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ADAPTING OECD AQUATIC TOXICITY TESTS FOR USE WITH MANUFACTURED NANOMATERIALS: KEY ISSUES AND CONSENSUS RECOMMENDATIONS

Elijah J. Petersen[1], Stephen A. Diamond[2], Alan J. Kennedy[3,*], Greg G. Goss[4], Kay Ho[5], Jamie Lead[6], Shannon K. Hanna[1], Nanna B. Hartmann[7], Kerstin Hund-Rinke[8], Brian Mader[9], Nicolas Manier[10], Pascal Pandard[10], Edward R. Salinas[11], Phil Sayre[12]

*** Corresponding author:** Alan.J.Kennedy@usace.army.mil; phone: 601-634-3344; fax: 601-634-2263

[1] Biosystems and Biomaterials Division, Material Measurement Laboratory, National Institute of Standards and Technology, Gaithersburg, MD, USA
[2] NanoSafe, Inc, Midwest, Duluth, MN, USA
[3] US Army Engineer Research and Development Center, Environmental Laboratory, Vicksburg, MS, USA
[4] Department of Biological Sciences, University of Alberta and National Institute of Nanotechnology, National Research Council, Edmonton, Alberta, Canada
[5] Office of Research and Development, National Health and Environmental Effects Research Laboratory – Atlantic Ecology Division, United States Environmental Protection Agency, Narragansett, RI, USA
[6] Center for Environmental Nanoscience and Risk, Department of Environmental Health Sciences, Arnold School of Public Health, University of South Carolina, Columbia, SC 29036, USA
[7] Department of Environmental Engineering, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark
[8] Fraunhofer Institute for Molecular Biology and Applied Ecology, Schmallenberg, Germany
[9] 3M, Environmental Laboratoary, St. Paul, MN, USA
[10] Institute National de l'Environnement Industriel et des Risques (INERIS), Parc Technologique ALATA, F 60 550 Verneuil en-Halatte, France.
[11] Experimental Toxicology and Ecology, BASF SE, Ludwigshafen, Germany
[12] formerly with Office of Pollution Prevention and Toxics, United States Environmental Protection Agency, Washington D.C., USA, phil.sayre@verizon.net

The unique or enhanced properties of manufactured nanomaterials (MNs) suggest that their use in nano-enabled products will continue to increase. This will result in increased potential for human and environmental exposure to MNs, during manufacturing, use, and disposal of nano-enabled products. Scientifically based risk assessment for MNs necessitates development of reproducible, standardized hazard testing methods such as those provided by the Organization of Economic Cooperation and Development (OECD). Currently, there is no comprehensive guidance on how to best address testing issues specific to MN particulate, fibrous, or colloidal properties. This paper summarizes the findings from an expert workshop convened to develop a guidance document that addresses the difficulties encountered when testing MNs using aquatic and sediment OECD test guidelines. Critical components were identified by workshop participants that require specific guidance for MN testing: preparation of dispersions, dose metrics, the importance and challenges associated with maintaining and monitoring exposure levels, and the need for reliable methods to quantify MNs in complex media. To facilitate a scientific advance in the consistency of nanoecotoxicology test results, we identify and discuss critical considerations where expert consensus recommendations were and were not achieved, and provide specific research recommendations to resolve issues for which consensus was not reached. This process will enable development of prescriptive testing guidance for MNs. Critically, we highlight the need to quantify and properly interpret and express exposure during the bioassays used to determine hazard values.

Introduction

The rapidly accelerating development and implementation of nanotechnology has inspired vigorous debate about the adequacy of current regulatory frameworks for assuring the safe deployment of manufactured nanomaterials (MNs) in the commercial marketplace.[1-4] A critical aspect of these debates is whether standard test protocols currently used in risk assessment are fully adequate for testing the hazard potential of MNs.[5, 6] Standardized testing protocols, and the guidance documents that describe them, are a critical component of risk assessment and regulatory processes that enable placement of chemical substances on the market. These test protocols describe specific techniques and methods for the collection and analyses of data with the goal of quantitatively describing, under controlled laboratory conditions, the release, fate, transport, transformation, exposure, and toxicity of chemical substances. The Organization for Economic Cooperation and Development (OECD) has promulgated internationally-accepted test guidelines (TGs) that are used for these purposes. A subset of these TGs focus on toxicity in aquatic, sediment and soil organisms and constitute the OECD's Test Guidelines Section 2: 'Effects on Biotic Systems'.[7-10]

Several recent publications focused on aquatic and sediment ecotoxicity assay methods commonly used in regulatory testing suggest that these methods are generally adequate for testing but MNs, but discuss the need for additional guidance to improve their applicability for hazard assessment of MNs.[8-14] The critical issue is that aquatic ecotoxicity testing with MNs involves exposure of test organisms to colloids or particle-sediment mixtures rather than solely to dissolved chemicals for which the OECD TGs were originally intended. MNs in test media typically undergo extensive agglomeration, settling, particle dissolution, and transformations during exposure and media renewal periods.[9, 15] These transformation processes depend, in part, on the MN intrinsic properties, MN concentrations, and media composition. The resulting variability in exposure presents unique challenges for exposure-response estimation. Alternate dose metrics based on particle number, surface area, or body burden, in addition to mass concentration, might be informative; however, metrics other than mass concentration are not generally considered within current risk assessment frameworks. Dissolution and ion release from MNs during testing, as often observed for silver and zinc oxide MNs,[16, 17] further complicates dosimetry, because the resulting exposures potentially involve both MNs and dissolved species. Concentration-dependent MN agglomeration, settling, and dissolution also present significant measurement and monitoring challenges, both logistically and methodologically. These MN behaviors often alter exposure levels beyond ± 20% of the initial (measured) or nominal concentration during an aquatic bioassay, a specification in many TGs hereafter referred to as the "20 % exposure specification". While MNs released from nano-enabled products may differ substantially from their as-produced form (e.g., CNTs released to the environment from polymer nanocomposites may be partly or fully encased in component polymers[18-21]), the focus in this manuscript is on as-produced MNs.

Herein, we discuss the findings of a workshop focused on drafting an OECD guidance document (GD) on Aquatic (and Sediment) Toxicology Testing of Nanomaterials, which provides necessary amendments to existing OECD aquatic toxicity test methods and is an OECD project approved in 2013. This meeting, held at the U.S. EPA in Washington D.C. in July, 2014, was attended by 23 experts from seven countries. We discuss, in depth, key limitations of current aquatic bioassay study designs for testing MNs and knowledge gaps that preclude or hinder the development of prescriptive, broadly-applicable aquatic toxicity standard tests for MNs, and suggest research to address these issues. Each of the following topics raised at the meeting are critically discussed: key considerations for testing the aquatic toxicity of MNs; the feasibility of conducting tests with MNs that meet the 20 % exposure specification; dosimetry and interpretation concerns for MNs; and challenges with testing MNs in sediments. We highlight issues where consensus was and was not reached during the workshop and subsequent discussions with workshop participants and recommend research to resolve topics where consensus was not reached. The discussions and viewpoints expressed by the workshop participants are summarized and inform, but are non-binding toward, the development of the OECD GD described above. The workgroup participants agreed to define MNs broadly as solid-phase substances having one dimension between 1 to 100 nm. While there are more detailed definitions (e.g. the European Commission-proposed definition [22]), our intent is to avoid limiting the workgroup findings to current MN definitions that may change. The more specific terminology used here (e.g. particle size, dissolution, agglomeration, aggregation, etc.) generally follow OECD documents on MNs.[23]

Key considerations related to NM aquatic toxicity testing

The importance of standard terminology

Workshop participants strongly agreed on the importance of using precise terminology when describing results from nanoecotoxicity tests. The absence of terminology in ecotoxicology TGs specific to (nano) particles, colloids, dispersions and suspensions further complicates conduct of standard aquatic ecotoxicity tests with MNs.[24] For example, MN suspensions have been erroneously referred to as dissolved MNs, rather than dispersed or suspended MNs. The operational definition of "dissolved" substances varies significantly among different fields, and there are environmental and mechanistic definitions that are partially related to the operational definitions;[25] a more detailed discussion of this topic is available in the Supporting Information. It is thus critical to make a distinction among the terms "suspension" and "dispersion" versus "solution." As the term "solution" suggests that the MNs are dissolved in the aqueous test media, the terms "suspension" and "dispersion" are favored. This is especially important as the 'true' dissolution of MNs into their component ions is an important process in environmental fate and ecotoxicology. For instance, some dispersed or suspended MNs will subsequently dissolve fully or partly to their constituent ions over the exposure time of nanoecotoxicity tests and this

must be accounted for in interpreting data. Consistent use of terminology can therefore minimize misinterpretation of reported results.

For the past two decades guidance for aquatic toxicity testing for hazard assessment has included a distinction in the terminology used to describe adverse effects. Intrinsic toxicity is derived from exposure to dissolved molecules and is distinct from adverse physical effects.[26] Physical effects can manifest as insoluble material attaching to the exterior of an organism as micelles, aggregated particles, or as a flocculent and lead to adverse effects from fouled respiratory surfaces, impaired mobility, and feeding (daphnids), or light attenuation (algae). Intrinsic toxicity is the focus of aquatic hazard assessment based on the concept that the dissolved molecule represents the most relevant exposure condition for aquatic toxicity testing and undissolved material is excluded from tests to avoid physical effects.[27, 28] Since aquatic exposures to MNs may include both dissolved and solid phases, additional effort is required to distinguish "intrinsic" toxicity from physical effects. In tests with MNs, particulate uptake has the potential to exert toxic effects which are not solely physical. Carefully designed control experiments are essential for making a distinction and avoiding misinterpretations,[29] and need to be incorporated into future work, including evaluating how and when to include the hazard from physical effects into aquatic risk assessment.

In addition, use of terms related to an 'equilibrium' being reached among multiple phases including organism tissues (i.e., bioconcentration factor, bioaccumulation factor, biota-sediment accumulation factor, etc.) is discouraged,[9] or at a minimum, need to be better qualified. Use of these terms may result in an inaccurate comparison between organism accumulation of MN and hydrophobic organic contaminants (HOCs) or dissolved metals. Bioaccumulation of HOCs is related to passage through biological membranes via passive diffusion, or active uptake through ion channels or carrier mediated transport.[30] For MNs, however, results show that absorption into organism tissues is typically limited. For example, ingestion of carbon-based MNs by aquatic organisms often leads to high ingested concentrations present only in the gut tract with non-detectable absorption into systemic circulation,[18, 31, 32] while many HOCs are concentrated in the lipid fraction of organisms.[33-36] In addition, changes in the octanol-water partition coefficients were not shown to correlate with changes in accumulation of multiwall carbon nanotubes (MWCNTs) by a benthic organism *Lumbriculus variegatus* or an earthworm *Eisenia foetida*.[37] An OECD document on sample preparation and dosimetry indicated that the OECD TG for octanol-water partition coefficients is unlikely to be directly applicable for use with MNs,[23] a conclusion also reached by others.[38]

MN behavior in test systems

The behaviors of MNs in aqueous media impact the accuracy and reproducibility of results derived from OECD ecotoxicity methods in that they are more dynamic and not predictable by traditional methods of partitioning and bioavailability. MNs are similar in concept to solid particulate chemicals or mixtures described as "difficult substances".[27] For example, MNs may agglomerate, settle from suspension and/or dissolve[18, 39] (Figure 1). Moreover, these behaviors

are greatly influenced by the test media and other factors such as the MN number concentration. Media with higher ionic strength, and especially higher concentrations of divalent and trivalent metal ions, will result in higher rates of agglomeration and MNs settling from suspension, with stabilization mechanisms playing a role.[40] Silver nanoparticles (AgNPs) provide an example of a MN that undergoes transformations in aqueous media; AgNPs may form silver chloride or silver sulfide particles if the media contains chloride or sulfur, and these modified particles can be significantly less toxic than unmodified AgNPs.[15, 41, 42] Silver nanoparticles also interact with natural organic material (NOM), oxidize, and dissolve,[15, 29] which influences surface chemistry, dissolution, aggregation and toxicity.[43-46] Formation of AgNPs from reduction of ions can also occur in aquatic media.[47, 48] Agglomeration and settling cause increased heterogeneity in the test vessel with higher mass concentrations toward the bottom of the container. The procedure used to disperse MNs in the aqueous media and the MN concentration dispersed can also impact the general dispersion stability and heterogeneity in the test container as well as the rate of agglomeration.[49] Thus, the assay results for MNs are often more sensitive to the dispersion and mixing steps than for tests with dissolved metals or HOCs. Additionally, washing procedures to purify MNs can influence chemistry and behavior where the coating is weakly bound to the MN surface.[50] All of these changes to the MN distribution could lead to inaccurate or inconsistent organism exposure.[29]

Monitoring and quantifying MN exposure

The current lack of widely available, routine measurement methods with known accuracy, precision, and method performance requirements for quantifying MN mass concentration and dispersion state in the test media further complicates MN testing. While quantitative measurements of the distribution of MNs in the test containers throughout bioassays are critical for understanding variable test results, such measurements are rarely performed (exceptions include[51-54]). When non-standardized methods are used, they are often experimental in nature and not easily implemented by testing laboratories. Describing quantification methods for each type of MN is beyond the scope of this paper, but is considered elsewhere.[55-58] Quantifying the MN concentration in the test suspension is most difficult for lower MN concentrations (i.e., $\mu g\ L^{-1}$) with most methods; while a promising recent study used atomic force microscopy to produce number concentrations down to $\mu g\ L^{-1}$ concentrations,[59] this process has not yet been standardized and is not available to most ecotoxicology laboratories for routine analysis. It is possible to measure the aqueous phase concentration of carbon nanomaterials (CNMs) when greater than 1 $mg\ L^{-1}$ using techniques such as UV/vis absorption spectroscopy[60, 61] and gravimetric analysis.[31, 62, 63] While some methods for quantifying lower CNM concentrations are described in the literature, these methods detect only specific types of carbon nanotubes (CNTs),[64] or additional work is needed to standardize the methods.[65-67] Metal and metal oxide MNs can be quantified in bulk by elemental analysis (e.g., ICP-MS) at low concentrations. Separation methods such as ultrafiltration, centrifugation and dialysis membrane techniques can be used to distinguish between unagglomerated, agglomerated, and dissolved MNs, but have not yet been standardized.[16, 29, 68, 69] The applicability and reproducibility of these separation methods

will be assessed by an OECD group developing a test guideline for measuring MN dissolution. Emerging techniques such as single-particle inductively coupled plasma-mass spectrometry (ICP-MS)[70-75] and liquid nebulization/differential mobility analysis[76] can distinguish among some of these different transformations for metal containing MNs but require standardization, have MN-dependent limitations because their lowest measurable MN size are above 1 nm, and thus, their practical application for routine hazard testing has not yet been demonstrated. Recently, Mader et al.[76] have addressed this issue by providing a framework for evaluating the performance of new MN measurements methods.

The role of standardized hazard testing in MN risk assessment

The different behaviors of MNs in comparison to soluble chemicals such as HOCs and dissolved metals have raised questions about the common practice of separately assessing hazard and exposure. While significant progress has been made toward understanding the environmental fate and transformation of MNs[15, 77-80] and obtaining the basic information required to estimate exposure,[81] work is still ongoing to develop models to predict the fate and hazard of MNs based on their composition and physicochemical characteristics.[82, 83] This knowledge, which informs and simplifies hazard testing for dissolved chemicals, is rarely available for MNs, suggesting that fate and exposure testing may need to be incorporated into hazard testing guidance for MNs. For example, the environmental relevance of testing the aquatic toxicity of MNs that rapidly settle out of suspension with pelagic organisms was debated during the workshop. The ongoing efforts at OECD to develop TGs and a GD on MN dissolution, dispersion stability, and environmental fate will inform these decisions, while the MN sorption to activated sludge TG also currently under development will enable more realistic estimates of surface water and terrestrial nanomaterial concentrations. At a minimum the toxicity of the corresponding dissolved bulk material (if available) should be determined for a complete interpretation of aquatic hazard data generated for MNs.[84]

Limit Testing

While the concept of limit testing is described in many OECD TGs, its applicability to MNs was not explicitly discussed during the workshop**.** The use of limit testing for assessing the hazard of MNs is complicated by many of the exposure issues described here for concentration-response (multiple exposure concentration) testing. Limit tests employ a recommended maximum exposure concentration to determine if a substance has hazard potential within reasonable limits. The goal is to identify a single high concentration of test substance at which no effects are observed, eliminating the need for further testing. OECD TG 218 & 219 (Sediment-water Chironomid testing with spiked water or sediment[85, 86]) describe the limit-test concentration as "…sufficiently high to enable decision makers to exclude possible toxic effects of the substance, and the limit is set at a concentration which is not expected to appear in any situation". OECD 218 sets this concentration at or below 1000 mg/kg sediment. Applicable aquatic TGs[93,101,130] recommend limit tests be set at 100 mg L^{-1} (or the highest soluble concentration, whichever is lower) for water only tests. For substances that form stable dispersions, an existing OECD GD[27]

(that does not specifically consider MNs) recommends a limit concentration of 1000 mg L^{-1} or the dispersability limit, whichever is lower. The application of limit testing based solely on mass concentration is potentially problematic for MNs as particle number concentration and surface area vary significantly for a given mass of material present at mean sizes between 1 and 100 nm. Other issues include varying MN transformation rates (i.e., dissolution, agglomeration) at different concentrations, and the potential for nanomaterial atypical dose response curves.

Potential modifications to test procedures

Adjusting media composition

A number of potential modifications to standard testing were considered for MN ecotoxicity testing to address the behaviors of MNs described above. One of these modifications is to prescribe a single test medium for each commonly used test organism for use with each bioassay method. Current TGs typically allow for flexibility in bioassay media selection in recognition of variability among various testing facilities. However, for MNs this flexibility can lead to difficulty in comparing test results and potentially a lack of agreement between labs that are using the same basic test method. Diluting test media (i.e., reducing ionic strength) or adjusting media pH away from the point of zero charge of the MN may reduce the rate of agglomeration and settling for many MNs,[87] but may be physiologically stressful for test organisms.[88] Thus, in selecting the standard test medium, there is a potential tradeoff between maintaining organism health and vitality and minimizing MN agglomeration and transformation rates. For example, *Daphnia magna* growth and reproduction are typically raised with greater water hardness,[89] but this leads to greater rates of MN agglomeration for charge-stabilized MNs resulting in lower or less consistent exposure. Choosing an alternate daphnid test species adapted to softer waters (e.g. *D. pulex*[88]) may be a viable alternative. Any modifications to the standard methodology which may alter the physiological stress responses of the test organism should be validated with a positive control experiment such as a reference toxicant test which can be found in OECD method validation studies and the open literature.[129] In addition, some MNs may yield acceptable assay variability in standard test media, and altering standard and historically used test media would limit relative comparisons to previous data generated using OECD ecotoxicity TGs. For MNs where dissolved metal ions may impact the toxicity (e.g., ZnO and AgNPs[17, 29]) it is important to exclude metal chelators such as EDTA as described in previous OECD documents for metal toxicity testing (e.g., algae testing[90]). While some studies have used chelators such as cysteine to eliminate the impact of released ions to highlight the impact of a MN itself, interactions between the chelators and the MN surface may impact MN behaviors and transformations.[91, 92]

Standardizing test vessels and systems

The selection of test vessels can also impact ecotoxicological results.[93-95] Increasing the consistency of the test vessel dimensions (material, size, aspect ratio, internal surface area) for

each test type and species is expected to reduce differences in the rate of MN agglomeration, settling, dissolution, or sorption, although it should be considered that a single test vessel type may not always be suitable for all types of MNs. A consistent test vessel for each test type and species should be selected from common commercially available products. Assay specific modifications should also be considered such as the impact of the agitating mode for the algae test on MN behaviors, and the grazing on the bottom of the vessel for the *D. magna* test.[90, 96, 97] Furthermore, interlaboratory comparison testing can be used to evaluate specific TG accuracy and precision among laboratories.[98-100]

Preparing initial MN dispersions

There are multiple approaches for preparing MN dispersions for aquatic toxicity testing, such as use of de-ionized (DI) water stock dispersions for spiking test media, sonication of MNs in the test media, and use of stabilizing agents. The approaches described in this section relate to preparing dispersions in DI water prior to mixing with the test media. It is often easier to produce stable dispersions of MNs in DI water as a result of the lower ionic strength and thus reduced agglomeration and settling rates. There are several potential approaches to disperse MNs in DI water that can be used individually or in combination: 1) use of commercial dispersants, capping agents, or solvents; 2) use of natural organic matter (NOM); and 3) sonication of unmodified MNs.

Many MNs are not stable in aqueous media in the absence of surface coatings or dispersants. When commercial MNs are synthesized with a dispersant or capping agent, they should be considered an integral part of the MN; control experiments can be conducted if it is important to elucidate the impact (stimulatory or inhibitory) of the dispersant or capping agent on the assay results.[29] Workshop participants discouraged use of additional synthetic organic solvents or dispersing agents, such as tetrahydrofuran (THF) or sodium dodecyl sulfate (SDS), when dispersing MNs due to high potential to confound results, as thoroughly discussed in previous papers.[12, 19, 101-103] However, if commercial products use synthetic solvents or dispersing agents in the MN formulation, then the bioassay should be conducted with the product as produced.[63] Thus, in these cases carefully designed control experiments (as described in[29]) are needed to elucidate the toxicity mechanism and avoid artifacts.

Ubiquitous natural dispersants such as NOM may be considered with the recognition of their potential to significantly alter MN dispersion stability and toxicity.[31, 32, 67, 104, 105] Environmentally relevant concentrations should be considered;[106, 107] however, to maintain a conservative approach for hazard assessment, only the lowest concentration necessary to achieve a stable dispersion should be used. Workshop participants discussed whether a standard NOM could be identified or used but no consensus was reached. It was agreed though that control experiments are essential to understand the influence of NOM on toxicity. This topic and discussion are covered in greater detail in the Supporting Information. Guidance on evaluating

effects of NOM on polymer toxicity,[27] and an existing USEPA guideline[108] may be of use in addressing this issue for MN.

Dispersion by sonication is implemented in the OECD MN dispersability and dispersion stability TG under development, but is known to generate oxidative species in solution as well as pyrolysis conditions. A variety of sonicator types and models exist and differ in power transformation efficiency and in the way in which the energy is delivered to the sample (e.g., sonication probes, bath sonication and cup horn sonication). The potential effect of sonication on MN surface chemistry and size should be evaluated as this procedure has been shown to destroy or damage CNTs[109, 110] if an ice-water bath is not used. Importantly, sonication may degrade molecules coating MNs,[111] and in some cases, the sonication process may alter the toxicity of surface coatings[29, 112] or add metal contamination through disintegration of the sonicator tip.[113] However, sonication may only provide short-term dispersion of some MNs, as agglomeration may reoccur after sonication ceases and during the bioassay.

Different approaches exist for dosing test media with MNs, such as creating a working stock for spiking test media and performing a serial dilution to create test concentrations, or direct addition of the test substance to the media to individually prepare each test concentration. If the agglomerate state of the MNs is not impacted by serial dilution, the stock approach may be appropriate; if the state of the MNs is impacted by dilution, individually preparing each concentration should be considered. While the approaches described thus far relate to the production of a stock MN dispersion, it may be advisable to follow a different approach if a MN has more than one potentially toxic component. This approach, typically used for testing chemical mixtures as the various components may be present at different ratios at different concentrations, is to prepare a separate dispersion for each concentration.[27] One example of MNs with multiple toxic components is CNTs that release toxic metals from the residual metal catalysts. If a stock dispersion is made, the concentration of released metal impurities will be higher in the stock dispersion because dispersed and settled CNTs will both release toxic metals. Dilutions made from the stock dispersion to obtain different dispersed CNT concentrations would have a different CNT to metal ion ratio than if separate dispersions were made for each concentration. If the primary toxic effect is driven by the dissolved metal impurity, a dilution series prepared from this stock dispersion may produce an acceptable dose response curve; however, the effect may be erroneously attributed to the CNT rather than the impurity. Preparing separate dispersions for each test concentration helps to distinguish effects due to the MN vs. impurities. However, preparing separate dispersions at low concentrations (< 1 mg L^{-1}) could lead to higher variability in assay results due to the inaccuracy of weighing small masses.

Preparing dispersions in assay chambers for organism exposure

After producing stock dispersions or dispersions for each test concentration using the procedures described in the proceeding section, it may be necessary to add the dispersions to the test media. If the dispersibility and dispersion stability TG is used to prepare the dispersion, it is important to

note that the TG is designed to test the stability of MNs in different aquatic media, not to prepare the best dispersion for ecotoxicity testing using other OECD methods.

After adding dispersed MNs to the test media, there are multiple options regarding when to test the ecotoxicity of the resulting suspension. One approach is to immediately add the dispersed MN to the test media. This approach may minimize the variability among laboratories in the initial MN dispersion that the organisms are exposed to if the dispersion procedure is robust. However, the MN settling rate during the course of the ecotoxicity assay may be quite variable due to factors such as different test media.

An alternative option for unstable MNs is to first add the dispersed MN to the test media or to sonicate the sample in the test media. Then, monitor the MN suspension stability with time to determine if, and wait until, a pseudo-steady state condition is reached, at which point the settling rate has reached a minimum (or acceptable level) or there is no longer detectable settling.[27] The MN suspension that has reached a pseudo-steady state could be transferred to test vials to start the bioassay. However, no consensus was reached in the workshop on a recommended maximum time limit to reach the pseudo-steady state. Measurements may be needed to assess if transferring the suspension will cause additional agglomeration, settling, and sorption to test containers, resulting in reduced exposure. Settled material included in bioassays may also act as a source for dissolved materials or resuspended particles and potentially alter system chemistry, e.g., oxidation or reduction states.[114] The approach described above is conceptually similar to water accommodated fraction (WAF) methods frequently used in petroleum testing.[28, 115, 116] Some similarities are that energy is first added to the system (e.g., by sonication for MNs and by blender mixing or slow stirring for petroleum) followed by a period of settling for MNs, or separation of petroleum, and collection of the MN dispersion or WAF, leaving behind the unsuspended material. In both cases, the goal is to produce repeatable water column exposures. However in both cases, physical effects or continued release of toxic components from the separated material are excluded from the hazard assessment. For example, physical effects of petroleum can be significant in oil spills, and Park et al.[117] demonstrated that removal of settled particles reduced the toxicity of Ag MNs to *D. magna* but not *Oryzias latipes*. Due in part to the many uncertainties associated with this approach, a consensus was not reached on the application of WAF approaches for MN hazard testing. It was, however, noted that WAF approaches are suggested for testing some difficult to test substances in existing guidance documents.[26]

Potential MN artifacts

When testing the potential ecotoxicological effects of MNs, a significant complication is that the MNs themselves may cause artifacts or misinterpretations in ecotoxicology assays.[29, 118-120] A comprehensive discussion of the potential artifacts and misinterpretations inherent to bioassay testing of MNs is provided in a recent publication[29] and is beyond the scope of this manuscript. Briefly, issues such as use of control experiments, evaluation of nutrient depletion caused by

MNs, MN interference with assay measurement (e.g., algal density), and inaccurate dosimetry quantification and metrics need attention to achieve consistent toxicological results. MNs may confound toxicity measurements by limiting the applicability of common approaches. For example, a recent study showed that Coulter counter and haemocytometer measurements of algal density after exposure to titanium dioxide or gold nanoparticles were impeded as a result of hetero-agglomeration between the algae cells and MN; fluorometric methods were found to be the most suitable.[119] Overall, multiple methods (e.g., Coulter counter and fluorometric analysis of algae), ideally using promulgated or standard test methods, should be utilized when available and careful consideration of relevant control experiments is critical.

Considerations for applying the 20 % exposure specification to testing MNs

OECD harmonized aquatic toxicity TGs discuss acceptable limits of variation in water-column concentrations and provide suggestions for approaches to maintain these limits. These are invariably set at 80 % to 120 % of nominal or initial (immediately upon dosing) measured water-column concentrations. The TGs vary in specifying whether changes in water-column concentration should be relative to nominal or measured values. Further, TGs vary in their prescription of what should be done if the 20 % exposure specification is exceeded. In some TGs, this outcome simply determines whether exposure-response analyses and reporting can be based on nominal rather than measured concentration values. [97, 121] In others, the need for more frequent substance quantification is discussed,[90, 122] but a specific schedule for these analyses, or an approach to determine the rate of concentration change, are not provided. In other TGs, it is suggested that the exposure system is preconditioned (to limit adsorption), media renewal intervals be shortened, or continuous renewal (or flow-through) systems be employed. It seems implicit in the TGs that variation in excess of ± 20% does not constitute test failure as long as diligent efforts were made to attempt to maintain consistent exposure and the exposure is quantified based on measured values, and that measurements are made frequently during a test or media renewal period. Beyond the TGs, there are documents[27, 123] that provide some consistency and guidance on exceedances of the 20% exposure specification. These GDs state that if concentrations remain within ± 20%, then results may be based on nominal or mean measured values, and if concentrations deviate by more than ± 20% then results must be reported based on measured values (geometric or time-weighted mean). It is also important to recognize that among these TGs and GDs, substance losses are generally attributed to their elimination from test systems (e.g. by volatilization and chemical degradation processes). In TGs and GDs where substance losses from the water column (but not from the test system) are observed, e.g. by settling or physical separation, it is recommended that insoluble components be removed by filtration, centrifugation, or other separation methods;[26, 27] this is potentially applicable to MN on a case-specific basis that ensures the worst-case, most conservative hazard result is generated, but consensus on this approach was not reached by the workshop participants.

Some advantages and disadvantages of the ± 20 % exposure specification are summarized in Table 1. Based on the literature and experience of workshop participants at the workshop, it was concluded that it is likely, for many MNs, that maintaining water-column concentrations within ± 20 % of the initial concentration during ecotoxicity assays with or without media renewal and without the use of dispersants or solvents will be difficult if not logistically infeasible, especially at higher (e.g., mg L^{-1}) concentrations. Even if a stable dispersion is initially prepared, it may not be possible to maintain consistent exposure if the changes to the state of agglomeration, particle dissolution and/or some other transformation of the particles continue to occur during the bioassay. Examples of rapid decreases in MN concentration and increases in agglomeration are shown in Figure 1. Clearly, it is important to consider whether the 20 % exposure specification should be applied to MNs and this suggests a need for guidance on how MN losses should be addressed and reported. Unfortunately, it is unclear from TGs what the basis or rationale for setting the level at ± 20 % is, other than the obvious goal of maintaining stable exposures, facilitating endpoint calculation, and avoiding overlapping exposure concentrations among treatment levels within a concentration series. Hence, it is difficult to assess whether this exposure specification would be more or less applicable to MNs compared with soluble chemicals. Regardless of the specific level of acceptable change in the aqueous concentration, the critical issue is how MN concentration (and other metrics such as particle size, particle count, or surface) should be quantified during testing. Approaches to calculate toxicity endpoints if there is greater than 20 % decrease in the aqueous phase concentration are discussed in the Supporting Information.

Dosimetry and interpretation

Dosimetry

An inherent hypothesis in nanotoxicology is that the size-specific properties that make MNs useful for technology applications will also be important for determining biological effects.[39, 124-131] However, a consensus on what particle-specific or unique effects that consistently apply to specific classes of MNs has yet to be reached.[132, 133] Various studies in the ecotoxicology literature have reported higher toxicity for smaller particles,[134-137] though size related toxicity is not always observed.[138, 139] It is widely recognized that the standard mass-only dose metric paradigm used in toxicology for traditional substances may not adequately represent exposure-response relationships for MNs.[39, 140, 141] The mass only paradigm is further compromised by decreasing suspended MN concentrations during bioassays, a scenario where a time weighted averaging approach more accurately reflects exposure concentrations but is seldom used in practice. There are numerous alternative dose metrics for MNs other than mass; the most commonly discussed are total available particle surface area and particle number concentration.[140] For example, Van Hoecke et al.[142, 143] reported that the available surface area (m^2 L^{-1}) of CeO_2 and SiO_2 MNs better correlated to growth inhibition of algal cells than mass concentration. For some soluble metal MNs (e.g., Ag, Cu), the dissolved fraction (and

dissolution kinetics) in test media also needs to be considered in dosimetry determinations.[137, 144-146] While some studies have reported toxicological response to correlate with certain MN properties, it has been difficult to confirm these trends across toxicological investigations. This is likely in part due to poor understanding of how the state of MN exposure differed (e.g., different states of polydispersity) between investigations because of challenges associated with measuring polydisperse MN suspensions in test media. Further, size-unique effects are suggested to be most likely to occur below 30 nm;[147] therefore, studies that have focused on size-related effects above 30 nm may not isolate particle-specific effects.

Aerosol science literature has addressed alternative dose metrics for particles (e.g., [148-151]), and several recent ecotoxicology studies reported an improved expression of dose response by surface area,[134-136, 152] ion release[136, 152] or particle number.[153] However, development of a standardized alternative dose metric for MNs for hazard assessments is encumbered for a number of reasons: (1) it is unlikely that any one alternative dose metric will provide an improvement over mass for all MNs in all test systems; (2) it is more difficult to directly measure surface area and particle number compared to mass concentrations at bioassay relevant concentrations and in bioassay media,[140] although methods are becoming available;[59] (3) unless size distribution data are known or measurable, polydisperse particle suspensions in test media will further complicate interpretation of exposure relative to effect; and (4) dynamic changes in dispersion stability or consistency (suspended concentration, agglomeration and dissolution) confound concise interpretation and render dose metric conversions from size and mass less accurate. Unless particle number concentration and/or size distribution are directly measured,[59] the uncertainty for surface area and MN number concentrations will be substantially higher than those based on mass concentrations. In this context, OECD recommended that particle counts, surface area, and mass should all be measured when feasible to allow calculation of alternative dose metrics.[23] These measurements should be monitored throughout the test in all test concentrations to account for concentration-specific change in dispersion characteristics.

Interpretation

Bioassays involving exposure to suspended MNs need to be interpreted based on multiple factors: their relevance and appropriateness for assessing the tested MN, the consistency of the exposure (stable concentration, agglomeration, and dissolution), whether maintaining a consistent exposure is possible in the bioassay method-specific test system, the accuracy of the representation of the exposure (e.g., was the frequency of characterization measurements sufficient to capture changes in exposure during the bioassay), whether nano-specific bioassay acceptability criteria (e.g., sufficiently consistent exposure concentration with respect to agglomeration and dissolution) are met, and whether the characterization and monitoring data during the bioassay are amenable to expressing data by an alternative dose metric. If the suspended MNs cannot be maintained within 20% of the starting value within the water phase (with respect to concentration, agglomeration, and dissolved fraction), it is difficult to employ any dose metric without complicated and potentially inconsistent conversions[150] and a time-

weighted mass approach may be a more expedient option to express dosimetry. While challenging calculations may be feasible in research, a more straightforward approach is needed for hazard and risk assessments. However, most of the historical literature used to determine regulatory hazard concerns for chemicals are mass-based and provide a critical benchmark against which to compare the toxicity of new MNs.

Sediment testing

Many of the considerations previously discussed for water column testing are relevant to sediment tests, with the notable exception that there is no need to remove insoluble test material according to standard assay protocols.[85, 154] While the latter is a major conceptual difference between tests of MN and traditional chemicals with pelagic organisms, it is not an issue in sediment testing. Some added complications are that MN interactions in sediments can significantly alter MN properties, and methods for quantifying concentration or other MN characteristics in sediments are very limited. However, given that most MN suspensions are generally not stable in environmentally relevant water chemistries (Figure 1), there was consensus from the expert workshop that consideration of sediment exposure and hazard is relevant and in many cases more representative of environmental exposure than aqueous tests. Current sediment toxicity standard methods for use with dissolved chemicals already acknowledge significant uncertainty regarding homogeneity, exposure, bioavailability, and synergisms. Thus, poorly understood bioavailability issues are commonplace in sediment testing and are not unique to nanoecotoxicology. An evaluation of available standardized sediment bioassay methods (OECD, EPA, ASTM, etc) suggested the test endpoints assessed in these methods will contribute valuable MN hazard information.[13] While it may not be currently feasible to rigorously characterize many types of MNs present in sediment, the consistency of sediment toxicity bioassays can still be generally improved by implementing standards for particle preparation, dispersion, spiking and equilibration in sediment.[11] Further, the use of a standardized (e.g., OECD) freshwater sediment in MN spiking studies would reduce variability in bioassay results relative to the use of field-collected sediments because sediment specific factors (e.g., organic carbon concentration) that can influence toxicity assay results are controlled. This discussion is divided into different important topics for MN sediment toxicity testing: (1) methods for consistently spiking sediment, (2) equilibration time, and (3) sampling and analysis of MNs in sediments during and after the test.

Methods for spiking and determining homogenization

Spiking of aquatic sediments is generally expected to be more consistent in terms of homogeneity if the materials are pre-dispersed into relevant water according to standardized methods rather than adding dry MNs to sediment.[12, 23] This is related to general difficulties regarding homogenizing chemicals into sediments.[155] If a MN is added to sediment in powder form (undispersed), it is likely that substantial clumping of particles within the sediment would

559 occur resulting in greater heterogeneity and therefore greater variability between bioassay test
560 replicates.[11]

561 As previously discussed, the use of a standardized sediment in MN spiking studies would likely
562 lead to more comparable results than the use of field-collected sediments. Two alternative MN
563 spiking methods have been discussed and used for sediment MN toxicity testing: (1) direct
564 addition of dispersed MNs to the sediment followed by homogenization[37, 156, 157] and (2) indirect
565 addition of MNs to the overlying water, followed by subsequent settling of the MN to the
566 surficial sediment.[12, 158, 159] In the literature, the direct addition method is much more frequently
567 used. Selection of one (or both) of these methods may relate to the test objectives, study system,
568 or functional ecology of organism used in the test or at the site of concern. For instance, a
569 testing laboratory may elect to use the direct addition method for an infaunal, deposit feeding
570 organism, which will feed on sediment below the sediment surface, while the indirect method
571 may be desirable for an epibenthic, surface deposit feeding or filter feeding organism, which will
572 interact to a substantially larger degree with the sediment directly below the water-sediment
573 interface. Research is needed to determine how to most consistently spike sediments (e.g.,
574 mixing method, duration) by these two spiking strategies so that particles are dispersed
575 throughout the sediment as homogeneously as practical to increase the inter-replicate reliability.
576 Additionally, research is needed to better understand how water exchanges, which are typically
577 performed during longer-term sediment toxicity tests, may impact MN concentrations and
578 distribution within (or on the surface of) the sediment.

579 **Equilibration time to reach a pseudo steady state time after spiking MNs**
580 It is well known that the time required to reach a quasi-steady state by equilibrium partitioning
581 for spiked sediment studies is important for determining bioavailability, especially for
582 hydrophobic compounds that take a long time period (weeks to months) to approach pseudo-
583 equilibrium in sediments.[155] Thus, two weeks[160] to four weeks[161, 162] on a roller mill is a typical
584 equilibration time to allow interactions between the spiked compound and ligands to approach
585 some level of steady state. However, currently available OECD sediment spiking methods
586 recommend 48 h equilibration.[85, 154, 163] As reflected by recommended ASTM and EPA
587 equilibration mixing times, a 48 h duration, while convenient, does not allow adequate
588 equilibration-reaction of metals in spiked sediment,[164] but may provide a worst-case scenario in
589 terms of greater MN bioavailability. While selection of equilibration times may be contingent on
590 experimental objectives, research is needed to determine how MN interactions with sediment
591 may change over time to determine the optimal equilibration time prior to test organism addition
592 and exposure.

593 **Sampling and analysis**
594 While current gaps in methods for MN characterization may limit determination of particle
595 characteristics following spiking into sediment, certain measurements may still be performed
596 such as use of ICP-MS to determine the total elemental concentration for metal and metal oxide
597 MNs. It is practical to take samples for such measurements from the whole sediment, sediment

porewater, and overlying water at test initiation and termination, as recommended in current OECD sediment testing guidance; however, MN-specific modifications of porewater separation methods may be needed to yield accurate results.

Workshop findings

While the findings discussed in this workshop primarily pertain to issues related to the applicability of OECD aquatic toxicity TGs, many of the findings also more widely apply to test methods for other documentary standards agencies (e.g., ISO and ASTM), test methods for terrestrial organism testing, academic research and regulatory decisions. The discussion of the workshop participants led to both convergent and divergent opinions on how the major issues impacting the consistency, environmental relevance and accuracy of aquatic bioassay results should be handled in aquatic toxicity testing. To the extent possible, it is desirable to minimize the amount of developmental work performed by commercial testing companies, such as assessing which procedure to disperse MNs in the test media or designing a complicated system to comply with the ± 20 % test specification,. A summary of issues for which workshop participants both achieved and failed to achieve consensus is summarized in Table 2; where consensus was not achieved, targeted research studies are recommended in the table. The research proposed is designed to support the development of precise guidance for conducting OECD aquatic toxicity TGs that will simplify this process for commercial testing laboratories and to help regulators interpret the results through the aquatic toxicity testing OECD GD, to be developed following this paper.

The workshop participants agreed that it can be acceptable to disperse particles in either working stocks (for spiking test media) or dispersing MNs directly into test media, as described above. The optimal method will be contingent on MN physicochemical properties, target concentration, media and bioassay method selection and preliminary data should be gathered prior to decision making. Synthetic dispersants should not be used to prepare MN suspensions for aquatic toxicity testing; however, if they are part of the (commercial) product formulation then the bioassay should be conducted with the as-produced material. This recommendation aligns with previous aquatic toxicity test guidance.[26, 123, 165] Natural dispersants such as dissolved organic carbon (i.e. humic acid) may be relevant but their impact on toxicity for MNs should be considered (e.g., for metal MNs); the total organic carbon concentration should be within the range of surface waters. Additionally, while particle stability is likely to be an issue, water column bioassays should be conducted with the goal of maintaining exposure consistency to abide by chemical hazard assessment practices (e.g. REACH[28]). However, alternative water column bioassay designs or sediment exposures should be considered for very unstable MNs, adapting guidance described in the difficult substances document.[27] For aquatic toxicity bioassays with MNs, an exposure chamber with consistent dimensions and one test media for each OECD test method/organism is desirable for MNs to increase test consistency. Standard testing endpoints and the number of test replicates should be applicable to MN testing. Some preliminary, but non-exhaustive,

experimentation to determine particle stability in the test media prior to organism testing would be informative for test design and reducing animal use in unsuccessful tests.

While the workshop participants did not come to consensus on whether the 20 % test specification in the water column can be consistently applied for MNs, the group agreed that an effort should be made to maintain concentration when logistically feasible. Consensus was not reached on whether inducing turbulence or using flow through systems should be employed to maintain particle concentration. Also no consensus was reached on whether to allow particle agglomeration, settling and dissolution kinetics to come to equilibrium before adding test organisms, as related to WAF testing. While some workshop participants agreed that pseudo steady state (or constant concentration) was likely to lead to greater test reliability and repeatability, there were divergent opinions on allowing pseudo steady state to occur and removal of the settled fraction of particles as it may not offer a worst case scenario; it should be noted that pseudo steady state may not occur in the aqueous phase for some MNs (e.g., complete settling from suspension, continual ion release due to adsorption to container or ligand surfaces). No consensus was reached on whether altering standard media to increase particle stability and ultimately maintain concentration was acceptable. While pH adjustments (within biological limits) away from the isoelectric point were generally more acceptable, there was concern that ionic strength dilutions would impact animal health and decrease comparability with historic datasets. While consensus was not reached on these items, suggestions for future research to help resolve the lack of consensus are provided in Table 2. Additional suggestions for future research to support more definitive suggestions for modifications to OECD aquatic toxicity test methods are provided in Table S1; the research topics in Table S1 are categorized by section of the manuscript while those in Table 2 are provided for each area for which consensus was not reached.

Following the consensus in Table 2 will help to substantially improve the reliability and data quality of nanoecotoxicology research and provide substantive improvements for regulatory testing. Facilitating the aquatic toxicity testing of MNs using standardized methods will help MN risk assessments to be conducted more efficiently. This will potentially allow MN enabled products to reach the market in a shorter time period, allow registrants to improve quality of data for fulfilling regulative information requirements, and will promote green product design by identifying MNs with potentially significant toxicological effects or with the potential to design more benign alternatives early in the development stages.

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Supporting Information Available

Supplemental discussions of definitions and measurements of "dissolved" substances, which type of NOM to recommend for aquatic toxicity testing, and the impact of calculating toxicity endpoints where the 20% specification is not achievable, and a table describing key additional research topics for each section of the manuscript. This information is available free of charge via the Internet at http://pubs.acs.org.

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Table 1. Arguments for and against implementing the ± 20 % test specification for aquatic bioassays testing nanomaterials that are not inherently stable in bioassay test media. It was generally agreed that attempts should be made to maintain concentration.

Advantages of 20 % test specification	Challenges related to applying the 20% test specification with MNs
Maintaining high and stable concentrations of nanomaterials will lead to more reproducible test results and agreement among laboratories.	Attempting to maintain stable concentrations of MNs that are inherently unstable in water lowers environmental relevance and does not account for MN transformation. The worst case scenario is not achieved if the transformation product is more toxic than the parent material (e.g., metals dissolution). It is generally not recommended that the toxicity of a parent material be tested if its half-life is less than 12 hours.[26]
Maintaining relatively stable exposure concentrations is consistent with the existing risk paradigm of assessing hazard independently from exposure. In this paradigm, hazard values are often interpreted in context with natural factors that affect fate and exposure.	It is difficult to impossible to maintain stability of nanomaterials that are not stable in test media. Even if concentration is maintained, the state of agglomeration and / or dissolution of the particles would likely change. Use of dispersants that would assist in maintaining stability is generally not favored.[9, 26]
Maintaining stable concentrations facilitates calculation of toxicity endpoints without need for weighted averages (or other methods).	Additional logistics added to maintain stability for unstable MNs (e.g., frequent water exchanges, flow through conditions, agitation) are more labor intensive, expensive, not tailored to particle delivery (e.g., clogging of tubing) and may result in repeated tests and increased costs.
	Water Accommodated Fractions approaches are already recommended for difficult to test substances such as partially miscible petroleum products.[26] This involves testing of the stabilized fraction that is more relevant to water column testing; testing of stabilized fraction is expected to allow for a more consistent exposure concentration and thus better facilitate calculation of endpoints. Excluding settled particles from bioassays may reduce variability by avoiding confounding, physical effects. However, excluding the settled particles may remove the physical effects and may not facilitate a worst case determination of the toxicity.

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1184 **Table 2. Summary of major issues discussed by workshop participants and where consensus was reached, was not reached,**
1185 **and research recommendations to fill knowledge gaps that prevented consensus.**

Issue	Consensus items from workshop	Items lacking consensus	Key Research Recommendations to address items lacking consensus
Is it feasible to consider hazard and exposure separately for MNs?	The focus of the guidance document is to increase the consistency of bioassay results used for hazard assessment. However, dispersion stability must be considered in bioassay method selection and monitoring. Effort should be made to maintain a consistent MN concentration when logistically feasible.	Designating a limit of acceptable exposure variability either at 20% (the ± 20% test specification) or some other level over the duration of the bioassay.	Approaches for maintaining MNs in suspension (e.g., frequent media renewal, flow-through delivery, and test media modifications). Testing of flow through systems should consider the potential for increased MN concentrations in the test system resulting from settled material not removed from chambers. Determine if maintaining stable concentrations reduces variability in test results when agglomeration and dissolution cannot be avoided. Investigation of time-weighted averaging and more complex approaches to express variable exposures. Determine the extent to which settled MNs influence ecotoxicity results. Research could also focus more broadly on quantifying the uncertainties that arise when exposure varies beyond specific thresholds (including ± 20%).
Dispersion methods	It is acceptable to disperse MNs in either working stocks (for spiking biological media) or dispersing MNs directly in the test media. Working stocks should be used only if there is a single substance in the NM that exerts toxicity. The optimal method will be contingent on target concentration, media and bioassay method selection.		
Addition of substances to enhance MN dispersion	Dispersants should not be used to prepare nanomaterial suspensions for biological testing unless they are present in the (commercial) product formulation. Natural organic matter (e.g. humic acid) may be used as a dispersant; however, control	The type of natural organic matter to recommend.	Impacts of different types of natural organic matter on MN stability and toxicity testing results.

Issue	Consensus items from workshop	Items lacking consensus	Key Research Recommendations to address items lacking consensus
	experiments are essential to understand the influence of NOM on toxicity.[9]		
Modifications to methods to address MN instability	Water column bioassays should be conducted to maintain consistency with chemical hazard assessment practices. However, alternative water column bioassay designs should be considered for very unstable MNs.	Whether to allow particle agglomeration, settling and dissolution kinetics to come to equilibrium before adding test organisms. It was agreed this could be presented as an option for non-dispersible materials along with caveats. Whether effects such as inducing turbulence, and flow through systems should be employed to maintain particle concentration.	Assess the reproducibility of test results when initial suspensions vs. pseudo steady state suspensions are tested and assess the relative impact of chemical vs. physical effects on MN toxicity. Assess the impact of approaches (turbulence and flow through systems) to maintain particle concentration on MN toxicity.
Standard test media and test chambers	One standard exposure chamber and test medium for each OECD test method/organism should be recommended for MNs to maximize test consistency. If test medium is modified (relative to current practice) a positive control test with a reference toxicant in the modified medium is recommended.	If it is acceptable to modify standard media to increase particle stability and ultimately maintain MN concentration. pH adjustments (within biological limits) away from the isoelectric point are more acceptable. However, there was concern that ionic strength dilutions could impact animal health and decrease comparability with historic datasets.	Research to support development of a single test medium for each TG that would lead to the most reliable ecotoxicity results for MN testing. Studies should quantify acceptable threshold for maintaining organism health and environmental relevance. Different types of test containers (size, type of material, geometry) should be tested to assess the robustness of the different TGs with regard to this parameter. The impact of the agitating media should be evaluated for tests, such as required by the algae growth inhibition test[90]. While using standard exposure chambers may increase hazard data consistency, the utility of chamber modifications for the purpose of environmental risk assessment needs further consideration.
Expressing and interpreting dosimetry	Preliminary testing is recommended to determine particle stability in the specific test system and biological test media prior to organism testing	Establish a standard dose metric and reliable analytical techniques for monitoring MNs. Without	It is important develop, validate and standardize analytical methods to directly measure particle number concentrations and size distributions in aqueous samples at toxicologically relevant concentrations (sometimes low $\mu g\ L^{-1}$). Best practices for calculating

Issue	Consensus items from workshop	Items lacking consensus	Key Research Recommendations to address items lacking consensus
	to inform test design, characterization monitoring frequency and reduce animal use by reducing the number of unsuccessful or unacceptable tests.	readily available direct measurement methods, it will be difficult to relate dose response to surface area or particle number metrics for heterodispersed suspensions of MNs that are unstable in biological media over time.	exposure-response values also need to be developed.
Sediment toxicity testing	Sediment toxicity tests are most relevant for MNs that are unstable in the media.	If very unstable MNs should only be tested in sediments (i.e., no water column testing).	Development of characterization methods for particles in the complex sediment matrix, especially for carbon-based MNs. For metal and metal oxide MNs, the development of methods to differentiate between MNs, dissolved metal ions, and MN agglomerates is needed. Investigating dosing directly to the sediment versus indirectly dosing the sediment through the overlying water (for a surficial sediment exposure) and the associated impacts homogeneity and toxicological results.

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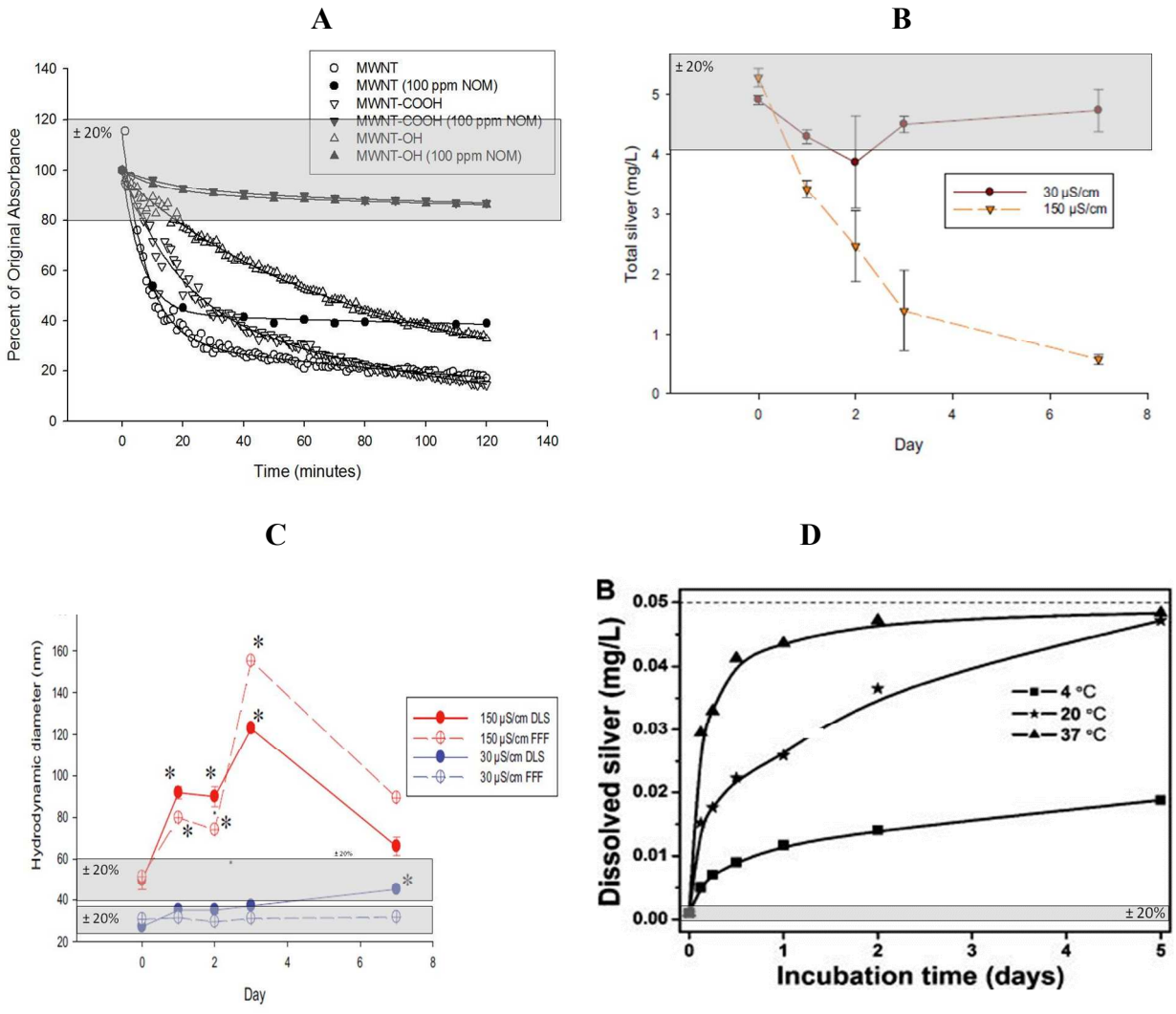
Figure 1. Examples of changes in nanoparticle stability (transformations) in environmentally relevant test media, with gray regions representing ± 20% of the original value. Different settling rates and stable concentrations of carbon nanotubes with different surface modifications and natural organic matter (NOM; 100 ppm concentration indicates 100 mg/L) (A), impact of greater ionic strength media on nanosilver concentration (B) and hydrodynamic diameter (C), and increasing dissolved concentrations of nanosilver with time (D). Further, test organisms may have an impact on nanoparticle stability; while graphene settling is relatively low in absence of test organisms (E), the presence of *Daphnia magna* increases settling (F). Error bars for parts C, E and F represent the standard deviation of triplicate measurements while the data points indicate the mean values. Figures are reprinted with permission from [16, 53, 61, 166]. Panels A, B and C were reprinted with permission from John Wiley and Sons (*Environ. Toxicol. Chem.* 2008, *27*, 1932-1941; *Environ. Toxicol. Chem.* 2014, *33*, 1783-1791). Panels D, E and F was reprinted with permission from American Chemical Society (*Environ. Sci. Technol.* 2010, *44*, 2169-2175; *Environ. Sci. Technol.* 2013, *47*, 12524-31).



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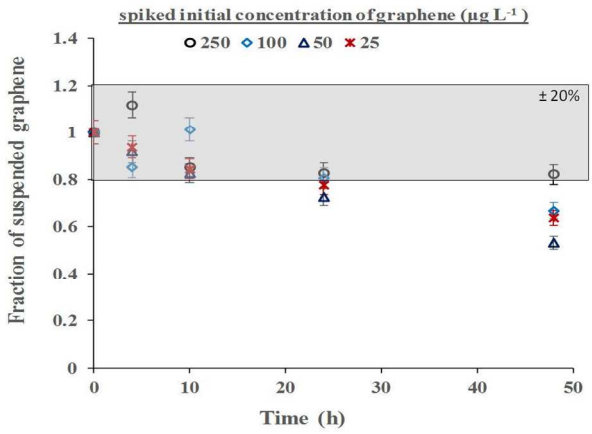

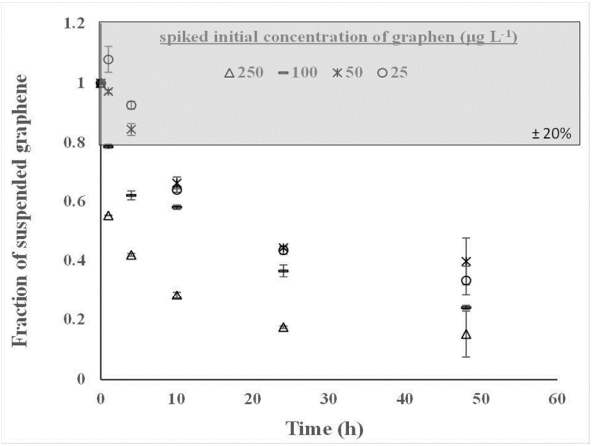

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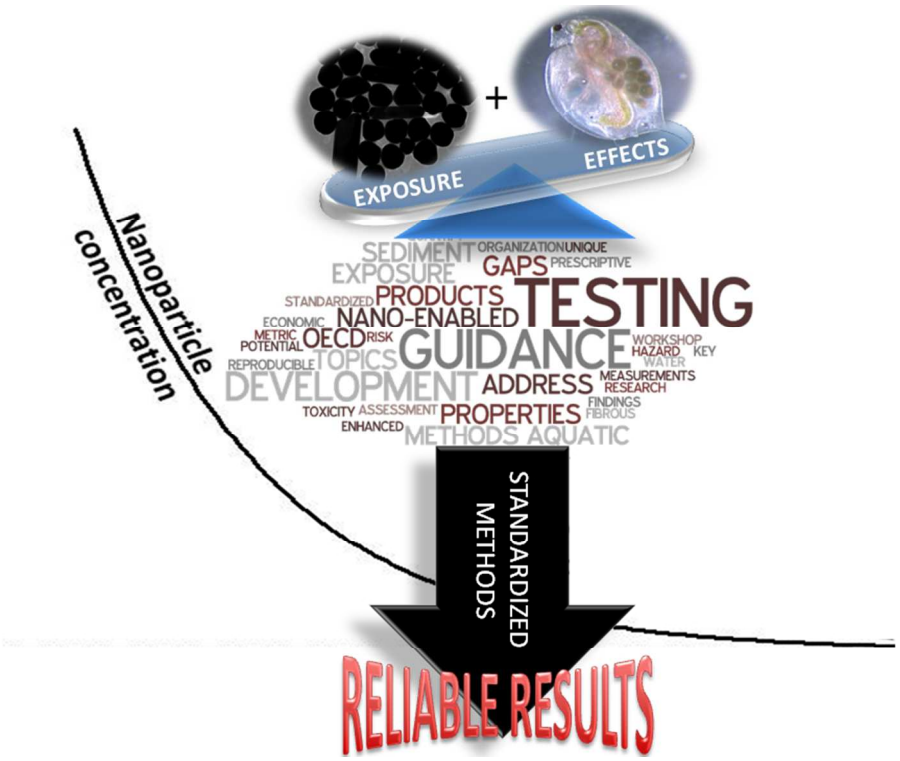

Improvements to bioassay testing through research, development and standardization.
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