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Nanomaterials in the Aquatic Environment: A European Union–United States Perspective on the Status of Ecotoxicity Testing, Research Priorities, and Challenges Ahead

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Abstract—*The European Union–United States Communities of Research were established in 2012 to provide a platform for scientists to develop a "shared repertoire of protocols and methods to overcome nanotechnology environmental health and safety (nanoEHS) research gaps and barriers" (www.us-eu.org/). Based on work within the Ecotoxicology Community of Research (2012–2015) the present Focus article provides an overview of the state of the art of nanomaterials (NMs) in the aquatic environment by addressing different research questions, with a focus on ecotoxicological test systems and the challenges faced when assessing NM hazards (e.g., uptake routes, bioaccumulation, toxicity, test protocols, and model organisms). The authors' recommendation is to place particular importance on studying the ecological effects of aged/weathered NMs, as-manufactured NMs, and NMs released from consumer products in addressing the following overarching research topics: 1) NM characterization and quantification in environmental and biological matrices; 2) NM transformation in the environment and consequences for bioavailability and toxicity; 3) alternative methods to assess exposure; 4) influence of exposure scenarios on bioavailability and toxicity; 5) development of more environmentally realistic bioassays; and 6) uptake, internal distribution, and depuration of NMs. Research addressing these key topics will reduce uncertainty in ecological risk assessment and support the sustainable development of nanotechnology. Environ Toxicol Chem 2016;35:1055–1067. © 2016 SETAC*

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Introduction

As nanotechnology continues to evolve, so do the test methods to assess the potential ecological effects of as-manufactured nanomaterials (NMs) and NMs after their release from products that incorporate them. The widespread use of NMs has inevitably resulted in their release into the environment, either as the original (as-manufactured) NM, or, more likely, as degradates of societal nano-enabled goods. Of particular interest is the aquatic environment, including sediments, which tend to be the ultimate sink for particulate contaminants. Once in the aquatic environment, NMs are highly affected by their surroundings and consequently undergo transformations (e.g., agglomeration, aggregation, dissolution, sulfidation). It is now clear that the fate and behavior of NMs depends both on their physical–chemical properties and on the characteristics of the receiving environment, including pH, temperature, concentration of

natural organic matter (NOM), ionic strength and salinity, and water hardness (presence of divalent ions such as Ca^{2+} and Mg^{2+}). Aquatic environments can contain substantial amounts of naturally occurring particulates such as organic particles/colloids (e.g., macromolecules of humic acid from degrading leaf litter) and minerals (e.g., iron particles from the weathering of rocks/soil). However, our knowledge is far from adequate in terms of identifying exposure or hazard to enable an environmental risk assessment of NMs that is as robust as those we currently prepare for traditional chemicals. A particular challenge for environmental safety is to understand how the myriad of naturally occurring particles (many at the nanoscale) interact with engineered NMs. One key concern is modifications to the NM surface by chemical reactions with the environment, including the adsorption of organic ligands, metals, and naturally occurring colloids. The formation of the so-called corona on the surface of NMs and its modification over time are poorly understood. Together, all of these environmental processes may alter the NMs, leading to very different physical–chemical properties of aged or released material compared with the original manufactured form. Furthermore, the surface coatings or the development of coronas may alter the bioavailability of NMs [1,2]. This creates increased uncertainty when the results of research conducted with as-manufactured NMs are used to predict behavior and effects in the environment.

There are also concerns about which aquatic ecosystems and compartments will be most at risk from NMs. For traditional chemicals, the regulatory testing strategy usually starts with aquatic tests in the water column [3,4]. However, because of the settling behaviors of particulates, benthic organisms and sediment systems are more likely to be exposed. Modeled average sediment concentrations of NMs often are several orders of magnitude higher than in the overlying water [5]; for example, the average concentration of carbon nanotubes in surface waters have been reported to range from 10^{-3} µg/L to 10^{-5} µg/L, whereas the concentrations in sediments were reported to be 1 µg/kg to 1 mg/kg, although these units are not directly comparable. One might argue that benthic organisms especially, and those in the water column, have evolved in the world of natural colloids and other particles. However, the unusual chemistries, reactivities, and shapes of engineered NMs may present different hazards. Natural colloids are also critical to many fundamental biological processes (e.g., biofilm formation, biocrystallization), and how engineered NMs modify these biological foundations of ecosystem function is poorly understood.

Currently, knowledge of biological effects in the aquatic environment is skewed toward studies on as-manufactured NMs in aqueous acute tests using pelagic organisms. This is clearly demonstrated by recent literature searches using the Web of Science (Table 1). Although more than 900 000 hits were recorded using *nano** as a search criterion, most published literature included the term *water*, with about 31 times fewer papers addressing sediments (Table 1). Clearly, only a small fraction of the published research involves sediments (Table 1). A comparison of hits using the terms *accumulation* or *effect* together with *nano* showed that there is a significant bias toward effect studies (20 times more).

Table 1. Literature search on nano-related published literature using Web of Science[a]

Search words	Hits
Nano*	952 650
Nano* water*	119 143
Nano* sediment	3876
Nano* effect*	291 579
Nano* effect* water	40 624
Nano* effect* sediment	575
Nano* accumulat*	13 616
Nano* accumulat* water	1969
Nano* accumulat* sediment	222
Organism groups	
Nano* alga*	3266
Nano* daph*	667
Nano* fish*	2314
Nano* benth*	323
Nano* benthos*	32
Nano* invertebrate*	369
Nano* polychaet*	59
Nano* oligochaet*	33
Nano* mussel*	533
Nano* snail*	190

[a]The search was conducted on 8 June 2015. Different search words are listed along with the number of published studies (hits) fulfilling the specific search criteria. An asterisk (*) refers to the end of the word being unspecific.

Environmental Fate of Nanomaterials

- The fate of nanomaterials (NMs) in the aquatic environment depends both on their physical–chemical properties and the characteristics of the receiving environment (pH, temperature, NOM, salinity, etc).
- The NMs may interact with naturally occurring particles, which likely modify the NM surface (e.g., creating a corona), thus providing the NM with modified physical–chemical properties that likely alter their fate and bioavailability.
- Because of the settling behavior of NMs, benthic organisms are likely to be exposed to a higher degree than pelagic organisms.

There is a need for studies on environmentally modified (aged/weathered) NMs, long-term chronic effects, bioaccumulation, and exposure of benthic organisms.

Furthermore, most published papers seem biased toward pelagic organisms, with fewer studies on benthic organisms. Of the benthic studies, the freshwater oligochaete *Lumbriculus variegatus* and insect larva *Chironomus riparius* and the estuarine polychaetes *Capitella teleta* and *Nereis diversicolor* have been the focus of some sedimentary studies [6–14]. Another Phylum that has been the focus of an increasing number of studies (although still in very low numbers) is the Mollusca, with the freshwater snails *Lymnaea stagnalis* and *Potamopyrgus antipodarum* and the marine mussel *Mytilus* spp. being the main taxa [15–17].

The number of studies on environmentally modified (*aged*) NMs, long-term chronic effects, bioaccumulation, and exposure of benthic (sediment) organisms is substantially fewer. It is recognized, however, that these studies are urgently required, to provide a comprehensive understanding of the potential effects of NMs after release into the natural environment. Moreover, the behaviors of NMs (e.g., dissolution, agglomeration) and their potential to cause artifacts in standard aquatic toxicity tests suggest that standard tests will likely need to be modified to test for potential ecological effects of NMs.

The European Union–United States (EU–US) Communities of Research were established in 2012 to provide a platform for scientists to develop a "shared repertoire of protocols and methods to overcome nanoEHS research gaps and barriers" [18]. The overall goal of the Ecotoxicity Testing and Predictive Modeling Community of Research (Ecotox Community of Research) is to encourage the evolution of hazard assessment methods and predictive models built on the foundations of fundamental research characterizing the fate (including aging) of NMs in different environmental compartments and the interactions of NMs with biota and ecosystems; knowledge of the state of the art of bioaccumulation, effects, and mechanisms and conveying this information to relevant stakeholders; and communication among regulators, experimentalists, and modelers to present the data or make them available in a useful format to help modelers, experimentalists, and risk assessors [18]. Based on ongoing work in the Ecotox Community of Research and 3 Ecotox Community of Research workshops (2013–2015), we provide an overview of the state of the art of NMs in the aquatic environment and discuss the challenges that lie ahead by providing suggestions for future research needs that will enable us to reduce uncertainty in ecological risk assessment and thus improve the quality of NM risk assessment.

The present study builds on our current understanding of as-manufactured NMs in addressing different research questions with a focus on ecotoxicological test systems and the challenges faced when assessing NM hazards (e.g., uptake routes, bioaccumulation, toxicity, test protocols, and model organisms), highlighting the main knowledge gaps, the challenges, and suggestions on how to focus future research.

Challenges in Aquatic Toxicity Testing of Nanomaterials

A key challenge in aquatic toxicology testing of NMs is that exposure often is not constant because particle settling and other transformations typically occur during the tests. In addition, methods to characterize and quantify NMs in experimental media and in environmental samples are time consuming, may require specialized equipment, or may not yet be available for complex matrices (e.g., sediment), thus creating significant uncertainty when trying to relate dose and organism response [19]. There may also be differences in results among laboratories, given that the dispersion methods used often vary (e.g., probe sonication or stirring in water) and there are many different forms of the same NM (e.g., graphene, graphene oxide, few layer graphene) that can be produced by different synthesis methods. Ecotoxicity testing of conventional chemicals, where there is adequate understanding of the contaminant fate and behavior, can often keep a reasonably constant exposure concentration throughout the bioassay. This is in clear contrast to the testing of particulate contaminants in general and NMs in particular. Furthermore, traditional aquatic testing often relies on steady mass concentrations of the test substance over fixed exposure times to deduce the exposure dose (i.e., concentration × exposure time = dose). This simple so-called two-dimensional approach may be problematic for use with NMs [20]. For example, in a mesocosm test with benthic and pelagic species, settling may result in increasing exposure concentrations for benthic species and decreasing concentrations for pelagic species. Such problems not only are of scientific concern but also have practical implications for testing strategies; for example, excessive aggregation might invalidate or limit the use of tests for screening high

Key Challenges in Testing and Assessing Nanomaterials

- Exposure is often not constant.
- Nanomaterials (NMs) are likely to agglomerate/aggregate after introduction to aqueous media and thus settle out of solution, resulting in a reduced aquatic concentration and increased sediment concentration.
- Nanomaterials undergo surface modifications (e.g., environmental corona development), which provide them with a new physical–chemical so-called identity, thus affecting fate and bioavailability over time.
- Methods to characterize and quantify NMs in experimental media and environmental and biological samples are time consuming, may require specialized equipment, or are not available for complex matrices (e.g., sediment).
- Artifacts may cause inaccurate results; thus, careful planning of control experiments is necessary.

concentrations of NMs. There also has been much discussion about dose metrics and whether to continue to use mass concentration for NMs or some other metric such as surface area or particle number concentration. However, there are examples in the literature illustrating both the classic concentration responses and nonmonotonic relations with NMs [19,21]. When possible, depending on sampling and analytical considerations, it may be useful to quantify NM concentration, particle number, and surface area. Furthermore, characterizing these metrics over time during a bioassay would provide insight into the integrated exposure that the organism experiences. This often proves a practical challenge because of lack of available methods.

Characterization methods

Regulatory testing requires that the concentration of the test substance is known, that its change during the bioassay is characterized, and that the exposure is confirmed by measuring the test substance in the exposure media and/or the organism. In addition, there is uncertainty about which types of characteristics of the initial material should be measured prior to and during toxicity testing. Standardized methods are available for some, but not all, NM characteristics, and each additional characterization technique raises the cost and increases the time required for the ecotoxicity test. One challenge is that there is a lack of characterization methods for detecting and quantifying NMs in complex environmental samples that are accurate, precise, and available for use in a standard laboratory (reviews of current methods are available [22,23]). For example, it is possible to detect NMs in tissues using advanced microscopic methods (hyperspectral imaging, confocal microscopy, or near-infrared fluorescence) depending on the NM properties. Electron microscopy can also provide unequivocal identification of intact NMs in tissues, and perhaps even localization/tissue distribution, but these measurements are challenging, time consuming, and expensive, and can usually only provide biodistribution information about a limited number of organisms or area of the organism. Furthermore, care should be taken when using electron microscopy only to identify NM, as artifacts are common [24,25].

There are some emerging approaches that hold significant promise for enabling these measurements but that are, at this stage, far from being standardized and widely available. One example is single-particle inductively coupled plasma–mass spectrometry (spICP-MS), an approach that has the advantage of providing a size distribution of the NMs in the tissue of interest [26]. However, such methods are limited to metal or metal oxide particles that will survive the chemical digestion processes needed to make a liquid sample for ICP-MS, and the detection of particles <20 nm is problematic with this method for some elements. Subcellular fractionation techniques may be used to examine the intracellular compartmentalization of metals administered in different forms (e.g., as metal salt and metal NMs) and can elucidate differences in handling and mechanisms of detoxification of internalized metals. The distribution of the metal among different subcellular compartments can reveal implications for cell and organism health. However, it is important to ensure that the subcellular fractionation procedure (i.e., centrifugation technique) is not altered by the presence of NMs. In addition, for metal NMs, it is often not clear whether the particulate form observed within the tissues was taken up as NM or as a soluble form, which was then precipitated in the tissues in particulate form. Although the latter is less likely, the inclusion of control experiments is important to test for this possibility [25].

Having readily available, quantitative methods for NMs in different matrices will provide insight into the potential effects of NMs. For example, linking NM exposure to organism body burden further clarified by quantitative measurements of NM distribution within the organism would likely lead to key mechanistic insights [14,27]. Furthermore, having reliable and rapid measurements of NM concentrations and transformations in different environmental media could enable more accurate characterization of the exposure dose and provide insight into the benefits of additional concentration metrics such as particle number and surface area. Although this is an important and interesting area, it does rely heavily on the availability of techniques that allow these measurements in aqueous samples. Another key area of research that would be feasible with improved analytical methods is the characterization of NM transformations and concentrations in soils and sediment. This remains a substantial research challenge for many NMs [19]. Finally, an important research area is the study of the fate and effects of NMs released from nano-enabled consumer products. Key research topics are summarized in Table 2.

Potential artifacts in nanoecotoxicity testing

One key consideration for testing the ecotoxicological effects of NMs is that they may cause artifacts as a result of their different properties and behaviors compared with stable, water-soluble chemicals. These potential artifacts and misinterpretations can occur at all stages of the testing procedure, starting from procuring the NMs (their physical–chemical properties sometimes dramatically differ from manufacturer specifications) to assessing their distribution in organisms or cells [3,4,25]. Many of these potential artifacts are illustrated in Figure 1. It may also be important to conduct control experiments to differentiate between direct toxicological effects from the NMs on the organisms and indirect effects such as nutrient depletion. Testing for NM artifacts is especially important for photoactive NMs, which may cause damage to biomolecules from light exposure during sample processing after the exposure assay is finished, and for NMs with strong absorbance or fluorescent properties that could impact assay measurements [3,4,28,29]. Including relevant control experiments (described at length in Petersen et al. [25] and also in Handy et al. [3,4]) during nanoecotoxicity testing will enhance the reliability of the data, facilitate standardization, and likely increase agreement among results obtained from different

Table 2. Key future research topics for nanomaterials (NMs)

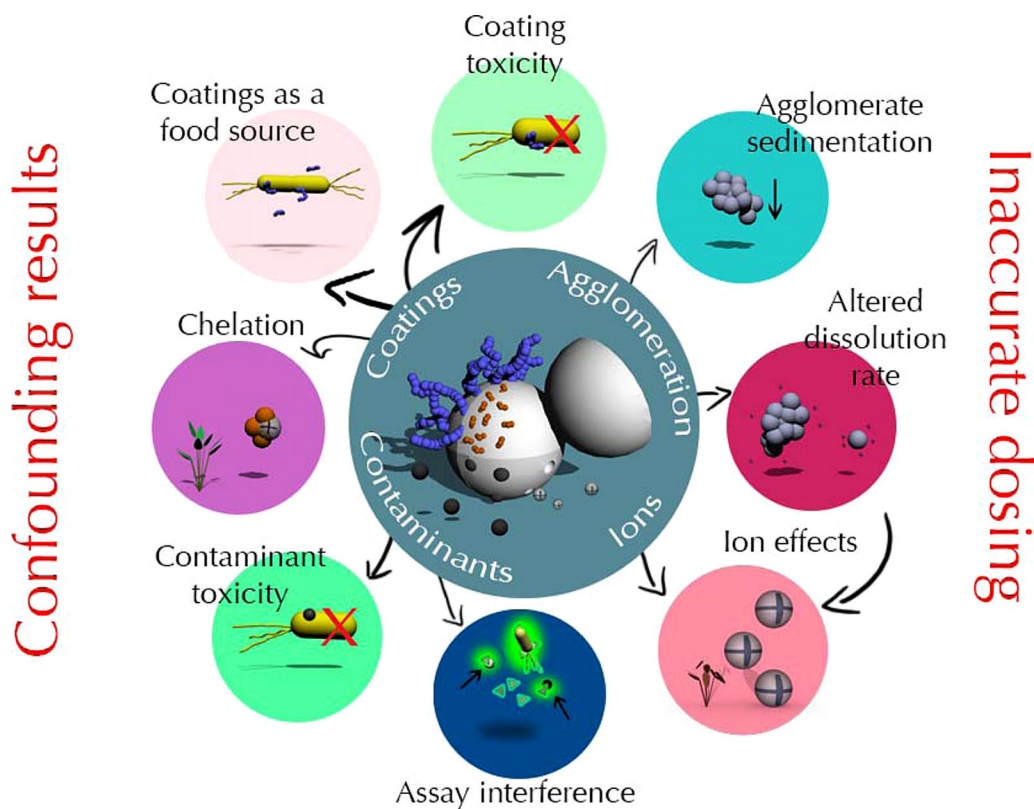| Overarching research topic	Future research areas
NM characterization in environmental and biological matrices	Continue developing characterization methods to analyze as-manufactured, aged, and weathered NMs in relevant environmental matrices, but especially for soils and sediments); however, a consensus has not been reached on how to prepare and test aged or weathered nanoparticles. These methods should be accurate, precise, and available for implementation in a standard research laboratory.
NM transformations in the environment	Environmental modification of NMs may affect their stability and fate after introduction to the natural environment. Differences and fluctuations in natural parameters such as salinity, ionic strength, organic matter, pH, temperature, and food availability, which undergo seasonally and yearly fluctuations, will affect corona development (both environmental and biologically mediated), for example, which may affect NMs' environmental fate (including the distribution between water and sediment compartments), thus affecting which organisms are at most risk for NM exposure. For metal NMs, mineralization or dissolution may lead to their removal from the water column, as would sedimentation. We therefore encourage studies characterizing changes to the NM, such as agglomeration, dissolution rates, corona formation, and reprecipitation both in the laboratory (i.e., in defined test media, and during the tests when the organisms are present) and in different aquatic environments (e.g., freshwater, estuarine, marine).
Alternative methods to assess exposure	Because of the challenges associated with quantifying NMs, and thus establishing exposure in complex media, it may be possible to instead determine exposure by measuring the test substance in or on the organism (e.g., body burden values), or by quantifying biomarkers of exposure. For body burden values, it is highly recommended to make similar measurements of ionic or bulk particle treatments for comparison and to use the same exposure concentration (or dose). Measurements of biodistribution of the NMs (and ionic and bulk particles if used for comparison) are highly desirable because NMs may not readily pass through the epithelial tissues in the gut tract or the surface skin or may be slower to absorb or adsorb compared with dissolved chemicals. A weight of evidence is needed employing different NMs and organisms to confirm the applicability of simple body burden measurements for NMs as a means to assess exposure by examining the theoretical basis (e.g., uptake mechanism, rate-limiting steps) that define accumulation. An alternative to measuring the NM in and on the organism is to determine its presence indirectly from biological responses of the whole organism, or key target organs/cellular compartments.
Influence of exposure scenarios on bioavailability and toxicity	Although it is well known that NMs will be transformed in the environment, the impact of long-term transformation processes on nanoecotoxicity has generally been less frequently studied. Some standardized test methods employ short-term exposures (e.g., 24–48 h), but these methods are not designed to detect delayed and chronic effects. We therefore recommend assessment of the influence of duration of exposure, including aging and development of environmental corona, and thus the relation between acute and long-term effects, for fate, bioaccumulation, and effects of NMs. Standardized test methods for chronic exposures could potentially be used, but modifications for NM testing would be needed.
Development of more realistic bioassays	For regulatory testing, exposure to traditional chemicals has mostly been via water, whereas the weight of evidence in the environmental risk assessment of NMs might suggest that sediment testing is most critical when the NMs are not stable in suspension. We therefore recommend rethinking the overall testing strategy for NMs to place more consideration on sediment tests and organisms that may be more appropriate for this mode of uptake compared with the base set of acute aquatic tests (algae, Daphnia, fish), although care should be taken to include water exposure as well when assessing toxicity to determine the most sensitive species. Increased realism should be considered through the use of micro/mesocosms and by including nano-enabled products in the mesocosm setup. Despite the challenges that typically are associated with mesocosm experiments—such as interpretation of results (i.e., multiple factors interacting, proper controls)—these studies can provide information regarding the impact of NMs and nano-enabled products on the interactions among organisms of different trophic levels or potentially trophic transfer. Food chain transfer studies that can be assessed using simpler experimental designs compared to the mesocosm setup, albeit substantially more complex than single organism testing, are encouraged to measure the transfer of NMs along a single food chain.
Uptake, internal distribution, and depuration of NMs	The majority of published data have reported total body burden; significantly less has been published on uptake and depuration kinetics and NM transformation and distribution in the organisms. Moreover, the mechanisms of translocation should be documented if uptake occurs. The impact of gut fluids and molecules on transformations and biodistribution of NM also should be studied. More work needs to be done to refine bioaccumulation tests to reflect exposure to particulate material rather than dissolved ions.

FIGURE 1: Potential artifacts in nanoecotoxicology testing. This schematic is intended to show the ways in which contaminants in the nanomaterials (NMs), release of dissolved ions, NM agglomeration, interactions between the organism and NM coating, or interference from the NM with the assay measurement (i.e., absorbance) can potentially cause inaccurate dosing or artifacts in nanoecotoxicology assays. Reprinted with permission from the American Chemical Society [25].

laboratories. Some control experiments include testing the potential effects of ions for NMs that dissolve in water, filtrate-only controls to test the potential impact of toxic impurities (e.g., metal catalysts on carbon nanotubes), testing of the same core materials of a larger size, and a coating control to assess whether the coating could have a toxic or stimulatory impact.

What Parameters to Measure and Report

One helpful step that will likely increase the reliability of nanoecotoxicology test results is to standardize the supporting measurements and data reporting. Some suggestions along these lines are provided in standard ecotoxicology methods for soluble, stable chemicals. For example, many Organisation for Economic Co-operation and Development (OECD) standard aquatic toxicity tests require measurements of the concentration of the chemical compound at the beginning and end of the experiment (e.g., OECD method 202, *Daphnia* sp. acute immobilisation test and reproduction test). The specification for many of these tests is that the concentration of the test substance should change by less than 20% (OECD) or 30% (International Organization for Standardization [ISO] and US Environmental Protection Agency [EPA] methods) during the exposure period. Thus, measuring the NM concentration at the beginning and end of an experiment is suggested as a minimum frequency. However, as described in the section *Challenges*

in aquatic toxicity testing of nanomaterials, quantitative measurements of NMs in water may be challenging, especially in the presence of natural organic matter or cellular organisms such as algae. In addition, NMs may undergo various changes during the aquatic toxicity test period (dissolution, agglomeration, etc.). Although it is well known that NMs will be transformed in the environment (e.g., oxidation of carbon NMs), the impact of long-term transformation processes on nanoecotoxicity results generally has been studied less frequently. One exception to this is the sulfidation of silver nanoparticles (AgNPs). This process occurs during transit through wastewater treatment plants and has been shown to dramatically decrease AgNP toxicity [30]. Monitoring these changes is even more complex in sediments as a result of analytical difficulties. Environmental modification of NMs may increase their stability in water, such as when graphene is oxidized [31]. Alternatively, for metal particles, mineralization or dissolution may also lead to their removal from the water column. Therefore, characterizing changes to the NM, such as agglomeration or dissolution rates in the defined test media, and during the tests when the organisms are present, may be critical to understanding the exposure and thus the subsequent toxic effect. Chemical oxidation and other phenomena related to particle stability also raise the issue of what aspects of the test media should be monitored. Often in traditional aquatic toxicity tests, the water measurements are restricted to pH, dissolved oxygen, and the general ionic composition and hardness of the media. However, other measurements may be

justifiable for NM tests. For example, would the measurement of redox potential or sulfur compounds give an accurate understanding of what chemical form organisms are being exposed to during a test with AgNPs? Would such additional measurements be justified in terms of time, cost, and resources for a regulatory test?

Quantifying changes to NMs in sediments during ecotoxicity experiments remains especially challenging. Currently, methods for characterizing exposure are limited to measuring total metal concentrations when metal-containing NMs are used. Often, the particle size distribution and changes in such distribution as a result of dissolution or aggregation processes cannot be measured readily in soil or sediment because of the large background of naturally occurring particulates. However, a thorough characterization of the sediment characteristics (organic matter concentration, particle size, etc.) used in nanoecotoxicity testing is important and considered critical for future modeling efforts. The debate concerning the use of standard artificial sediments (according to OECD protocols) versus natural sediments continues. The latter confer additional reality to the tests and also allow for results to be more widely applicable. The use of standard artificial sediments, however, facilitates laboratory comparability, and this line of thought is not different for NMs compared with hazard testing of conventional chemicals [3,4].

Which model organisms to use

Rapid agglomeration and settlings of some NMs suggests that testing pelagic organisms may have less environmental relevance than testing benthic organisms. Although all pelagic organisms will be exposed to NMs and their transformation products in the water column, the group of filter feeders (e.g., *Daphnia magna*) will be exposed to NMs and their agglomerates in the water column while filtering water for food. For animals that breathe in water, the gills or other respiratory surface are vulnerable to chemicals because of the anatomical features that enable respiration to occur, including a large surface area, small diffusion distances to the internal body fluid (e.g., blood), and high blood flow (perfusion of the respiratory surface). This vulnerability also applies to NMs. Another consideration is mechanical suffocation (nonchemical toxicity) in aquatic organisms; however, measurements to quantify this effect are not currently included in regulatory tests. Benthic species (both epi- and infaunal) will be exposed either via direct body contact with sediment-associated NMs (i.e., bound to sediment particles, from porewater and overlying water while irrigating) or through ingestion of settled NMs associated with the sediment, biofilms, or other food sources. For regulatory testing, these issues are pragmatically framed around the notion of exposure routes (water, food, sediment) for traditional chemicals, and the weighting of evidence in the environmental risk assessment might be more toward the results of (for example) sediment testing where effects on the benthos are a concern. For NMs, the overall testing strategy may need to be adjusted so that more consideration is given to soil/sediment tests compared with the base set of

acute aquatic tests (algae, *Daphnia*, fish [4]). However, such thinking is based on nearly 100 yr of epithelial biology where substances are taken up by ubiquitous active solute transporters, facilitated diffusion, or passive diffusion depending on the membrane biology, water permeability, and anatomy of the biological barrier/organism. This has arguably led to a selection of regulatory test organisms in which these features are well known. However, NMs bring new challenges to epithelial biology. Most materials are too large to use solute transporters or simple diffusional processes, and internalization via endocytosis and related mechanisms has not been documented. However, with the huge diversity of biological barriers in the animal kingdom alone, there is no guarantee that the traditional test organisms used in regulatory ecotoxicology are the so-called best or most representative organisms to use to account for this mode of uptake. Current legislation is geared toward protecting most of the organisms most of the time; and without a biological barrier or uptake information on NMs across a range of phyla and life stages, we may not achieve this with our current test organisms or bioassays. Work on marine species and other organisms currently not used in regulatory ecotoxicology is needed to identify vulnerable anatomical features or groups of organisms.

Using the Organisms to Measure Exposure

The difficulty in measuring NMs in exposure media and complex environmental matrices has already been discussed

Overall Considerations and Suggestions Related to Improving Nanomaterial Ecotoxicity Testing

- The overall testing strategy may need adjustment so that more consideration is given to:
  - sediment systems compared with the base set of acute aquatic tests (algae, Daphnia, fish), although care needs to be taken to compare nanomaterial (NM) sensitivity between pelagic and sediment-dwelling organisms; and
  - more complex ecotoxicity testing, such as long-term chronic exposure, increased environmental realism (e.g., mesocosms), and testing with aged/weathered NMs.
- Acknowledging the challenges associated with confirming exposure, alternative or complementary approaches could be used to estimate exposure, such as by measuring organism NM body burdens or by biological response assessment. Both of these approaches require implementation of a reference substance, such as the ionic form of NMs that dissolves or a larger or different shape of the particulate form of the same chemical substance.

in the 2 previous sections, yet regulatory tests require some confirmation of the exposure. Of course, for traditional chemicals, an alternative approach is to define the exposure by measuring the test substance in or on the organism (e.g., apparent bioaccumulation, net uptake) or by quantifying biological responses that are well known to be associated with the exposure (i.e., biomarkers of exposure). The following sections explore these 2 approaches and whether they can be applied to NMs.

Confirmation of exposure through body burden assessment

Bioaccumulation terminology for dissolved chemicals may be misleading for NMs. There are several important differences between uptake of NMs and traditional dissolved chemicals that complicate usage of the same terminology. Mainly, the uptake of NMs does not reach a steady-state equilibrium condition, and concepts that rely on steady-state concentrations (ratios) between the external compartment and the organism (i.e., bioconcentration factor, biota sediment accumulation factor) are in most cases not appropriate for use with NMs unless caveats are included to clearly distinguish the difference from traditional dissolved chemicals [3,4,19]. Instead, terms such as body burden, which do not make assumptions about equilibrium being reached, or the biodistribution in the organism, are encouraged. Overall, this is an area where consensus has not yet been reached in the nanoecotoxicology field. However, a prerequisite for regulatory use would include defining a test or measurement that is analogous to the concept of bioaccumulation for dissolved chemicals. While almost all studies on this topic have demonstrated a lack of NM absorption across epithelial cells, a study with *Drosophila melanogaster* fed with single-wall carbon nanotube–spiked food showed that only a small fraction (10^{-8} of the total dose of ingested nanotubes) was translocated to other tissues in the organism [32]. Overall, NMs do not readily pass through the epithelial tissues in the gut tract or the surface skin [33], or may be slower to absorb compared with solutes, so further work on the time scales of such tests will be needed. Wray and Klaine [34] examined the influence of particle characteristics (AuNP surface charge, size, and shape) on total body burden in *D. magna* and found no evidence that AuNPs were absorbed across epithelial membranes, a result similar to other studies with carbon nanomaterial [23,35,36]. These authors discuss the possibility that a part of the ingested NPs may adsorb to gut structures (e.g., microvilli) and that these have a slower transport out of the gut compared with NPs that are not in contact with gut structures. In any case, clear terminology should be used so that such measurements for NMs are not confused with those for soluble chemicals with very different properties and biokinetic principles. Moreover, NMs may undergo surface transformations in the gut (e.g., coated with a protein corona), with implications for uptake and depuration kinetics in predator organisms. However, only a few studies have been published on trophic transfer [37], so more information is required to address this question.

Body burden assessment. Although bioaccumulation constitutes an important part of risk assessment, there is not much information in the literature on NM bioaccumulation. Most of these studies have reported total body burden after the conclusion of the experiment, while only a limited number have focused on uptake and depuration kinetics and NM transformations in the organisms [8,9,14,16,17,27,36,38,39]. Most likely as a result of limitations in availability of analytical methods and instruments, even fewer studies have been published on internal distribution of NMs after exposure [6,24,35,40], or on trophic transfer [27,37]. A weight of evidence is needed with different NMs and organisms to confirm the utility of simple body burden measurements for NMs and the theoretical basis (uptake mechanism, rate-limiting steps, etc.) that define the validity or utility of the approach.

Use of reference substances in body burden–related assessments for NMs. One approach that has been used to determine the NM component of ecotoxicity for NMs is to compare toxicity results from NM exposure with the toxicity of the ionic form for NMs that dissolve or of a larger bulk form (e.g., micron scale) of the same chemical substance. This approach provides a means to compare bioavailability and toxicity of NMs with the conventional form of the same chemical substance. Some studies have observed nano-related effects (including both effects on different endpoints and more pronounced effects on the same endpoints) at the whole-body level and at the subcellular level, whereas other studies have shown higher toxicity from the bulk or ionic form (see Mouneyrac et al. [41,42] for examples on metal NPs in sediment systems). In trout, for example, the target organs for nano-Cu are broadly the same as for $CuSO_4$, but the rate of appearance and severity of organ pathologies may be different [43,44], and toxicity may be at least partly caused by dissolved ions for NPs that dissolve during the test period.

In principle, the reference treatment does not need to relate just to the chemical substance (e.g., dissolved vs particulate), but could be extended to the different forms (crystal structures of the same chemical), sizes, and shapes of NMs. In an aquatic water column test, or cell culture media, such reference substances may be less difficult to measure. The matrix of soils or sediments presents a difficult challenge (for the reasons described in the previous 2 sections). However, if we move our thinking away from the test media to the organism itself, measurements may be less problematic (decreased particulate background noise within the organism compared with sediments). A body burden test system with reference chemicals or treatments would require some consistency in the exposure dose. The same concentration of the compound should be included in all treatments. For these types of experimental setups, different forms of well-defined test substances (e.g., NM, bulk, ionic metal, different NM sizes and shapes) will be needed so that concentrations may be reliably compared. For example, the use of mass concentration (e.g., mg/L) of a metal may require correction for surface coating (oxide formation) or the presence of organic matter that changes the molecular weight of the

primary particle. These are not minor considerations when the organic surface coating on a 20-nm metal particle might occupy 30% or more of its mass. Interestingly, gut epithelial cells can distinguish between crystal structures of the same NM and selectively take up certain crystal forms (e.g., of titania [45]). How and why this occurs is unclear, but it raises the concern that risk assessments may need to consider crystal structure as well as size when exploring the bioaccumulation potential of NMs.

Confirmation of Exposure Through Biological Response Assessment

Internal distribution in organisms and biomarkers of exposure

The alternative to measuring the test substance itself in and on the organism is to determine its presence indirectly from biological responses of the whole organism, or preferably key target organs/cellular compartments. Such ideas are well established for soluble chemicals. For example, the liver is a central compartment for the metabolism of organic chemicals, where chaperone molecules serve to modulate metal concentrations in the blood and inside cells. However, to use biomarkers of exposure for NMs, at least 2 fundamental pieces of information would be needed: First, where does the NM go inside the organism (choice of target tissue/cells)? Second, what does it do when it gets there that provides a unique biological signal of the presence of the material? The former is dogged by the ever-changing corona on the surface of the NM, dissolution, and reprecipitation (e.g., in the gut) and how this might influence uptake and biodistribution. In sediment tests, for example, it might be expected that the NM corona and speciation will alter in the sediment matrix, leading to measurable differences in bioavailability. Increasing evidence suggests that metal NMs are available for uptake via the dietary route of exposure (diet and sediment) and that sediment-dwelling organisms may accumulate metal NMs. However, the digestive anatomy (chemical environment of the gut) is well known to alter the uptake kinetics of metals and organic chemicals. The effect of the gut lumen chemical environment on corona formation, dissolution, and reprecipitation on NMs also needs to be studied. This cannot be done in isolation from the mechanical anatomy of the gut, as some of this biology is specifically designed for sorting food by particle size. For example, polychaetes have a conveyer-belt feeding manner whereby all particles are transported through the worm and defecated. Mollusks, in contrast, have an internal sorting mechanism in the gut and digestive diverticula whereby smaller particles will be retained in the digestive gland and larger particles will be transported in the intestine. The underlying science for understanding the relation between particle size and digestive physiology for accumulation is poorly developed, and our ability to predict ecological consequences of different NMs is therefore limited. Similar information is needed for fishes and other vertebrate animals.

However, a prerequisite is to understand what corona forms in the exposure media, then in the mucous epithelia of the organism (uptake surface), and then the blood (extracellular fluid) and the tissues (intracellular environment), as well as how this changes over time (degradation/dissolution) within each of these compartments. For fish, NMs might also adsorb to the outside of the gill, and so a measurement of these tissues might provide a more relevant exposure concentration, even if a bioaccumulation parameter cannot be determined.

Determining a biological signal that indicates the presence of NMs may be less problematic from the perspective of an analytical biochemistry challenge. Biomarkers are often geared toward the mechanism of toxicity (biomarkers of oxidative stress, ionoregulatory disturbance, etc.), not the physical form and shape of the material. Nonetheless, modifications of existing biomarker screens could include the use of phagocytosis and endocytosis-related assays to confirm the presence of particles [3]. Some information exists suggesting that subcellular endpoints, especially oxidative stress, may be more sensitive for NMs than other more conventional contaminants. For example, Cong et al. [46] reported that sediment-associated AgNPs did not impact whole-body endpoints such as mortality and growth in the polychaete *Nereis diversicolor*, whereas subcellular endpoints were more responsive (e.g., lysosomal damage, DNA damage determined using a comet assay). A limiting aspect for biomarkers is crystal structure and particle shape is that our understanding of biocrystallization and how cells sense crystals is far from adequate for toxicological applications.

Incorporating Increased Environmental Realism in Nanoecotoxicity Testing

Although most ecotoxicity studies with NMs have examined the impact on individual organisms, alternative approaches such as mesocosm studies can provide a more complex system, which better simulates the environment [37,41]. These studies can provide information regarding the impact of NMs and consumer products containing NMs on the interactions among organisms of different trophic levels or potentially trophic transfer [47]. However, a limitation of mesocosm studies is that it can be challenging to unequivocally interpret the results as a result of the complexity and multiple factors interacting. In addition, it is often challenging to quantify NMs in the complex matrices (e.g., sediment) that are typically present in mesocosm experiments. It is also possible to study food chain transfer in simpler experimental designs, albeit substantially more complex than single organism testing, by measuring the transfer of NMs along a single food chain [27].

Furthermore, most NM tests to date have been conducted using NM synthesized in-house or procured from the manufacturer. For example, Natalio et al. [48] tested the impact of paint with and without vanadium pentoxide (V_2O_5)
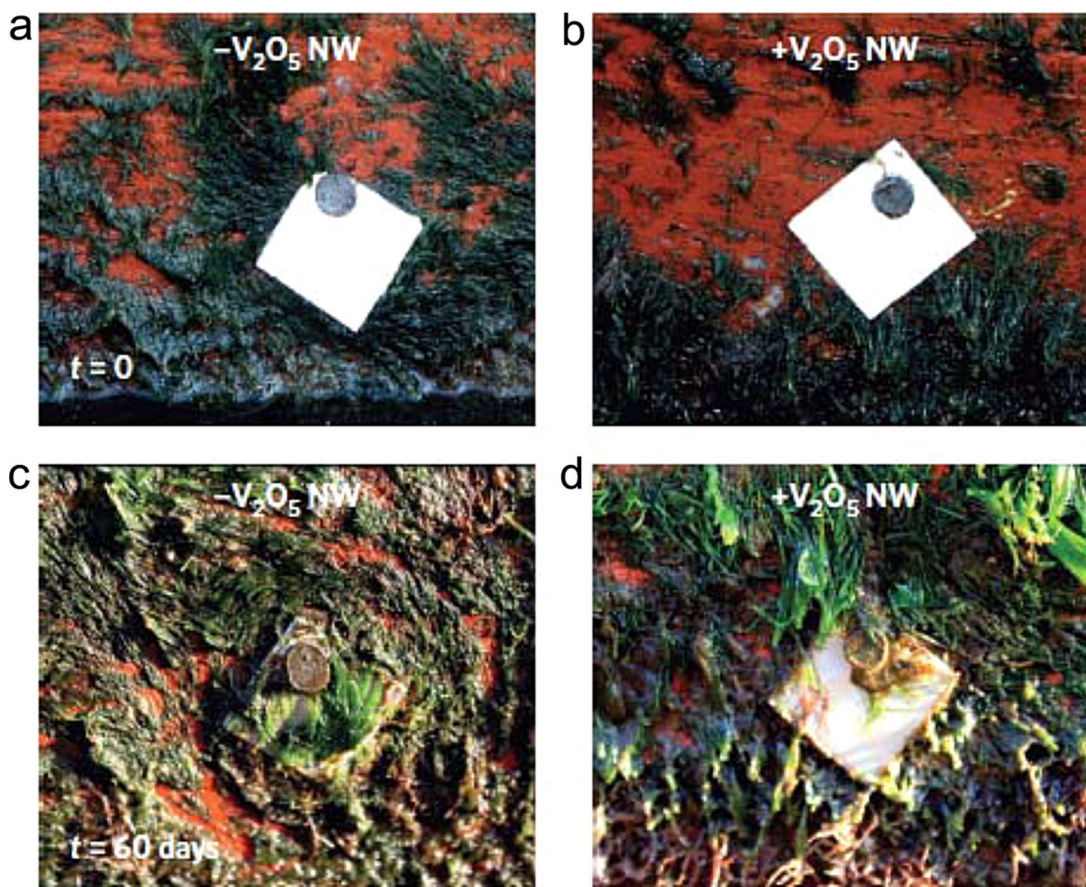
FIGURE 2: Effect of nanoparticles on biofouling in situ [48]. Digital image of stainless steel plates (2 cm × 2 cm) covered with a commercially available paint for boat hulls (a) without vanadium pentoxide nanowires ($-V_2O_5$ NW) and (b) with vanadium pentoxide nanowires ($+V_2O_5$ NW) immediately after fixation ($t=0$; top row) and after 60 d ($t=60$; bottom row). The painted stainless steel plates with no V_2O_5 NWs suffered from severe natural biofouling (c) whereas biofouling was complete absent on plates with V_2O_5 NWs (d). Reprinted with permission from *Nature Nanotechnology* [48].

nanowires on antifouling on boat hulls (Figure 2). Although approaches like this have resulted in significant increases in the scientific understanding of the potential effects of these materials in the aquatic environment, assessing the impact of NM aging and transformations on their toxicity requires more research. It is also important to consider the form in which NMs will actually be released into environmental compartments from consumer products. Carbon nanotubes, for example, may be partly encapsulated by polymers if they were released from a polymer nanocomposite [49,50]. Thus, the form that may reach the environment after usage or disposal of consumer products may differ from that which is most frequently tested by scientists. However, the exact form of the released particle may differ based on the product application, and information about the nanoparticle by itself remains valuable for assessing the potential impact of NM spills. In addition, there have been few measurements of NMs in field samples, and it is thus challenging to know exactly what form is present at the highest concentration in the environment. This raises questions concerning mesocosm simulations: What is the realistic test concentration? What is the form we should test (i.e., aged, with/without corona, size, mono-/polydispersed)? Should we apply NMs to the water and then follow them to the sediment and eventually to the food chain? Will a freshwater, marine, or estuarine system be

the most realistic test scenario, or do we need all 3, as they each represent unique chemical–physical parameters as well as biological components? A discussion of the appropriateness of this type of mesocosm setup for NMs is needed, and careful consideration should be given to these factors when mesocosm studies are designed and performed. Additional research is needed to test the ecotoxicity of NMs released from consumer products (e.g., Figure 2) [48], and this is now starting to take place [47].

Putting it all Together Through Nanocategorization and Modeling

There is a strong desire to find categories that can be used to group NMs [51,52]. This would enable risk assessment of an NM with unknown toxicity using fate and hazard data determined for other NMs in the same group, a process that could be similar to read-across and grouping strategies for dissolved chemicals. There is still much debate regarding grouping and categorization of NMs, and at this point there is no agreement. Categorization of NMs has recently gained traction for use with human health toxicity [53–55] but has not yet been developed to the same extent for ecotoxicity,

although some inroads have already been made in the environmental area [56]. The progress continuously being made in this area, together with the development in NM quantitative structure–activity relationships, can support the development of safe products such as through Safe by Design [57].

Where to Focus Future Research to Reduce Uncertainty in Ecological Risk Assessment

Validated bioassays, hazard assessment tools, and especially predictive models remain to be developed and tested for NMs. Although we have learned much over the last decade, it is still critical that underpinning research continue to be conducted that explores the fundamental principles defining the consequences of the interactions of NMs with biota (e.g., bioavailability, internal deposition, deleterious effects, and bioaccumulation). Because of the complexity of nano-research, efforts should take an interdisciplinary approach to move the research forward and should be founded on current and emerging research needs (e.g., follow technology and production closely).

An enhanced understanding of the underpinning science will lead to more environmentally realistic and implementable approaches ensuring the safe use of NMs and thus the potential benefits of products of nanotechnology. Our specific recommendations for future research areas are centered around 6 main topics (Table 2): 1) NM characterization in environmental and biological matrices; 2) NM transformation in the environment and consequences for bioavailability and toxicity; 3) alternative methods to assess exposure; 4) influence of exposure scenarios on bioavailability and toxicity; 5) development of more realistic bioassays; and 6) uptake, internal distribution, and depuration of NMs. Based on our current understanding of fate and effects of as-manufactured NMs, we recommend studying the effects of aged and weathered NMs, as-manufactured NMs, and NMs released from consumer products when addressing these 6 topics, which are further described in Table 2. It should be noted that the term *weathered* needs to be properly defined, as it currently is interpreted differently among stakeholders in this field. Although testing the effects of as-manufactured NMs is the most straightforward (albeit still challenging), testing the effects of particles released from consumer products or those altered in the environment is more environmentally realistic. Research addressing these key topics will reduce uncertainty in ecological risk assessment and support the sustainable development of nanotechnology.

Data availability

The present study is not built on datasets but on published literature.

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Recommendations for Overarching Research Topics to Reduce Uncertainty in Nanomaterial Environmental Risk Assessment

Emphasis should be placed on studying the ecological effect of aged and weathered nanomaterials (NMs), as-manufactured NMs, and NMs released from consumer products in addressing the following:

- characterization and quantification of NMs in environmental and biological matrices;
- transformation of NMs in the environment and consequences for bioavailability and toxicity;
- the development of alternative methods, from conventional ones, to assess exposure;
- the influence of exposure scenarios on bioavailability and toxicity;
- the development of environmentally realistic bioassays; and
- the uptake, internal distribution, and depuration of NMs.

Because of the complexity of nanosafety research, an interdisciplinary approach is key to moving this area forward.

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