitive to ENMs such as mungbean (*Phaseolus radiatus*), thale cress (*Arabidopsis thaliana*), and tomato (*Lycopersicon esculentum*) [97]. Alternatively, scientists could turn to biochemical or metabolic measurements that tend to be more sensitive, such as chlorophyll levels [97], respiration [100], or nitrogen fixation by legumes [101].

BIOACCUMULATION TESTS

The potential for bioaccumulation is an important aspect of hazard assessment. For traditional chemicals, most bioaccumulation tests typically involve exposing the test organisms until steady-state concentrations of the test substance are achieved between the external media and the tissues of the individuals. This information enables calculation of bioconcentration factors (BCF). Whereas the idea of equilibrium states may apply to chemicals in aqueous solution, it is difficult to see how this theory applies to colloid chemistry, where the system is dynamic. Traditional equilibrium chemistry, therefore, is at worst totally inappropriate to apply to ENMs or at best requires heavy modification. This notion questions the fundamental validity and purpose of performing standard BCF tests with ENMs (Fig. 4). For example, even if one considers the simplest possible model, a two-compartment model (the external environment and the whole organism), in which the net flux of the substance into the organism (accumulation) is defined by the uptake (k_1) and elimination (k_2) rate constants, many unknown parameters exist for ENMs (Fig. 4). In external media, knowing the free solute concentration is essential to calculating uptake rates, and although uptake rates may be estimated from total concentration, this overall approach may be inappropriate for ENMs, which are not soluble. Free ion activity models also routinely use thermodynamic stability constants of the chemical species to estimate likely losses by adsorption and competition for ligands on the surface of the organism [102]. This has not been measured for ENMs, and it may be more appropriate to use the DLVO theory (DLVO is named after the researchers: Derjaguin, Landau, Verwey, and Overbeek; see Handy et al. [1]) instead to estimate the stability of particle dispersions [1].

Similarly, from the biology perspective, uptake rates and elimination rates of solutes are usually fitted to well-known models such as Michaelis-Menten kinetics or the Fick equation for diffusive fluxes. This is not an arbitrary fitting of mathematical models but has a long-established and sound basis in the underlying biology (i.e., the mechanistic details of moving solutes on solute transporters). The precise pathways for ENM uptake and excretion are yet to be confirmed, but even if the major pathway for uptake is endocytosis (see Handy et al.



Data Required for Modelling

External Environment

Need to know information for solutes	Do we know this for ENMs?
Thermodynamic stability constants of the chemical species present.	No, not measured. May need to do this for surface chemistry-but how?
Free ion or free solute concentration.	Not appropriate, ENMs are not aqueous solutions but emulsions or dispersions.
Total concentration, preferably molar.	Not appropriate, only mass concentration (mg/L).
Effects of temperature, pH, DOC, Ca, etc., on free solute concentration.	No, not measured yet.
Effect of flow dynamics on removal of the substance from the external media (losses from bulk solution).	Yes, sometimes, but using a different set of rules to solutes.

Organism

Need to know information for solutes	Do we know this for ENMs?
Surface adsorption of solute on to exterior of the organism (binding, but not true uptake).	No, not measured yet. Need a sensitive detection method for ENMs. Not clear that ENMs will fit the rules of adsorption chemistry for solutes.
Total concentration, preferably molar, in the organism.	Only mass concentration (mg/L).
Uptake rate measured and fitted, e.g., to Michaelis–Menten kinetic parameters (K_M and V_{max}).	No, not measured. Michaelis–Menten kinetics may not be appropriate.
Elimination rate measured as above, and net flux calculated.	No, not measured yet. No proof that unidirectional fluxes = net flux for ENMs.

Fig. 4. Examples of the fundamental conceptual problems for bioaccumulation tests and the measurement of bioconcentration factors (BCF) using engineered nanomaterials (ENMs). A simple two-compartment model of the external environment and the whole organism is shown, where the net flux of the substance into the organism (i.e., accumulation) is defined by the uptake (k_1) and elimination (k_2) rate constants. Many unknown parameters exist in both the environmental compartment (left table) and the organism (right table) for ENMs. See text for details. DOC = dissolved organic carbon.

[48] for discussion), the appropriate kinetic model for these mechanisms remains to be described for use in BCF-like calculations. If the efflux component uses a completely different mechanism, the simple rule that influx should equal efflux to achieve a steady-state net flux requires some mechanistic understanding of how these pathways talk to each other inside the cell to control the net flux. Given these substantial theoretical problems and knowledge gaps, we are far from being sure that BCF and other accumulation tests are appropriate for ENMs.

In addition to all the practical problems concerning maintaining and verifying exposures (above), the assumption that some kind of steady-state bioaccumulation of ENMs will occur has yet to be demonstrated unequivocally for most materials. Even if the tests were conducted, such as the OECD 305 fish bioaccumulation test, which takes up to 90 d, currently limited technical ability exists to measure ENMs in tissues routinely. Only a handful of studies have investigated the accumulation of ENMs in organisms. The data so far suggest that invertebrates such as *Daphnia* and earthworms do not readily take up and accumulate CNTs. Furthermore, the CNT may remain in the gut lumen, also making it difficult to purge the gut contents so that accurate internal body burdens can be obtained [62,103,104]. Similar observations have been made in the gut of *Daphnia* for TiO_2 [105]. However, trout at least, do show TiO_2 accumulation in the internal organs from the water [13] and from dietary exposure [106]. The patterns of accumulation in trout were broadly similar to those of other toxic metals.

For most materials, routine methods for detecting ENMs in tissues are needed urgently and are being developed [25]. Whether the bioaccumulation tests in the current hazard assessment strategy are technically feasible, or scientifically appropriate for ENMs, remains unclear. Developing new tests and concepts based on the colloid behavior and uptake of ENMs as part of a revised approach would be prudent.

BACTERIAL TESTS FOR ENMs

Numerous regulatory ecotoxicity tests use bacteria in the test matrix as part of microbial biodegradation assessment (OECD 301, 302, 304 [5]). These tests use a mineral medium or sometimes a soil. All of the arguments presented for soil organisms and test validation would therefore apply here. The biodegradation tests would seem appropriate for carbon-based ENMs, which presumably could be metabolized eventually to carbon dioxide and water. However, the tests would be inappropriate for metal ENMs that are already in an elemental state.

Perhaps a more important question is whether the overall hazard assessment strategy needs to take into account the effects of ENMs on microbes. The suggestion of including a benthic grazing test to the base set of ecotoxicity tests (discussed previously) could apply equally to microbes given their importance to biofilms at the base of food webs. Bacteria have been shown to be very sensitive to exposure to a variety of ENMs, including carbon nanotubes, ZnO, CdSe, and TiO_2 [107,108]. Silver nanoparticles have been studied extensively in the laboratory and have significant inhibitory effects on bacterial growth and activity [107,109,110], although Ag ions released by the NPs could have contributed to this toxicity [111,112]. Alterations to microbial communities could have significant effects on biogeochemical cycling and other critical ecosystem services [113]. Therefore, including a bacterial test early on in the testing strategy may be advisable. Testing programs also could include protocols for testing either isolated cultures or natural populations of bacteria and methods for assessing effects on nutrient cycling.

CONCLUSIONS AND RECOMMENDATIONS

The overall conclusions of this analysis of discussions at the SETAC workshop is that the process of hazard assessment remains useful for ENMs. The need remains, however, to modify the testing strategy and many of the test methods to account for ENM behaviors such as aggregation and agglomeration. In particular, including a benthic test (e.g., invertebrate grazer test) to the base set of acute protocols in addition to the algal growth, *Daphnia*, and fish test would reflect the environmental importance of particle settling in the hazard assessment strategy. Although all of the major categories of tests (aquatic, sediment, soil, and so forth) require modifications to work optimally with ENMs, the original concern that we would have to start over with completely new tests is not supported by experiences at the bench or by published results.

In regulatory acute aquatic tests involving organisms in the water column, the modifications are relatively modest but would greatly improve control over the exposure with ENMs. This is therefore good news for the regulators. In tests lasting more than a few days (weeks, months, such as chronic aquatic tests), however, the significant logistical issue arises of the extra time and effort needed to maintain the exposure concentrations with the current quality criteria for nominal concentrations in testing systems. More flexibility in the target values for exposure concentration needs to be balanced against these practical difficulties in regulatory testing. Measuring exposure to ENMs in soils and sediments is particularly difficult and sometimes not technically possible. The technical barriers on measuring ENM exposure are challenging but tractable problems, and the chemistry community has dealt with developing new methods to detect new chemicals many times before. Indeed, many of the challenges for water samples are on the verge of being overcome, although the techniques are still far from routine applications [25].

The data so far do not support the need for a wholesale rethinking of endpoints. Many of the existing endpoints appear to work for ENMs, but increased sensitivity is needed in some tests. In particular, the utilization of *C. elegans* in the soil tests and using metabolism endpoints in plant growth tests may improve sensitivity. The *C. elegans* tests also may be a good alternative to the much longer earthworm tests. Maintaining the exposure is a challenging practical problem, and anything that can be done in the testing strategy to shorten tests by using an alternative test organism would be beneficial. Pilot studies to measure the fate and behavior of the ENM in the test system also may save time in the long run.

One area that involves significant scientific concern is the bioaccumulation tests, such as fish bioaccumulation test, OECD 305, and marine invertebrate grazer tests. These tests are not currently practical in the routine regulatory test laboratory because of a lack of simple, rapid tissue detection methods. Concern has been raised that the notion of a bioconcentration test with ENMs is flawed theoretically. Research is needed to explore this and, if necessary, to design a new test.

The practical recommendations for the researcher at the bench from the workshop include checking all of the routine measurements and assays used within a test protocol for interference from the ENM before starting the test. The magnitude of this problem may be material-specific, so checks should be made for each ENM tested. Permitted variations in regulatory

tests that are relevant to the behavior of colloids should be standardized. These include the levels of lighting and shaking or agitation of test vessels, especially in the algal growth inhibition tests [114–116]. Avoid procedures in which sonication of complex test media may produce ROS, because this will help reduce variability in test results. Do incorporate controls for the effects of dispersants in the test, but be prepared to run a full dose series of the dispersant controls if concern arises that the dispersant-to-ENM ratio will influence the shape of the material. This is important for SWCNT. The use of dispersants should be minimized for solution handling purposes. The addition of dispersants might alter the surface of the material and therefore the environmental relevance of the test. Continue to take samples of test media to confirm the exposure by measuring mass concentration, while the scientific debate on the best metric to define exposure dose is ongoing. Whenever possible, however, also take samples of media for particle size distribution and zeta potential measurements. In complex media where exposure cannot be easily measured, such as soil, consider collecting the test organisms instead to confirm the exposure. Alternatively, use test media where the chemistry may be more readily measured, such as the use of hydroponics instead of soil for some terrestrial tests.

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