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GUIDANCE DOCUMENT ON AQUATIC AND SEDIMENT TOXICOLOGICAL TESTING OF NANOMATERIALS

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NO. 317

GUIDANCE DOCUMENT ON AQUATIC AND SEDIMENT TOXICOLOGICAL TESTING OF NANOMATERIALS

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

Environment Directorate
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Foreword

1. Guidance for the ecological toxicity testing of manufactured nanomaterials (MNs) was identified as a priority goal by the OECD's Working Party on Manufactured Nanomaterials (WPMN). On behalf of the WPMN, an expert meeting on ecotoxicology and environmental fate of MNs took place in January 2013 in Berlin. During the meeting attendees identified the need for development of an OECD Guidance Document (GD) for aquatic ecotoxicity testing of MNs. To that end, a Standard Project Submission Form (SPSF) was submitted to OECD and the WNT in November 2013. Following review by National Experts, the SPSF was revised and approved by the WPMN and the WNT-26 in April 2014.

2. In February 2014, a coordination meeting focusing on the Test Guidelines (TGs)/GDs in development on dispersion, stability and dissolution and considerations on ecotoxicity testing of MNs took place at the University of Vienna, Austria. The experts discussed planning, cooperation, and overlap of the different activities. The consensus decision was these documents under preparation should leverage, coordinate and reference one another to the degree practicable. In July 2014, an OECD workshop was specifically convened to consider issues in, and approaches to drafting this GD. The workshop was held at the U.S. Environmental Protection Agency (EPA) Headquarters in Washington D.C. and was attended by 23 experts from seven countries. Meeting participants agreed that the format of the GD to be drafted should generally follow that of the document; “Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures”, which was recently revised in 2018 (OECD GD 23, 2018), but with significant modifications to specifically consider the hazard implications related to the particulate nature of MNs. A follow up workshop to coordinate multiple TGs and GDs on MNs was held at the German Environment Agency (UBA) in Dessau, Germany in January 2015 and also provided a foundation for the content in this GD. Finally, discussions from the ProSafe meeting hosted at OECD Headquarters (Paris, France, November 2016), specifically the EcoEffects breakout group, were integrated into this GD.

3. This document was led by the United Sates and Canada and benefited from the inputs of the Joint WNT/WPMN Expert Group on Fate and Ecotoxicity of Manufactured Nanomaterials. This Guidance Document should be considered as a living document and subject to amendment and refinement as new information becomes available, and/ or new Test Guidelines are developed.

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Glossary of Terms and Nomenclature used in the Guidance Document

agglomerate (ISO/TS 80004-4, 2011) Collection of weakly or loosely bound particles or aggregates or mixtures of the two in which the resulting external specific surface area is similar to the sum of the specific surface areas of the individual components.

aggregate (ISO/TS 80004-4, 2011) Particle comprising of strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components.

disperse The act of suspending manufactured nanomaterials into media often through use of energy (e.g., sonication, mixing, vortexing, etc) in an attempt to make a stable dispersion.

dispersion Heterogeneous mixture of at least two substances which can not or can only hardly be dissolved or react with each other. One or more substances (dispersed phase; here the nanomaterials) are dispersed in the other continuous substance (dispersion medium; here the stock media or test media).

dissolution Process under which a substance dissolves.

dissolution rate The amount of substance dissolved (solute) into a solvent over time.

manufactured nanomaterial (MN) (ISO/TS 80004-1: 2015) Nanomaterial intentionally produced for commercial purposes to have specific properties or a specific composition.

medium (test medium) In general, refers to the aqueous medium used for organism testing according to an OECD Test Guideline. This medium is (a) formulated to meet test organism requirements; (b) it is used as the performance control; and (c) it is the aqueous media into which the MN is dispersed (or spiked).

medium (stock medium) In general, the aqueous medium into which the MN test material is initially dispersed into prior to spiking into the test medium used in toxicity testing. This medium is often ultrapure water, which may increase the dispersion and stability of the MN.

nanomaterial (ISO/TS 80004-1: 2015) Material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale.

nanotechnology (ISO/TS 80004-1: 2015) Application of scientific knowledge to manipulate and control matter in the nanoscale in order to make use of size- and structure-dependent properties and phenomena, as distinct from those associated with individual atoms or molecules or with bulk materials.

nanoscale (ISO/TS 80004-1: 2015) Size range from approximately 1 nm to 100 nm.

NOTE 1: Properties that are not extrapolations from a larger size will typically, but not exclusively, be exhibited in this size range. For such properties the size limits are considered approximate. NOTE 2: The lower limit in this definition (approximately 1 nm) is introduced to avoid single and small groups of atoms from being designated as nano-objects or elements of nanostructures, which might be implied by the absence of a lower limit.

primary particle size The non-aggregated size of individual particles.

size (RTI/NIH, 2019[4]) The physical dimensions of a particle determined by specified measurement conditions.

size distribution (RTI/NIH, 2019) Refers to a group of particles of differing sizes. When a group of particles are of differing sizes, they might be described by particle size distribution.

solubility The quantity of solute that dissolves in a given quantity of solvent to form a saturated solution.

stability: The measure of a MN dispersion that quantifies the rate of change of the MN physico-chemical parameters in dispersion (e.g., concentration, agglomeration, dissolution, or other selected characteristic). Within the present GD, stability is understood as dispersion stability. A dispersion is defined as stable if the mass concentration does not derivate more than 20% from the initial value due to sedimentation within a relevant time scale. A deviation from ± 20% initial value should be evaluated with respect to the uncertainty of the analytical method used to evaluate the stability. The total mass concentration is the primary parameter to interpret in context with a ± 20% stability target. However, other MN exposure metrics (e.g., the extent of agglomeration, dissolution, or change in other MN characteristics, zeta potential) in addition to total concentration may also be evaluated relative to the ± 20% variability target, where practicable.

surface area (RTI/NIH, 2019). The quantity of accessible surface of a powdered sample when exposed to either gaseous or liquid adsorbate phase. Specific surface area is a fundamental characteristic of nanoparticles defined as the total particle surface area of a material per unit of mass. Surface area may also be a measure of exposure, defined as the total particle surface area per unit volume of liquid media or per unit mass of sediment.

test material The MN, including formulation specific additions, including coating, dispersant, etc.

Working party on manufactured nanomaterials (WPMN) (OECD, 2019a) The Working Party on Manufactured Nanomaterials (WPMN) is a subsidiary to the OECD's Joint Meeting of the Chemicals Committee and the Working Party On Chemicals, Pesticides And Biotechnology (Joint Meeting) overseeing the Chemicals Programme. The WPMN's responsibility is to promote international co-operation amongst countries on the human health and environmental safety implications of manufactured nanomaterials (limited mainly to the chemicals sector). It ensures that the approach to hazard, exposure and risk assessment is of a high, science-based, and internationally harmonised standard.

1. Introduction

1. Currently available OECD TGs address safety testing of chemicals in its broadest sense with respect to physical-chemical properties, effects on biotic systems (ecotoxicity), environmental fate (e.g., degradation and bioaccumulation), health effects (toxicity), and other areas such as pesticide residue chemistry and efficacy testing of biocides. These TGs are internationally accepted as standard methods for safety testing and provide the common basis for the mutual acceptance of test data (OECD, 2019b). These TGs provide guidance for professionals working in industry, academia and government on the testing and assessment of chemical substances. The subset of TGs that address ecotoxicology testing are the OECD Guidelines for the Testing of Chemicals, Section 2: Effects on Biotic Systems. OECD aquatic and sediment TGs are intended to be applicable to different types of chemicals (e.g., mono-constituent or multi-constituent substances, mixtures of chemicals, pesticide formulations, cosmetic products, etc.).

2. GD 23 (OECD GD 23, 2018) provides additional guidance for testing difficult-to-test substances and mixtures (defined in Table 1 of GD number 23) but does not specifically address MN-specific test difficulties. It has become clear over the last decade that MNs present significant challenges in ecotoxicity testing, particularly in regulatory testing where the accuracy and repeatability of test results are essential, but there is lack of consensus between experts regarding the acceptability of the removal of solid phase particulates (the settled or suspended MNs themselves) from the test in order to reduce variability or fit cleanly within the traditional approach for aqueous phase exposure (based upon the bioavailable fraction). The WPMN, as part of its ongoing MNs work (Rasmussen, 2016), has addressed this issue by reviewing OECD TGs for their adequacy in testing MNs (OECD, 2009a), generating interim guidance on test dispersion and sample preparation[1] for its Sponsorship Programme (OECD, 2012a), and welcoming a workshop focused on development of guidance on aquatic and sediment testing (Petersen, 2015a). The role of the GD on Aquatic and Sediment Toxicological Testing of Nanomaterials is to provide guidance for adapting existing OECD TG methods (Table 1) to improve the accuracy, repeatability and reproducibility when testing MNs (and possibly other particulate substances).

3. The need for MN-specific hazard testing guidance is implicit from the handling of undissolved substances as described in OECD GD 23, 2018, which generally implies effects are best described by the dissolved fraction of the test substance. OECD GD 23, 2018 generally suggests removal of settled or undissolved material from media prior to testing to eliminate the potential for solid-phase effects on test organisms. OECD GD 23, 2018 outlines two exceptions: (1) when there is a specific regulatory relevance; or (2) where the test substance has an inherent tendency to form an aqueous dispersion or emulsion such as surfactants and detergents. Minimization of effects from settled or undissolved material is also discussed in ECHA (ECHA, 2017a; ECHA, 2017b). Emerging discussion on sample preparation for testing MNs in ecotoxcological tests can be found in ECHA (ECHA, 2017a; ECHA, 2017b) and for nanomaterials (OECD, 2012a). Since testing of MNs inherently involves expanded consideration of the hazard of particulate, undissolved (or dissolving) materials in media, this GD addresses a current gap in the OECD GDs and TGs. However, in some risk assessment and management contexts, it is important to discriminate the difference between the chemical toxicity versus biological effects resulting from the physical effects of MNs. This document gives additional guidance on this separation through characterization and specific performance controls.

[1] OECD, Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials [ENV/JM/MONO(2012)40]

2. Scope

4. This document provides guidance for aquatic (including sediment) ecotoxicity testing of MNs for the purposes of determining their hazard. The definition of MNs as having one dimension between 1 and 100 nm is generally adopted. However, the guidance provided here should also be relevant for colloidal (nano) particles (e.g., 1 to 1000 nm) with primary or aggregate/agglomerate sizes greater than the range for MNs. The guidance focuses on freshwater aquatic and sediment toxicity test methods described by the OECD Guidelines, including those summarized in Table 1. More specifically, this GD addresses practical aspects of carrying out valid tests with MNs including interpreting and reporting the results, and addresses the specific issues listed in Table 2. It also addresses modifications or additions to OECD TG procedures intended to incrementally improve the accuracy, intra-laboratory repeatability,[2] inter-laboratory reproducibility[2] and intra-laboratory reproducibility[3] of test results. While the focus was specifically on adapting the OECD TGs for reliable MNs testing, the methods and principles discussed herein are likely more widely applicable to aquatic toxicity test methods published by other organisations.

5. This GD considers initial characterization of test materials, preparation of test dispersion, monitoring MN behaviour in the test dispersion throughout the duration of the test, quantifying exposure and exposure-response. It focuses specifically on issues unique to testing MNs, and does not reiterate issues that apply equally, and are well recognized, for conventional ("non-nano") substances (which, where present, also need to be considered when testing MNs). These include, but are not limited to, variation in exposure caused by the presence of test organisms, presence or absence of ligands such as natural organic matter or the use or interpretation of endpoints other than those that reflect population-level responses to exposure (e.g., physiological, genomics). This GD does, however, consider the potential effects of undissolved particles and dynamic (i.e., changing) exposure conditions, including but not limited to release kinetics of MN dissolution (i.e., ion release from the particles) throughout the duration of the test. While these factors are not traditionally directly considered in standard hazard testing, they are inherent issues requiring consideration that increase the variability in MN hazard testing.

6. The focus of this GD is on measurements of worst-case hazard using traditional population level endpoints including survival, growth, reproduction, etc., and does not provide guidance on making formal risk assessments. Since the focus is on the most conservative assessment of hazard, the guidance within involves efforts to disperse MNs into laboratory media that may not always be realistic to environmental dispersal. Environmental realism is to be integrated with the exposure assessment component of the MN risk assessment and is beyond the scope of this GD. This GD does consider relevant methods for testing the hazard of MNs based upon their initial dispersibility and subsequent stability in relevant test media.

[2] Intra-laboratory repeatability was defined as the value below which the absolute difference between two single test results obtained at the same laboratory, under identical conditions, may be expected to lie with a probability of 95%. (OECD, 2005)

[3] Inter- or intra-laboratory reproducibility was defined as the value below which the absolute difference between two single test results obtained at different laboratories or at the same laboratory using a specific protocol, under reproducible conditions, may be expected to lie with a probability of 95%. (OECD, 2005). Note that TGs may have been published subsequent to finalization of this GD

Table 1. Relevant OECD Ecotoxicity Test Guidelines (TG) for which this Guidance Document applies.[4] This document is likely applicable to supplement other existing toxicity test standards for the purpose of testing of manufactured nanomaterials. TGs are organized first by test media (water vs. sediment) and then organism type (fish, invertebrate, plant).

Exposure	Organism	Species	TG	Title	TG specific considerations (Section 6.4)	Feeding considerations (Section 6.3.3)
Water	Invertebrate	*Daphnia spp.*	(OECD TG 202., 2004a)	*Daphnia* sp. Acute Immobilisation Test		
	Invertebrate	*Chironomus spp.*	(OECD TG 235., 2011c)	*Chironomus* sp., Acute Immobilisation Test		
	Fish	*Cyprinus carpio, Danio rerio, Lepomis macrochirus, Oncorhynchus mykiss, Oryzias latipes, Pimephales promelas, Poecilia reticulata*	(OECD TG 203., 2019)	Fish, Acute Toxicity Test		
	Fish	*Danio rerio*	(OECD TG 236., 2013b)	Fish Embryo Acute Toxicity (FET) Test		
	Algae	*Anabaena flos-aquae, Desmodesmus subspicatus, Navicula pelliculosa, Raphidocelis subcapitata (*formerly *Pseudokirchneriella subcapitata), Synechococcus leopoliensis*	(OECD TG 201., 2011a)	Freshwater Alga and Cyanobacteria, Growth Inhibition Test	X	
	Plant	*Lemna sp.*	(OECD TG 221., 2006)	*Lemna* sp. Growth Inhibition Test	X	
	Plant	*Myriophyllum spicatum*	(OECD TG 238., 2014b)	Free *Myriophyllum spicatum* Toxicity Test		
	Invertebrate	*Daphnia magna*	(OECD TG 211., 2012b)	*Daphnia magna* Reproduction Test		X

[4] Note that TGs may have been published subsequent to finalization of this GD.

Exposure	Organism	Species	TG	Title	TG specific considerations (Section 6.4)	Feeding considerations (Section 6.3.3)
	Fish	*D. rerio, O. mykiss, O. latipes, P. promelas*	(OECD TG 210., 2013c)	Fish, Early-life Stage Toxicity Test		X
	Fish	*C. carpio, D. rerio, O. mykiss, O. latipes, P. promelas*	(OECD TG 212., 1998)	Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages		X
	Fish	*D. rerio, O. mykiss, O. latipes*	(OECD TG 215., 2000)	Fish, Juvenile Growth Test		X
	Fish	*P. promelas*	(OECD TG 229., 2009b)	Fish Short Term Reproduction Assay		X
	Fish	*O. latipes, D. rerio, P. promelas*	(OECD TG 230., 2009c)	21-day Fish Assay		X
	Fish	*O. latipes, D. rerio, Gasterosteus aculeatus, P. promelas*	(OECD TG 234., 2011b)	Fish Sexual Development Test		X
	Fish	*O. latipes*	(OECD TG 240., 2015a)	Medaka Extended One Generation Reproduction Test (MEOGRT)		X
	Amphibian	*Xenopus laevis*	(OECD TG 231., 2009d)	Amphibian Metamorphosis Assay		X
	Amphibian	*X. laevis*	(OECD TG 241., 2015b)	The Larval Amphibian Growth and Development Assay (LAGDA)		X
	Invertebrate	*Potamopyrgus antipodarum* & *Lymnaea stagnalis*	(OECD TG 242., 2016b) (OECD TG 243, 2016c)	*Potamopyrgus antipodarum* Reproduction Test & *Lymnaea stagnalis* Reproduction Test	X	X
Sediment	Plant	*Myriophyllum spicatum*	(OECD TG 239, 2014c)	Water-Sediment *Myriophyllum spicatum* Toxicity Test		
	Invertebrate	*Lumbriculus variegatus*	(OECD TG 225, 2007)	Sediment-Water *Lumbriculus* Toxicity Test Using Spiked Sediment		X

Exposure	Organism	Species	TG	Title	TG specific considerations (Section 6.4)	Feeding considerations (Section 6.3.3)
	Invertebrate	*Chironomus riparius, C. yoshimatsui, C. tentans (now C. dilutus)*	(OECD TG 218, 2004b)	Sediment-Water Chironomid Toxicity Using Spiked Sediment		X
	Invertebrate	*Chironomus riparius, C. yoshimatsui, C. tentans (now C. dilutus)*	(OECD TG 219, 2004c)	Sediment-Water Chironomid Toxicity Using Spiked Water		X
	Invertebrate	*Chironomus sp.*	(OECD TG 233, 2010)	Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment		X

Note: The table indicates whether the method is for water-only or sediment testing, acute or chronic duration, and identifies the applicable test species.

Box 2.1. Specific Limitations on Scope

This GD reflects broadly-accepted ecotoxicological principles that have been applied to historically tested substances. It is important to recognize, however, that MNs present novel scientific and technical issues that were not foreseen. New MNs continue to be developed and their use is difficult to predict. Furthermore, MN analytical and characterization techniques and their availability continue to expand. For these reasons many aspects of the guidance provided here cannot be prescriptive. Specific areas where the level of prescription is limited include selection of specific analytical approaches, frequency of sampling, numbers of samples and analytical replicates, or for imaging techniques, the number of images and measurements to be made. The GD does describe approaches for preparing MN suspensions in test media or sediment. However, due to the diversity of MNs that may require testing, this GD offers flexibility, with allowance for case-specific final decisions on methodology. In addition, suggestions are provided for additional experiments and measurements that may be helpful to elucidate the toxicity mechanism such as by measuring the dissolved ion concentration if this fits the purpose of the ecotoxicity test. Literature review, consideration of published standard methods and consultation with analytical experts on the latest methods is recommended. In some cases, specific situations, and geographical regions, consult with regulatory authorities (if possible) may be desirable to determine data acceptability requirements prior to expending resources on testing.

7. This document is divided into the following three main sections:

1) Section 5: Test dispersion preparation
2) Section 6: Conduct of the test
3) Section 7: Data analysis and reporting (Nanomaterial-specific)

8. Development of separate but interrelated MN-specific TGs and GDs was underway during the drafting of this GD. These GDs and TGs focus on testing to determine MN characteristics and behaviours and should be consulted for additional guidance on fate and toxicity testing of MNs. The flowchart in Figure 1 provides context for how these other documents may be used in aquatic hazard testing of MNs.

- Test Guideline on Dispersion Stability of Nanomaterials in Simulated Environmental Media (TG on dispersion stability); (OECD TG 318., 2017);
- Test Guideline on Dissolution Rate of MNs in Aquatic Environment (TG: Dissolution[5]); in ring testing, validation;
- Guidance Document for the testing of dissolution and dispersion stability of nanomaterials, and the use of the data for further environmental testing and assessment strategies (GD 318, 2020: Dispersion and Dissolution);

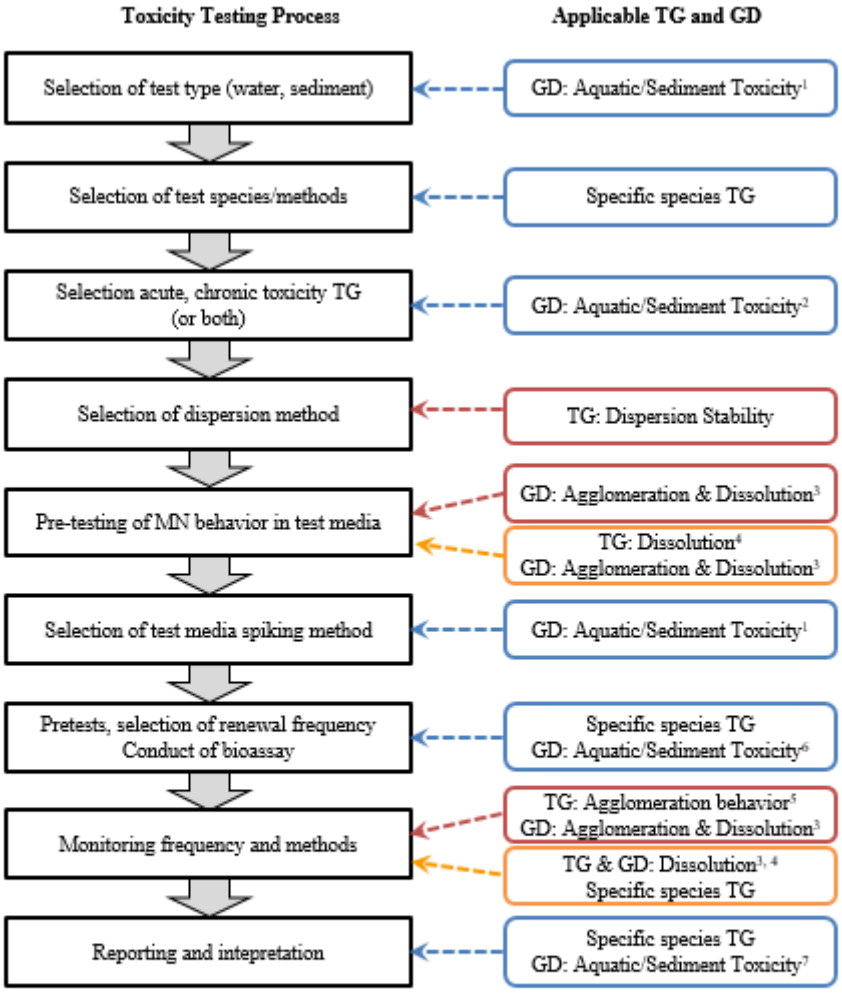
[5] Under development

Table 2. Characteristics and properties of MNs that are difficult to test within ecotoxicity test performance. While some considerations listed here are also applicable to conventional substances (and associated guidance documents), they are particularly important to consider for manufactured nanomaterials.

Property	Nature of Testing Difficulty
Particle size and size distribution, shape (e.g., spherical, fiber), agglomeration/sedimentation during ecotoxicology tests	● MN characterization (as-received and under test conditions) ● Achieving, maintaining and measuring exposure conditions ● Light attenuation/scatter ● Chemical versus physical effects ● Interaction with other particles (homo/hetero-agglomeration) ● Interaction with food particles
Dissolution (rate) of particles	● Quantifying and characterizing dissolved and particulate MN exposure ● Potential for multiple modes of action (i.e., from MNs and ions)
Toxic below MN analytical detection limits	● Quantifying and characterizing exposure ● Inability to characterizate particle transformations at low mass concentrations
Oxidation mediated by surface area	● Toxicity of modified structures or breakdown products
MN-specific transformation/bio-degradability	● Stability of pristine vs. transformed nano-scale particles ● Changes may occur during storage of the MN ● Toxicity of modified MNs or transformation/degradation products ● Changes (e.g., desorption or degradation) may occur to MN coatings or functionalizations
Particle adhesion to container walls	● Maintaining exposure concentrations ● Test vessels (material type, shape, etc.) ● Quantifying and characterizing exposure
Adsorption of media components	● Depletion of nutrients in test dispersion
Light-absorbing/reflecting	● Reduction of available photon energy (e.g. for plant growth) ● Light attenuation
Engineered/Natural	● Distinguishing naturally occurring nanomaterials from MNs added to complex matrices such as sediment when determining the experimental exposure
Photoactivated toxicity enhanced by particle surface exposed area	● Photon source, photon dosimetry, exposure metrics

Note: The table focuses on issues specific to testing of MNs and does not cover all general issues to toxicity testing of traditional substances, which are beyond the scope of this document. Some issues may also be applicable to conventional substances and are addressed in associated guidance documents such as GD 23 (OECD GD 23., 2018)

Figure 1. Flowchart describing connection to other Manufactured Nanomaterial (MN) specific documents (Test Guidelines (TGs) and Guidance Documents (GDs))



[1] See section 5
[2] See section 6
[3] OECD TG under development
[4] OECD GD 318
[5] OECD TG 318
[6] See sections 5 and 6
[7] See section 7

Note: The **process boxes and solid arrows** describe the basic steps during testing. The **rounded rectangles and dashed arrows** indicate the MN-specific GD or TG that may inform each process. Determination of test type, species selection and testing duration decisions are beyond the scope of this GD and may be determined based on specific regulatory requirements.

3. Background

3.1. Rationale for document

9. Reviews of OECD harmonized (and other) guidelines have consistently concluded that those TGs are generally applicable to testing MNs if several inadequacies are

addressed (Handy, 2012a; Handy, 2012b; Kühnel, 2014; Diamond and Johnson, 2009; Hansen, 2017; Brinch, 2016; Khan, 2017). These conclusions have been confirmed in technical, laboratory-based tests, and evaluations of specific TGs (e.g. OECD TG 210., 2013a; Shaw, 2016; OECD TG 201., 2011a; Bondarenko, 2016; includes several non-OECD tests, and OECD TG 201., 2011a; OECD TG 202., 2004a; OECD TG 210., 2013a;; OECD TG 225., 2007; Hund-Rinke, 2016; and Cupi, 2015).

10. The inadequacies identified generally relate to the particulate (colloidal) and dynamic nature of MNs (Holden et al., 2016) and the absence of guidance for consistently producing and sufficiently characterizing test dispersions before and during the ecotoxicity test. Requirements for measuring and providing MN-specific information including primary particle characteristics, agglomeration and settling behaviour, and transformation (e.g., dissolution of particles) are currently absent in TGs. In addition, the frequency of these measurements, their use in quantifying exposure-response relationships (e.g. EC/LC50 values, NOEC values, etc.) or use of other, particle-specific response metrics such as surface area or particle number (Hull, 2012) are unaddressed. Providing such guidance is complicated by the limited availability of protocols or methods that are applicable to a broad range of MNs and various test organisms, including different endpoints.

11. For these reasons, adapting current TGs for a specific MN necessitates a step-wise approach beginning with assessment of existing knowledge followed by varying levels of developmental work; this process will be dependent on the specific MN to be tested and the TG used. These needs are addressed in this GD and may be applied to OECD aquatic and sediment TGs.

4. Analytical and measurement techniques

12. There are numerous techniques for measuring and quantifying the physical, chemical, and behavioural characteristics and properties of MNs. While some of these techniques are well-established and, in some cases, published as standardized methods with varying degrees of resolution and applicability (e.g., dynamic light scattering techniques: (ISO 22412:2017); surface charge: (ASTM E2865-12., 2018); particle size distribution: (ASTM E2834-12., 2012), many remain experimental, needing further development, or require equipment not typically available to toxicity testing laboratories (Baalousha, 2012; Baalousha, 2014; Goodwin DG, 2018; Laborda, 2016; and Petersen, 2016). The use of various methods for physicochemical characterization of MNs based on the OECD testing programme has also been evaluated (Rasmussen, 2018). It should be recognized that the development and standardization of methods for MNs quantification and characterization, as well as any consideration to reporting requirements (OECD, 2016a), are proceeding rapidly, thus attempts to provide detailed descriptions of current methods would quickly become outdated. In particular, there are several OECD projects currently focused on developing TGs for characterization of MNs. In general, analytical methods must suit the tested MNs and biological test system and therefore should be selected by the user on a case-specific basis. Important considerations include the applicability domain, ease of use, availability of relevant instrumentation, and whether documentary standards and reference materials exist for the method.

13. Analysis and measurement of test materials have two basic phases:

1 Characterization of the material as produced (or received) prior to its dispersion in ultrapure water or test media, or mixing in sediment.

2 Characterization of the test material after it is added to (A) ultrapure water used to make a stock dispersion; and/or (B) test media, including monitoring test

material characteristics and behaviours periodically over the duration of the test.

14. As with historically-tested substances, quantifying mass concentration of MNs in test dispersions over the duration of the test is required. While mass concentration has limitations for understanding the state of the MNs, it is currently the most logistically feasible measurement for MNs during toxicity testing and is also the exposure metric most commonly included in literature reports. However, other exposure metrics (e.g., surface area and particle number) and calculation of exposure-response values may be considered where feasible. If these supplemental measurements are made, they may prove informative where mass concentration-based metrics do not correlate well with test organism responses. This may in turn reduce the need for repeated or future testing, allowing a reduction in animal use. Given that nuances in how these additional dose metrics are computed (e.g., whether the mean particle size or particle size distribution is used) can have a profound impact (Petersen, 2019), it is critical to carefully detail how these dose metrics were measured or derived.

15. Frequent sampling of test dispersions may be required due to MNs agglomerating and settling from the water column, resulting in significant variations of exposure concentrations (regardless of the metric used). This issue is discussed in depth in Section 5. In general, sampling frequency for characterization must be sufficiently robust to determine the actual exposure that the test organism experiences over the duration of the test to assure that exposure-response calculations accurately reflect toxicity endpoints.

4.1. Characterization of as-produced test material

16. The as-produced test material may be a dry powder or a wet preparation. Prior to addition of the test material to a working stock or test media, the test material should be analysed if supplier information is insufficient for test performance or the subsequent result interpretation. To confirm or supplement information provided by the supplier, measurements should include, depending on the relevance, the elemental composition, purity, crystallinity, primary particle size and size distribution, morphology, agglomerate or aggregate size distribution (if applicable), and may include other surface properties, such as specific surface area, coating, functional groups, etc. The characterisation may, however depend on the chosen method and can lead to discrepancies among results. Therefore, the characterisation method must be reported. It is important to note that MNs should be handled carefully, and work should be conducted with the appropriate safety protocols to minimize exposure (Environment Canada, 2010). For as-produced, wet preparations, it is logical to determine the form of the main ingredient element (e.g., particulate versus dissolved for metals), and assess presence of stabilizing agents and other additives or impurities (Hull, 2009) that may confound or alter the exposure, prior to testing. All of this information is critical for identifying batch to batch variation and will provide the basis for comparative and predictive ecotoxicology of MNs. Robust characterization prior to toxicity testing is particularly important if the tested MN is to be spiked into a complex matrix, such as sediment, where characterization during the assay may not be feasible. A minimal list of characteristics to be measured when feasible and methods for doing so are listed in Table 3. It must be recognized that additional characteristics and methods may be required or at least desirable (e.g., (ECHA, 2017a) and that these will necessarily be identified on a case-by-case basis.

17. More detailed background information on characterization of MNs for biological testing is available in the document “Physical-chemical properties of nanomaterials: Evaluation of methods applied in the OECD-WPMN testing programme” [ENV/JM/MONO(2016)7] and also a recent publication ((Rasmussen, 2018). It should be noted though that these documents focus on characterizing the starting material and not necessarily on characterization of the MNs in test media.

4.2. Characterization of test material in stock and test dispersions

18. Material dispersions in both the stock dispersion and test media should be monitored to confirm that they can be consistently prepared. It is critical that the stability of test dispersions (other than stock dispersions) be evaluated in the test media added to the same type of vessels that are to be used in ecotoxicology testing. The rationale for using the same type of vessels as for the toxicity testing is that the amount of MNs that settle to the bottom of the container can depend on the shape of the container (e.g., test tube versus petri dish) and sorption to the sidewalls can also vary based on the composition of the container (Sekine, 2015).

19. While there is evidence that the presence of test organisms can alter particle behaviours in these assays by increasing settling such as by increasing agglomeration during passage of MNs through the organism digestive system (Patra, 2011; Tervonen, 2010), including the organisms in MN preliminary stability studies is optional. Excluding organisms in pre-tests accommodates acquisition of basic MNs fate information and supports a reduction of animal use in testing. It should be noted that reducing the number of vertebrate animals should be prioritized, while pre-tests with invertebrates such as Daphnids or algae can be considered. Obtaining an understanding of organism impacts on MN behaviours can also be achieved later in ecotoxicology testing through the monitoring procedures described below.

20. While total measurable mass concentration is the typical parameter used to determine “stability” for traditional (or soluble) substances, other kinetic processes (e.g., agglomeration, dissolution, etc.) should also be considered, characterized and reported to the extent practicable in MN hazard testing. A deviation from ± 20% of the initial value should be evaluated with respect to the uncertainty of the analytical method (percentage error in the measurement) used to evaluate the stability. Where the deviation exceeds ± 20% (also taking into account the measurement error in the analytical method), the exposure concentration of MNs in dispersion should be determined at frequencies sufficient for quantifying exposure using averaging, time-weighted averaging, or geometric mean approaches (Simpson, 2003; Kennedy, 2017). To allow more expeditious and economical testing, primary focus should be given to measuring and monitoring mass concentration (total and dissolved where applicable) (Section 7.2.1), with secondary focus given to monitoring agglomerate size when this is feasible such as in test media. It is preferred to have the MN present in the test media dispersion as agglomerates that are as small as obtainable or as individual particles to achieve a worst-case scenario for hazard assessment for pelagic testing (Section 5.3); for sediment testing, these small agglomerates or individual particles will be spiked to the sediment (Section 5.6). For example, the amount of characterization in a complex matrix such as sediments may be limited to mass concentration (or nominal concentration only if the MN is carbonaceous, and hence, analytical methods are not available); thus, it is critical to robustly characterize the as-produced material as described in Section 4.1. The frequency of sampling, number of replicates per sampling point and the particular measurements (of both the stock dispersion and test dispersion) will be determined from preliminary stability studies described in Section 5.2; it should be noted that higher concentrations may impact the dissolution rates and dispersion stability in comparison to lower concentrations for a particular aqueous media. A minimal list of characteristics and material behaviours to be measured, including methodologies, are provided in Table 3. It must be recognized that characterization techniques are constrained by the MN composition and concentration. For example, to date there is no standard method to directly measure the mass of carbon nanotubes, especially in complex matrices. Additionally, some MNs may be toxic at low concentrations (e.g., nanosilver) at which size measurements may not be feasible because of instrument detection limits. In such cases, measurements from more concentrated stocks should be reported and nominal concentrations in the test media may be the only option until more sensitive (or specific) techniques become available.

21. For several metal and metal oxide MNs, e.g. nano-scale silver, copper oxide, and zinc oxide, it has been well-documented that exposure of test organisms to both particle and dissolved forms of the material may occur (Navarro, 2008; Felix, 2013; Ma, 2014; O'Rourke, 2015; Kalman, 2015; and Khan, 2015). In such cases distinction between both forms may become essential and additional measurements will then be required that involve separation of released ions and undissolved MNs by centrifugation, filtration, or dialysis followed by analysis of concentrations of both components and determination of particle size (Liu, 2010; Kennedy, 2015). This is further discussed in the separate OECD GD titled "Guidance Document for the testing of dissolution and dispersion stability of nanomaterials, and the use of the data for further environmental testing and assessment strategies" (Section 2; Figure 1). In addition to metals, the dissolution of organic compounds may be considered, where applicable. It is important to note that cell suspensions, which are used in several TGs, can substantially impact the MNs dissolution rate. The large total surface area of the suspended cells and the concomitant increased uptake of dissolved substances may result in dissolution kinetics that may differ substantially from estimates made from the test dispersion in the absence of cells. As with other characterization needs, the frequency of sampling, separation, and analyses of dissolved and particulate forms will be based on results of preliminary stability studies described in Section 5.2.

Table 3. Generalized MN characterization considerations and methods for (A) as-produced test material and (B) test material in stock dispersions and test dispersions. The listing is not intended to be exhaustive or prescriptive to allow case-specific flexibility.

Table A. As-produced Test Material

Characteristic or Property	Applicable Analytical Method(s)	Guidance for Use
Elemental composition and concentration	Applicable method is material dependent. Relevant methods include the following: Mass spectrometry Inductively coupled plasma-mass spectrometry Atomic absorption spectroscopy Absorbance/fluorescence spectroscopy Electron microscopy (with EDS for elemental identification)	Collect and analyse samples to determine the presence and concentration (where appropriate) of test material, confirm values provided by material supplier, and confirm consistency among different batches of test material. Selection of preparation techniques, power analysis to determine the number of samples and replicates, numbers of images to collect (for microscopic analysis techniques) and analyze will be determined on a test material case-by-case basis. More heterogeneous or complex materials will require more samples, images, and analyses. Size should be determined using at least two different analytical methods. References that provide guidance on making sample size and analysis decisions are listed below. Some techniques (e.g., inductively coupled plasma-mass spectrometry) only work after sample digestion. Additional guidance should be sought from analytical experts, standard organizations, and the scientific literature.
Particle morphology	Electron microscopy Atomic force microscopy	Same as for Elemental composition and concentration row
Particle size (including primary particles, agglomerates, and size distributions)	Differential mobility analysis Nanoparticle tracking analysis Dynamic light scattering Electron microscopy Atomic force microscopy Centrifugal liquid sedimentation Single particle inductively coupled plasma-mass spectrometry Asymmetric flow field flow fractionation	In addition to the guidance provided in the Elemental composition and concentration row, some of these techniques (e.g., differential mobility analysis) can only be used for dispersions, while other techniques (e.g., electron microscopy) typically require deposition on a substrate prior to analysis.
Particle specific surface area	Gas absorption (dry samples) Dye absorption (wet samples)	Surface area may be directly measured for primary particles or agglomerates, or calculated from primary particle characteristics (Hull, 2012), (Mottier, 2016), contingent on feasibility.

Note: It is impractical to provide greater specificity since MN characterization methods and reporting requirements are developing rapidly and this table would quickly become outdated. However, information about current methods is available (OECD 2016a; Rasmussen, 2018). It is critical to note that some of these techniques may not be applicable to certain test media and this needs to be evaluated.

Table B. Test Material in Stock Dispersion and Test Dispersion

Characteristic or Property	Applicable Analytical Method(s)	Guidance for Use
Mass concentration of test material (may include particle and dissolved forms)	Mass spectrometry Atomic absorption spectroscopy Absorbance/fluorescence spectroscopy Inductively-coupled plasma-mass spectrometry Single particle inductively coupled plasma-mass spectrometry	The frequency and number of samples to be analysed to quantify the mass concentration of the test material will be based on preliminary stability and dissolution studies (see Section 5.2).
Separation techniques where particles and dissolved forms are present	Dialysis Ultra-centrifugation Filtration / ultra-filtration	Same as for Mass concentration of test material row. In addition, the separation process should remove MNs as small as possible, and the methodological details should be reported. The recovery of the dissolved species must be measured to assess losses of ions during the separation processes.
Particle size (including primary particles, agglomerates, and size distributions)	Differential mobility analysis Nanoparticle tracking analysis Dynamic light scattering Electron microscopy Atomic force microscopy Centrifugal liquid sedimentation Single particle inductively coupled plasma-mass spectrometry Electron microscopy Atomic force microscopy	Selection of a specific method will be dependent on the material to be tested. The number of samples processed will be based on analytical needs. The accuracy and precision of the measurements should be determined from lab and/or matrix spiking utilizing well-characterized reference materials and comparing to the values reported. Particle size measurements should be required when feasible because material interaction with test media may result in alteration of as-delivered morphology and particle morphology may suggest a specific mode of action. Agglomeration may vary based on the test media. At least two complementary methods should be used to determine size. A Standard Reference Material (SRM) or other traceable, well characterized reference standard should be used to evaluate the method's accuracy and precision.
Charge based dispersion stability	Zeta Potential (electrophoretic mobility)	Samples from the dispersion are taken to determine charge, based on electrophoretic mobility. Particles in dispersion with charges departing from the isoelectric point (0 mV) may be judged as having greater electrostatic stability. This measurement is not a good indicator of steric stability. This measurement is specific to a particular test media.

Note: It is impractical to provide greater specificity since MN characterization methods and reporting requirements are developing rapidly and this table would quickly become outdated. However, information about current methods is available (OECD 2016a; Rasmussen, 2018). It is critical to note that some of these techniques may not be applicable to certain test media and this needs to be evaluated.

5. Test dispersion preparation

22. The overall objectives in preparing test dispersions are to achieve exposures that can be accurately quantified over the duration of the test, to minimize changes in the suspended concentration during the assay, and to improve test reproducibility. Modifications to test media preparation and/or monitoring described in TGs may be required where exposure concentrations vary during the testing period. (OECD GD 23., 2018) indicates that modification of test design should be considered where a test substance concentration varies by ± 20% or more, relative to the actual initial concentration, during the test or dispersion renewal period (OECD GD 23., 2018). While mass concentration is the primary exposure metric for MNs (due, in part, to limitations in characterization methods or logistical considerations), variability in other exposure metrics (e.g., surface area concentration, particle number, particle or agglomerate size, etc.) may be important in testing MNs and can be reported. Some changes to the test substance mass concentration are MN or particle specific (e.g., dissolution or a decrease in the aqueous-phase concentration from agglomeration and subsequent settling), while other mechanisms that may decrease the concentration (e.g., adsorption to tubing or the sidewalls of containers) are not MN specific.

23. Modifications to address exposure variability include increasing the frequency of test dispersion renewal, introducing stabilizing substances (although this could impact test results, as discussed below), or measuring test substance concentrations (or other characteristics) at intervals sufficient to adequately quantify exposure (e.g. using geometric means or time-weighted averaging) for use in estimating exposure-response values. An improved understanding of the stability of the test substance and consistency of exposure under the test conditions should therefore be obtained before commencing the test. A preliminary assessment of substance stability in the test system will provide the basis for consistent testing, reduce the probability of failed or inconclusive tests, and inform the necessary frequency of MN characterization monitoring and thus reduce costs and the number of animals used. Information on needed modifications to test dispersion preparation can be obtained from 1) review of existing data on the physical and chemical properties and behaviours of the MN and; 2) results from preliminary stability studies performed under the anticipated assay conditions (e.g., using test dispersions, exposure vessels, and renewal strategies relevant to the specific TG). Changes to the test media or frequent media renewal may adversely impact the health of the test species, and this should be minimized when possible. Overall, measurements of dissolution and stability can be performed using OECD TG 318 (OECD TG 318., 2017) and other OECD GDs and TGs that are under development (previously described in Figure 1). Recommendations for how to monitor the test dispersion are described in Section 5.5.

24. The approaches for preparing test dispersions described here are intended as general guidance. Frameworks are described for evaluating MNs with different dispersion and dissolution behaviours (Kennedy, 2017) and for evaluating the validity of aquatic nanotoxicology data generation intended to support regulatory decisions (Hartmann, 2017).

5.1. Review of existing information on the test material

25. Information relating to physical/chemical properties, fate, transport and environmental toxicity of a test substance will be informative in selecting a test media, preparation method and test system that will minimize exposure variation or provide for adequate exposure monitoring. However, given the broad and expanding range of MNs being developed, it is unlikely that detailed fate, transport, or test dispersion preparation (or other test methods) specific to the MN to be tested will be available (Petersen, 2015a), (Callaghan, 2017). Where such information is available (e.g. Tantra, 2015; Martin, 2017), its direct applicability to the material to be tested should be carefully evaluated. Minor differences in MN production method, use of stabilizing agents, coatings, and

functionalization, and storage and handling can significantly alter material behaviour in test systems (Kittler, 2010; Kennedy, 2012; Gorham, 2014; Afshinnia, 2017; Ortelli, 2017). It is also likely that the available information and data were acquired under test conditions (test media, dispersion method, physical conditions, etc.) significantly different than those to be used with the selected TG. For these reasons, the applicability of existing methods should be confirmed, and preliminary testing should be commensurate with the apparent comparability of the materials to be tested and the specific TGs to be used. It is suggested that dispersion methods described in OECD TG 318 (OECD TG 318., 2017) be investigated as a starting point for test dispersion preparation (with the recognition that the methods described therein are not specific to hazard testing).

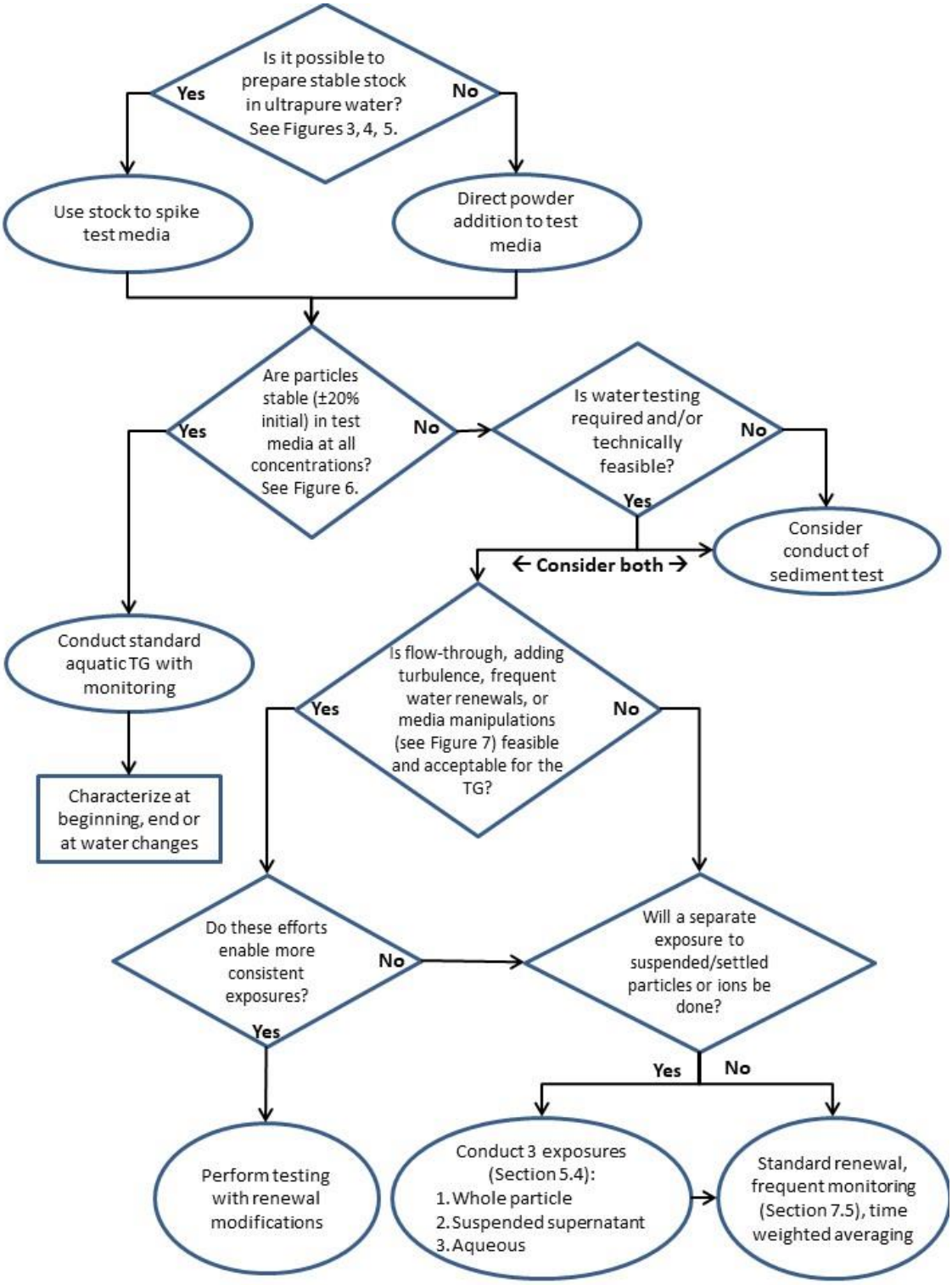
5.2. Preliminary dispersion stability assessments (discretionary "pre-tests")

26. Stability pre-tests are discretionary but may save time and resources related to determination of particle behaviour, frequency of water changes, or sample monitoring, number of replicates and number of animals used during the ecotoxicology tests. The choice of test method and subsequent decision on the exposure medium depends on the required ecotoxicity endpoint and the specific MN of interest. A series of interrelated flowcharts and diagrams is included to provide overarching guidance on selection of methods and approaches for preparing and monitoring stock and test media dispersions.

- ***Figure 2***: An overarching flowchart that outlines potential test method decisions for preparing dispersions based on dispersion stability
- ***Figure 3***: An overview flowchart outlining approaches for spiking test media
- ***Figure 4***: A detailed diagram of the spiking approaches in ***Figure 3***.
- ***Figure 5***: A flowchart outlining stock dispersion preparation and assessment of its stability for repeated use
- ***Figure 6***: A flowchart outlining approaches for assessing stability of test media dispersions
- ***Figure 7***: Flowchart to provide guidance on water exchange and test media manipulations.

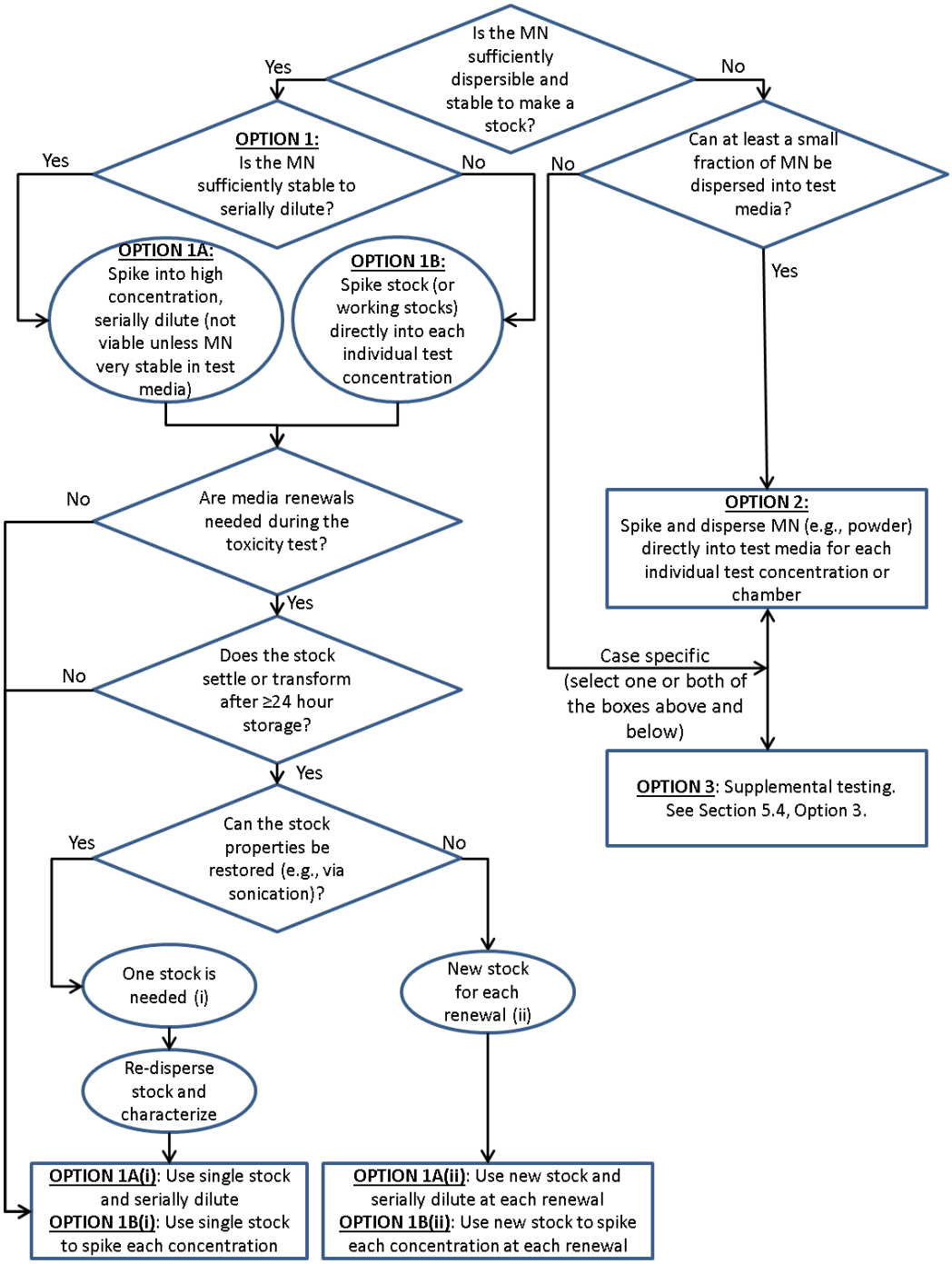
Figure 2. Overarching flowchart for selecting methods for preparing MNs to be tested.

A stability assessment is recommended for each individual exposure concentration.



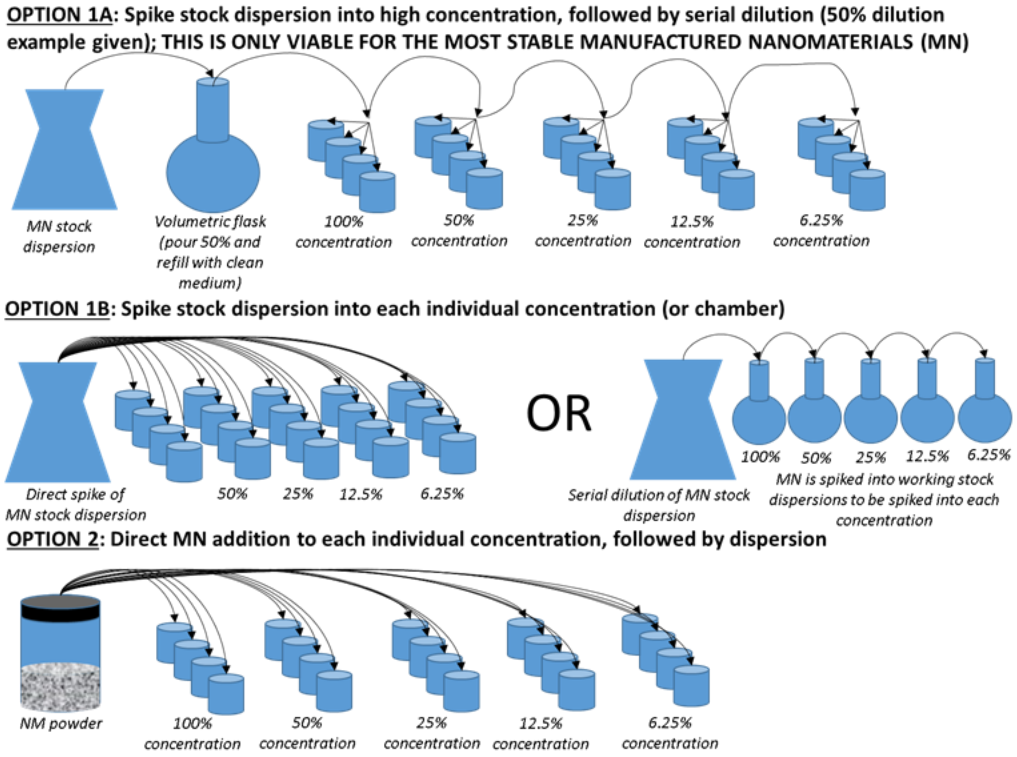
Note: The content is not intended to be prescriptive and is provided as general guidance.

Figure 3. Flowchart to inform decisions on how to prepare MNs stock dispersions and spike MNs into test media.



The content is not intended to be prescriptive and is provided as an example of a method selection process. See also Figure 5. The frequency of testing the exposure concentration for the different options is described in Figures 5 and 6.

Figure 4. Examples of methods for spiking Manufactured Nanomaterials into test media in the exposure vessels.



Note: The content is not intended to be prescriptive and is provided as an example of a method selection process. The need for equilibration time prior to test organism addition should be determined on a case-by-case basis

Figure 5. Flow chart to inform development and testing of stock dispersions to be used in preparing test dispersions

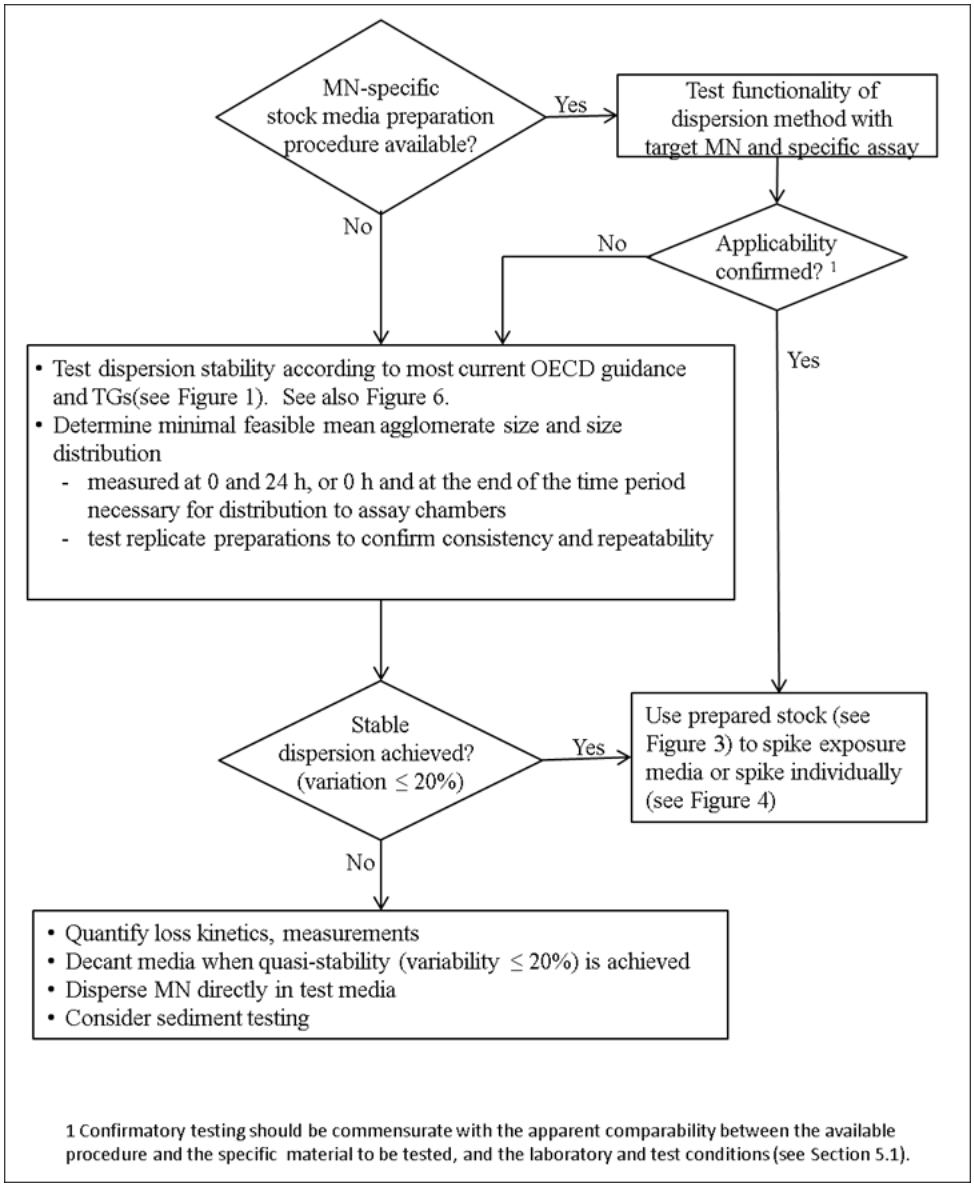


Figure 6. Flow chart for determining test dispersion stability.

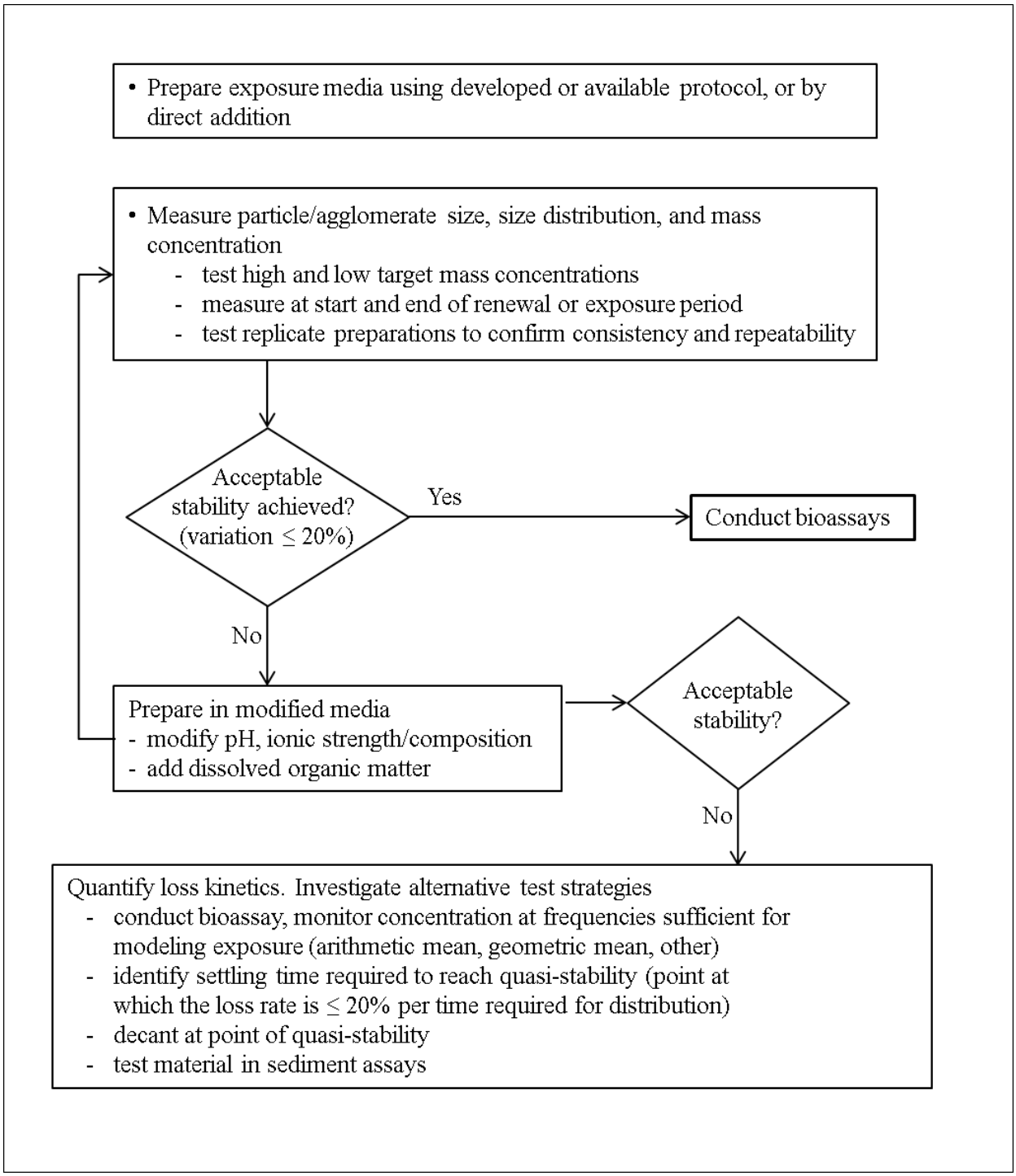
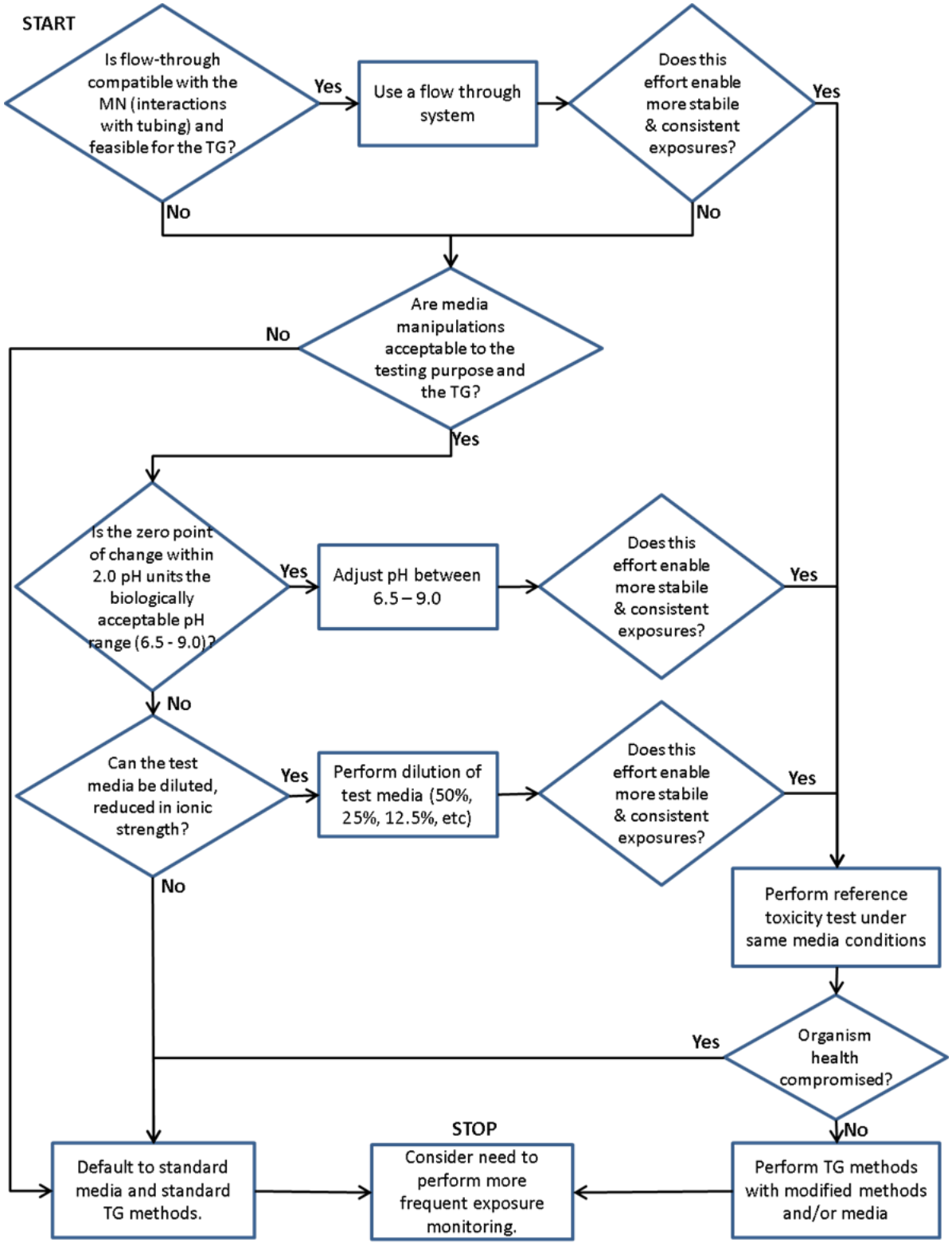


Figure 7. Flowchart to provide guidance on water exchange and test media manipulations. Note that dispersants such as natural organic matter (NOM) can be considered on a case-specific basis (see Section 5.3).



27. The preliminary assessment of particle stability, as it is defined in the glossary, may have four different outcomes:

1) sufficient MN stability in test dispersion to allow consistent organism exposure: e.g., MN is dispersible and is sufficiently stable (e.g., ± 20% within work day[6]) after addition to the test media to provide a consistent organism exposure;

2) test dispersion preparation methods can be developed to achieve sufficient stability (see Figure 3, Figure 4, Figure 5, Figure 6);

3) MNs are not sufficiently stable (change ≤ ± 20%) in dispersion but the exposure can be adequately quantified by measurement and monitoring at different time points during the toxicity test to calculate exposure-response relationships using time-weighted or other averaging techniques (see Figure 2, Figure 5, Figure 6);

4) MNs are not sufficiently stable and settling rates are too high (e.g., <20% of the original MN remains in dispersion after 6 hours[4]) for water-column exposures and for time point sampling to be practical. The relevance of water-column versus sediment testing should be evaluated based on the purpose of testing of the assessment (see Figure 2).

28. Where settling rates are high (Outcome 3 above), it will be necessary to employ an exposure monitoring schedule sufficient to quantify changing exposure concentrations (Figure 6). Generation of such data will more robustly represent the exposure, using time-weighted averaging or other techniques (Simpson, 2003; Petersen, 2015a; Kennedy, 2017).

29. Some MNs may not be dispersible or may immediately settle from dispersion (Outcome 4 described above) despite attempts to prepare and/or maintain an acceptable dispersion (e.g., sonication, test media adjustments, turbulence, etc.). In such cases, the appropriateness and feasibility of water column testing versus sediment testing may be considered. It is important to note that limited stability as a result of rapid agglomeration or an inability to disperse the MN in the test media cannot be equated with “no exposure” because exposure to epibenthic and benthic (sediment) organisms can still occur; exposure to pelagic organisms may also occur depending upon the experimental setup. Figure 2 provides a conceptual (non-binding) flow diagram of a decision-making process. It should be recognized that dispersion stability may refer to material characteristics, properties, or behaviours other than, or in addition to, mass concentration; these include agglomeration and/or dissolution.

30. For MNs that dissolve, the kinetics of dissolution (ion desorption or release from particles in dispersion) should be considered to account for organism exposure to released MN constituents. An OECD TG on dissolution kinetics in environmental media as well as an accompanying GD are under development and may support such measurements (Figure 1). Selection of specific characterization measurements to assess dissolution will depend on the MN to be tested, the TGs to be used, and specific testing objectives.

5.3. Dispersion methods (for both stock and test media dispersions)

31. Where possible, methods for dispersing MNs described in the MN-specific dissolution and dispersion stability TGs and related GD (Figure 1) should be used in toxicity testing. In addition, several recent efforts have been made to harmonize dispersion protocols for biological testing of MNs (Taurozzi, 2011; Taurozzi, 2013; Hartmann, 2012;

[6] Discretionary numbers provided are arbitrary and only used as considerations to categorize these outcomes. The 20% values are consistent with TGs on aquatic toxicity testing and 6 hours is consistent with dispersion stability determinations in TG 318.

DeLoid, 2017). If stock dispersions are to be used repeatedly, sufficient characterization is needed to identify acceptable ranges for particle size, stock dispersion concentration, etc., to assure consistency among spiking events (Coleman, 2015).

32. The water used to prepare test media dispersions may be ultrapure water, reverse osmosis water, or deionized water. Such water is likely to have less potential to generate reactive oxygen species (ROS) relative to higher ionic strength waters during sonication (DeLoid, 2017) and lower ionic strength water is better for dispersions of electrostatically stable MNs. In cases where it is most feasible to disperse MNs directly into the test media (see Figure 3 and Figure 4), the potential for ROS generation and persistence should be considered, even if they are only generated during a brief period.

33. The method selected for dispersing and spiking MNs into water should be thoroughly described and reported as the procedure may often be case specific. The following procedures offer a starting point:

a) pre-wetting prior to adding to stock or test media

b) as powder, paste, drop-wise, monitoring the rate of pouring or addition

c) with continuous stirring or agitation

d) mixing period prior to introducing higher dispersion energy (if needed)

34. For the purposes of toxicity testing, the method used to disperse MNs should be optimized to achieve the smallest agglomerate size, narrowest distribution and adequate dispersion stability to serve as a reliable stock media or consistent test media concentration. If a similar dispersion quality can be obtained using two different approaches, it is recommended to use a lower sonication energy, to minimize potential MN degradation during sonication, and to use an approach that avoids potential cross contamination such as the release of particles from a probe sonicator (Betts et al., 2013). Methods that do not directly involve probe contact with the sample (e.g., bath sonication, cup-horn sonication, agitation, stirring, rolling, vortexing) are desirable as they may use lower energy and reduce cross contamination potential. Cup-horn methods allow dispersion at higher energy relative to bath sonication but still do not directly contact the sample. Specific guidance on sonication methods is beyond the scope of this GD and is described elsewhere (e.g., cited references below). In many cases the higher energy of probe sonication (OECD TG 318., 2017) may yield the optimal dispersion, providing a benefit over lower energy methods. The amount of energy applied should be reported to allow for replication and comparison (Taurozzi, 2011; DeLoid, 2017). It is recommended to perform both size analysis and measure the dissolved fraction before and after sonication since this process can accelerate dissolution rate. The use of sonication should not alter the test material or medium. Further guidance should be consulted, such as references listed as follows for bath sonication (Taurozzi, 2011; Wu, 2014; Crane, 2008; Hartmann, 2015), cup-horn sonication (IUTA., 2014), and probe sonication which may yield more reproducible dispersions for some particles (Taurozzi, 2011; Wu, 2014; Crane, 2008; Handy, 2012a; Hartmann, 2015). The following conditions during sonication should also be considered:

- The use of an ice bath to cool test media which can decrease the rate of NM transformation/degradation by sonication processes
- Temporal monitoring for temperature, pH, dissolution, energy application, dispersion properties
- Determination of necessary energy allocation and dispersion time requirements to acquire minimum agglomerate size or particle size distribution stability (see (Jiang, 2009) for an example of an assessment of the optimum sonication time and energy).

35. ***Media adjustment, addition of stabilizing substances***: Overall, it is only appropriate to modify the test media if a change less than 20 % for the MN mass

concentration in the test media cannot be achieved by suitable dispersions or periodic media renewal. If test media manipulations described in the following paragraphs are unable to achieve greater stability, then return to the original test media and use robust monitoring of the MN exposure concentration.

36. It is well known that changing aqueous media (e.g., pH modifications, ionic strength manipulations) can influence the stability of MN dispersions (Petersen, 2015a) and may also impact the MN dissolution rates. In addition, it may be advisable to decrease the concentration of chelating agents in the test media such as EDTA to decrease the rate of MNs dissolution (Hund-Rinke, 2016). General considerations for performing test media adjustments are provided in Figure 2, Figure 6, and Figure 7. Modification of the standard media may not be desirable if the results are to be compared with data of substances tested in the same media/conditions. However, if the objective is to optimize dispersion stability during the toxicity test for a worst-case exposure, then pH modification is preferred over ionic strength adjustments (Petersen, 2015a; Kennedy, 2017). As illustrated in Figure 7, the pH may be adjusted at least 2.0 units from the isoelectric point of the test material, but the adjusted pH needs to be within the biologically acceptable range (e.g., 6.5 – 9.0); it is important to recognize that low pH media may promote greater metal bioavailability. A pH manipulation of the test media, compared to ionic strength adjustments, would minimize ionoregulatory stress that could increase sensitivity of organisms to the MN (especially for AgMNs and other metal and metal oxide MNs) since the amount of acid or base added to change the pH within this range would be relatively small (Harmon et al. 2015). Ionic strength reductions can be achieved by dilution with ultrapure water or prepared with alternative salts to increase dispersion stability (divalent ions reduce the stability of electrostatically stabilized MNs more than monovalent ions at the same molarity; (Huynh, 2011). If the test water is modified, it is recommended that control and reference toxicant tests be performed to determine if test organism health (e.g., reproductive output, growth yield, etc.) is compromised by media modifications. For example, reducing the ionic strength of the test media may increase a test organism's sensitivity to MNs (e.g., silver) that cause ionoregulatory stress in more hypotonic solutions (Harmon AR, 2017). In such cases, a supplemental reference toxicity using a dissolved toxicant with a comparable mechanism of toxicity to the focus MN (e.g., $AgNO_3$ for AgMNs) may be employed to understand if the test organism was made more sensitive due to dilution of medium ionic strength. Such changes to an organism's baseline sensitivity and the potential for decreased environmental relevance of modified test media are arguments against adjusting test media. If any change is made to the test media composition, it must be reported.

37. It is preferred that the addition of stabilizing substances be avoided, if other options exist, since they may substantially alter the organism exposure to and toxicological response from MNs (Kennedy, 2012; Jung, 2015; Gao, 2012). It may be acceptable on a case-by-case basis to use natural organic matter (NOM), or dissolved organic matter (DOM), as a stabilizing substance in test media where site-specific environmental relevance is sought, or when there is a strong desire to stabilize the test material (Tejamaya M, 2012). Any use of a stabilizing agent should be discussed with regulators (if possible) and be clearly disclosed in reporting. The minimum concentration of DOM that will achieve the desired stability should be determined during test dispersion development. If DOM is used to test MNs that can dissolve, it is important to consider ligand and other interactions that might affect the toxicity of dissolved species. For example, when the silver ion released from nano-scale silver interacts with DOM, its bioavailability and toxicity are altered (Kennedy, 2012; Fabrega, 2009). If DOM is used to stabilize a MN, an additional test without DOM should be conducted to address potential for reduction of ion toxicity and the more conservative hazard value from the two tests should be reported. Selection of necessary controls (e.g., DOM-only; absence of MN) should also be conducted to understand the impacts of the stabilizing agent on the test organism (Table 5). The type of DOM used may also impact toxicity test results and therefore should be characterized and reported. This is discussed in more detail in Section 6: *Conduct of the test*.

38. The presence of additives in the as-delivered MNs formulation may affect the toxicity of the MN. When testing formulations, it is recommended not to use serial dilutions since different components of the formulation may have different solubilities or stabilities, which could lead to an inability to accurately dilute the formulation. Instead, the required concentration of the formulation should be added to each test container separately and then dispersed.

5.4. Preparation of test media dispersions

39. Test media dispersions may be prepared by direct addition and dispersion of MNs in test media, or by dosing test media using a stock dispersion. The approach to be used can be revealed by the stability of the test MN dispersions determined in discretionary pre-tests as described in Paragraph 28. Options for test dispersion preparation are described below and in Figure 3.

1) **Option 1**: If a MN is sufficiently stable in ultrapure water, a stock dispersion may be prepared and used. Options 1A and 1B are detailed below and illustrated in Figure 4. When test dispersion renewals are needed, the feasibility of storing and reusing stock dispersion should be considered, as discussed below. Option 1A (serial dilution of MNs) is generally not recommended except for sufficiently stable (± 20%) MNs. Therefore, Options 1B and 2 are generally more applicable to MNs.

A. **Option 1A**: If the MN is stable enough in test media for consistent mixing while diluting and dosing test media, a stock dispersion may be generated and used to spike the highest exposure concentration followed by a serial dilution to create lower test concentrations. This methodology is illustrated in Figure 4.

B. **Option 1B**: If the MN is not sufficiently stable for consistent serial dilution mixing in test media dosing, a single stock dispersion using media in which the MN is stable (e.g., ultrapure water) may be generated and used to spike each individual concentration directly using different volumes. Alternatively, the stock dispersion itself can be serially diluted into working stock dispersions to spike each individual concentration to maintain the same spiking volume. This methodology is illustrated in Figure 4.

40. The stability of the stock dispersion should be evaluated if it is to be reused for media renewal during the test.

i) The original stock dispersion may be reused if the MN dispersion can be successfully maintained (e.g. based on concentration, agglomerate size, dissolved content, or other measured characteristics) which may require the application of energy (e.g. stirring, agitation, sonication, etc.). While this may be possible for MNs that do not dissolve (e.g., carbon-based, TiO_2, gold), it may not be feasible to maintain and reuse stock dispersions of MNs that dissolve.

ii) The original stock dispersion may not be reused if there is potential for irreversible MNs transformation (e.g., coating disruption, altered agglomeration, dissolution, etc.) during storage and / or from application of additional energy. In these cases, new stock dispersions should be generated for each renewal (and characterized and reported to confirm consistency with previous stock dispersions).

2) **Option 2**: If only a small fraction of the MNs are stable in the test media, it may only be practical to add the as-produced MN (e.g., powder) directly to the exposure media for each test concentration or test media. This approach may be needed when simple volumetric addition or dilution of the stock dispersion

into the test media causes significant changes in MN characteristics or where serial dilution yields non-homogeneous dispersions (e.g. for lower-stability materials). This methodology is illustrated in Figure 4.

3) **Option 3**: If it is not possible to disperse the MNs in stock dispersions, the relevance and validity of water-only toxicity testing should be examined based on the purpose of the testing. If required, a supplemental approach may be applicable where a relatively small portion of the added MN (potentially including dissolved ions) remains in dispersion, relative to a larger fraction that settles from the test media. In this case, it may be desirable to determine the toxicity of the fraction that remains suspended by generating dispersions followed by a settling period and collection of the water-column dispersion (excluding settled material). The particle size distribution and mass concentration of the MN that remain in the water must be characterized using methods in Section 4.

If water-only toxicity testing of non-dispersible MNs is required (Option 3), a weight of evidence approach can be executed by performing testing to generate multiple lines of evidence to better understand the hazard of low stability MNs. At a minimum, the whole MN sample should be tested (condition (i) in Table 4), while conditions (ii) in Table 4 and (iii) in Table 4 can be conducted to better understand the cause of the hazard and potentially reduce variability related to dynamically changing dispersions. It is also important to recognize that sediment toxicity testing is particularly relevant for rapidly settling MNs (see Section 6.1).

Table 4. Description of different hazard testing approaches and objective of testing.

Sub option	Description of approach	Objective of testing
(i) hazard testing of whole MN sample (suspended particles, settled particles, dissolved fraction)	Spiking of MN into each individual test media concentration (since serial dilution would introduce inconsistencies) and testing the hazard through monitoring of suspended particle and dissolved concentrations and settled particles (if possible)	This is the recommended default option for testing of MNs, with the potential to also conduct tests focused on determining the cause of toxicity under more controlled scenarios
(ii) hazard testing of suspended sample (suspended particles, dissolved fraction)	Testing the supernatant of the particles after a prescribed settling period determined from discretionary pre-tests, after which the supernatant is removed and tested (settled material is excluded)	When the goal is to reduce variability in the test and only the portion of the MN that remains in the water column for ecotoxicity assessment is considered (note: this approach will not address the effects of the unstable particulate fraction)
(iii) hazard testing of the dissolved fraction only	Testing after removing any undissolved particulate material using appropriate separation techniques (e.g., ultracentrifugation or ultrafiltration) and testing the hazard of the supernatant (or filtrate)	When determining the contribution of the dissolved fraction is needed for comparison to established toxicity thresholds established for dissolved chemicals (note: this approach will not address the effects of the particulate fraction)

41. Dispersion method development begins with evaluation of material stability in ultrapure water for generating a stock dispersion that can be used throughout the duration of the test (Figure 5). The tested stock dispersion is then diluted in test media at target concentrations, followed by monitoring of test dispersion characteristics at each exposure concentration over the duration of the test or at renewal periods within the exposure vessels. It may be determined for some MNs that a relatively unstable stock dispersion can be used to spike test media in the exposure vessels if dosing proceeds rapidly or with continuous energy (e.g., sonication, stirring) applied to the stock dispersion to maintain the dispersion; this scenario might occur where dilution of an unstable dispersion results in satisfactory levels of stability at assay concentrations and/or when individual concentrations are low and it is not feasible to accurately weigh powder. This outcome will be more likely at lower MN concentrations since lower MN concentrations agglomerate more slowly. Given the well-documented variability in behaviour of MNs in aquatic media, and the diversity of test media used, it is not possible to define specific methods for generating a dispersion of all MNs in all test media at all exposure concentrations (Callaghan, 2017). While general methods for optimizing MN dispersion for ecotoxicological tests have been discussed by (Hartmann, 2015) there still remains a need for preliminary stability studies, as outlined in Figure 5. It must be recognized that rapid MN settling may result in an exposure period to suspended particles shorter than the full test period, resulting in the potential for significantly underestimating toxicity of the tested MN. Exposure to settled particles may occur depending upon the hazard testing approach taken (Table 4) and experimental setup. Overall, it is critical to quantify the exposure concentration because reporting only the nominal concentration limits the validity of this data.

5.5. Monitoring test dispersion stability

5.5.1. Stability of stock dispersion

42. Prior to toxicity testing, the stability of the stock dispersion should be measured over the duration of its use period when conducting a specific toxicity TG (Figure 5). Stability determination should include suspended concentration, agglomerate size, and dissolution (fraction of total concentration in the dissolved form). At a minimum, stock dispersion stability should be sufficient to allow for dosing of all test media concentrations in the vessels without a significant change in stock dispersion concentration, agglomeration and dissolved fraction (and possibly other characteristics). Stability testing should demonstrate that this objective can be met under test conditions for preparation of all test concentrations. If the stock dispersion will be used for several renewals over the duration of the test, its stability (or potential for re-dispersion) should be confirmed at each renewal time. If exposure dispersion renewals are required, the stock dispersion should also be characterized at the time of each use. If these stock dispersion characteristics cannot be restored, a fresh stock dispersion should be prepared prior to spiking test media in the exposure vessels; the characteristics of the fresh stock dispersion must be comparable to any previous stock dispersion used in the test. Characterization of each stock dispersion used (whether resuspended or freshly prepared) should be clearly reported.

5.5.2. Stability of test media dispersions in the exposure vessels

43. It is important to improve quantification of MN exposures during tests, which may require more frequent sampling and analysis for MN dispersions that agglomerate and settle more rapidly (Figure 6), to improve interpretation of test results. General guidance on summarizing exposure information over the course of the toxicity test is as follows:

- When the test objective is to monitor MN stability in dispersion, sampling should be taken from the midpoint of the exposure vessel (in the x, y and z directions) to avoid the vessel walls and the bottom; this represents exposure to the suspended material. However, when the objectives are to characterize the whole MN exposure in the vessel (including mass balance), homogenization or re-dispersion of the MN is appropriate.
- When the dispersed concentration (or other metrics) does not exceed ± 20% variability, it is preferred to use averages of the initial and final values to confirm that amount of variability observed during the exposure period.
- When the variability in suspended concentration (or other metrics) exceeds ± 20%, it is preferred that multiple sampling points to characterize the exposure be taken. If only two sampling points are taken, exposure should be quantified using geometric averages; when more than two sampling points are taken, time-weighted averaged exposures are preferred (Petersen, 2015a); (Kennedy, 2017). It is important to recognize that MN stability will be affected by the presence of test organisms. Thus, pre-tests to determine sampling and analysis intervals may be performed with test organisms present in the exposure media. However, consideration should also be given to minimizing the number of organisms required in preliminary testing, potentially by reducing the number of replicates or concentrations tested.
- These considerations may apply to measurements other than mass concentration (e.g. agglomerate size, surface area), although it is not practical to stipulate when these metrics should be used, or how they relate to exposure metrics or risk assessment. These metrics might help to provide insight into the toxic effects observed and enable comparisons among different MNs. Such methods of measurement are listed in Table 3, and should be at least reported where practical.

5.5.3. Frequency of sampling exposure dispersions

44. Sampling frequency will depend on multiple factors, such as MN concentration, the rate of change determined in discretionary pre-tests, analytical budget, logistics associated with sampling and measurement, and the purpose of testing. The water handling methods described in some TGs may impact MN agglomeration and settling if they involve continuous stirring or a flow through (e.g.,(OECD TG 203., 2019) versus static test media (e.g., OECD TG 202., 2004a) and therefore impact the sampling intervals. For static systems, it is important to include early sampling points for MNs that quickly settle. If the monitoring frequency is insufficiently robust to characterize the MN exposure, the test results may be rejected.

45. It is reasonable that MNs with low dispersibility or stability in environmentally relevant media (in the absence of stabilizing agents) will settle to the sediment. Exceptions may include very low ionic strength waters with a low concentration of suspended particles and very low concentrations of MN where homoagglomeration and heteroagglomeration may be minimal. While sediment testing may be relevant to MNs that are relatively stable in water, it is especially relevant for MNs that are not dispersible in water and for MNs with lower than 20% stability (as described for pelagic toxicity testing above) over the prescribed test method renewal period (Figure 2). Many of the previously discussed complications associated with accommodating consistent MNs exposures in water are less likely to require as much consideration in ecotoxicity testing in the sediment matrix. For example, there is less need to account or separate exposures to suspended versus settled MNs, once the MNs are in equilibrium with the system; the sediment matrix itself is likely to dramatically alter bioavailability of the applied MN and therefore organism exposure (Petersen, 2015a; OECD TG 218., 2004b; OECD TG 225., 2007). Quantifying homogeneity, bioavailability, exposure and synergisms of traditional substances in the sediment matrix is complex and often poorly understood (Baalousha, 2015) dealing with such uncertainties in sediment ecotoxicological tests is commonplace in standardized guidance, making these tests perhaps more amenable (in practice) in their current form for testing of MNs than water-column ecotoxicological tests. As with water-column ecotoxicological tests, the general toxicological endpoints assessed in standard sediment ecotoxicological tests are expected to apply to MN hazard testing (Diamond and Johnson, 2009). While current analytical techniques for robust characterization of MNs are limited in the sediment matrix (Petersen, 2015a; Petersen, 2016; Goodwin DG, 2018), there are relatively straightforward approaches for preparing, dispersing, spiking and aging MNs in sediments for improving homogeneity and the consistency of sediment ecotoxicological testing (Coleman, 2018). General considerations for test selection are provided in Figure 2 and demonstration is provided in (Kennedy, 2017)

5.5.4. Use of a standardized sediment

46. It is well known that sediment characteristics (e.g., grain size, organic matter content, etc.) will dramatically influence substance interaction, equilibrium partitioning to the pore water, and bioavailability. Therefore, if different laboratories use different sediments, whether field-collected or laboratory formulated, the results of sediment toxicity tests for the same substance are expected to differ. Testing a substance or MN in field-collected sediment may have site-specific relevance but may produce results that have limited applicability to other sediment sites due to incomparable characteristics (grain size, organic carbon content, percent solids, etc.). Consequently, when the goal is to improve the consistency of sediment toxicity test results between tests (within a single laboratory) or between testing laboratories, there are advantages to performing MN spiking studies using a standardized formulated sediment (prepared with peat, kaolin clay and sand), that can satisfy specific test organism requirements. Examples of standardized sediments are described in current OECD TGs (OECD TG 218., 2004b; OECD TG 225., 2007) and (OECD TG 239., 2014c). Use of sediments other than the standard sediments, should only be used in justified cases, such as when formulated sediment is unsuitable to meet test organism requirements or for site-specific relevance. In such cases, the characteristics of

the selected sediment should be rationalized in clearly written documentation. Overall, these considerations are not specific to MN testing.

5.5.5. Steps to spike sediments with MNs

47. MNs are generally obtained in one of two forms; a dry powder or an aqueous dispersion. Handling of dry powders is discussed in the next paragraph. For an aqueous dispersion, methodological considerations are less involved as simple dispersion and spiking into the test matrix are relatively straightforward. However, aqueous dispersions may not be practical for spiking sediments as greater material masses are often needed for sediment testing (limit tests for sediment and water are 1000 mg/kg and 100 mg/L, respectively; (OECD TG 218., 2004b; Baumann, 2014; Griffitt, 2009; OECD TG 239., 2014c) and would not be stable in aqueous dispersion. Limit tests (further described in Section 6.2.2) are referenced here to illustrate that a relatively greater mass of material is typically required to observe effects in sediment testing.

48. Different techniques have been considered for spiking MNs into solid phase environmental media such as sediments. Current scientific opinion is that powder MNs should be first dispersed into water to achieve optimum homogeneity in sediment (Handy, 2012a; OECD, 2012a). A powder added directly to a wet sediment may clump and generally be heterogeneously distributed in the sediment matrix (OECD, 2014a), leading to inconsistent exposure between test organisms and experimental replicates within a treatment. Therefore, the recommendation is to first disperse the MN as a liquid stock dispersion prior to spiking into the sediment medium. Previous guidance for preparing MN stock dispersions for water-column testing (Section 5.2) is also relevant for sediments. Another possibility that can be considered, and has been applied in spiking soils with MNs (Hund-Rinke, 2012), is spiking the dry sand prior to the preparation of the artificial sediment.

49. While an indirect method of adding MNs to the overlying water and allowing settling onto the sediment surface has been used in the literature (Stanley, 2010); (Waissi-Leinonen, 2012), directly spiking MN into sediment is recommended to optimize consistent and repeatable tests. Directly spiking sediment with the test substance is a common method in the literature and standardized testing for sediment ecotoxicity testing, and is described below. This method may be most representative where sediments are well mixed or in dynamic systems, and when the desire is to generate toxicity information for infaunal, burrowing, sub-surface, and deposit feeding organisms. Additional background on ecological relevance is provided in (Coleman, 2018)

5.5.6. Homogenization of spiked sediments

Technique

50. There are techniques available in standard OECD, ASTM and USEPA guidance for directly spiking substances to sediment that can be applied to MNs. Briefly, the test substance (i.e., dispersed MNs) is added to the previously homogenized test sediment gradually while the sediment is mixed via overhead impeller. The material continues to be mixed for a set period of time (e.g., 4 hours) to further homogenization. Specific methods for spiking and homogenizing MNs into solid phase environmental media are being investigated and disseminated (Miglietta, 2015; Coleman, 2018; Handy, 2012a; Handy, 2012b), but have yet to be standardized.

Analytical confirmation

51. Analyzing and characterizing MNs in sediments is challenging and methods for doing so remain limited. Thus, reportable information may be limited to measured total MN concentrations per unit sediment volume (e.g., mg/kg). However, information regarding particle/ion partitioning and bioavailability may be derived from sampling of sediment interstitial (pore) water, with the recognition that methods used to isolate pore water may inadvertently alter or remove MNs and are not yet standardized. For metal and metal oxide

MNs in sediment, the minimum characterization information should be collected and determined from the entire sediment matrix, overlying water and porewater (to estimate a bioavailable dissolved fraction) at test initiation and termination (and between water exchanges, where applicable). However, for carbon-based MNs and for MNs where the concentrations of MNs added is technically indistinguishable from the background concentration of the metal (e.g., Ti for TiO_2 MNs), only nominal concentrations may be feasible, which further emphasizes the importance of robust characterization information for the as received MN and stock dispersion discussed in Section 4. Data for metal and metal oxide MNs provides information regarding the nature and stability/consistency of the exposure, including whether the exposure to MNs is being impacted by dispersion, re-dispersion, and subsequent loss of the dispersion to the water column during overlying water exchanges. Alternatively, extra (surrogate) replicates which are meant to be sacrificed each sample day could be prepared.

52. For direct sediment addition, homogenization should be confirmed immediately following the spiking and mixing event. Spiking, homogenization and equilibration methods in the literature may vary. For example, organic compounds (Rosen, 2005) and MNs (Stanley, 2010; Coleman, 2013; Coleman, 2018) have been homogenized into sediment for 1 to 4 hours using overhead mixers. The consistency of the homogenization should be confirmed at a minimum by verifying consistent texture (i.e., visually looks the same throughout). Following homogenization, sediment aliquots should be collected from 3 or more different locations from the sediment (e.g., center surface, center mid-depth, center bottom, edge-mid-depth) and analyzed to determine mean (bulk) concentrations and standard deviations to determine the coefficient of variation. Clearly such an assessment is expedient for only certain MNs (e.g., metal nanoparticles) for which well established and practical techniques such as ICP-MS are available. The acceptable variability should be determined on a project specific basis, although low variability (e.g., coefficient of variation ≤ 20%) is desirable. The analytical methods should be clearly reported, including limitations and interpretation of what state of the MN the analysis may be representing (e.g., total concentration, particulate concentration, dissolved concentration).

5.5.7. Storage, aging, equilibration

53. It is well known that equilibrium between test substances and sediment is not immediate. Multiple studies have shown that bioavailability of substances decreases with increasing storage and equilibration time after being spiked into sediment. For example, hydrophobic substances may take weeks to months to come into a state of pseudo-equilibrium with the sediment and sediment porewater (Kukkonen, 1998). However, the opposite result with MNs becoming more bioavailable has been observed for one study with AgMNs in soils (Diez-Ortiz, 2015). Consequently, some standard guidance for spiking sediments suggests two to four weeks storage time (ASTM E139-94., 2000; U.S. EPA., 2001). Equilibrium or mixing times for traditional substances in OECD TGs are typically 48 hours. While a shorter duration is convenient for generating data more quickly, such durations may not approach a full equilibrium-reaction (Simpson, 2004) and may not be representative of real-world conditions where equilibrium partitioning is quasi-stable. However, in some situations a shorter equilibrium duration may be considered worst-case, since generally the spiked substance should be more bioavailable. These considerations are relevant to MNs, as interaction time with sediment organic matter, sulphides, and surface adsorption to sediment particles will change bioavailability and lead to MN transformations (e.g., sulfidation). This may impact the toxicity beyond solely a change in the exposure concentration of released ions; for example, sulphides in sediments reduces the toxicity of nanosilver and nanocopper (Wijnhoven, 2009; Coleman, 2013; Ma, 2014). The stability of the coating around MNs may also degrade with storage and environmental interaction time (Sharma, 2014; Kittler, 2010), leading to changes in exposure over time. A hard recommendation for a prescribed storage time after MN spiking of sediment cannot currently be provided given a lack of scientific support in literature. While no recommended equilibrium periods for MNs exist, knowledge of dissolution kinetics of the particular metal or composite MN will be helpful in determining an appropriate equilibrium period (Xiao,

2016). It is suggested that sediment be stored for at least 48 hours and that the selected equilibration/storage time be clearly reported with full discussion of the implications on data interpretation for risk assessment.

6. Conduct of the test

6.1. Test Guideline selection

54. Prescriptive guidance on which TGs to select for testing a particular MN is beyond the scope of this GD. However, since MN dispersion potential and dispersion stability in test media or matrix are a major emphasis in both testing relevance and exposure monitoring frequency throughout this GD, a brief discussion of test type (pelagic versus sediment) is warranted (Figure 2). Testing of MNs may fall within any of the following three scenarios: (1) acute and chronic water column ecotoxicological testing for stable (e.g., ≤ 20% change) MN dispersions; (2) only water column testing for MN dispersions that are partially stable (> 20% change) where the level of stability may assist in determining the need for acute and/or chronic tests on a case-specific basis; and (3) sediment toxicity tests for MNs that are not dispersible, or are partially stable in the test dispersion; in some cases it is possible that sediment testing may also be required for MN dispersions for the first two scenarios. Thus, there may be overlap between scenarios; for instance, in Scenario 1 sediment testing may also be needed, or in Scenario 3, water-column testing may be of lower relevance but still may be required to make a risk management decision. Selection of TGs for a given MN should be made on a case-by-case basis taking into account the purpose of the testing and regulatory requirements, and can be informed by results of discretionary stability pre-tests described above (Section 5.2) using discussed methods (Section 5.4) and may be informed by the literature, if available.

6.2. Procedural Modifications

6.2.1. Modification of test procedures based on particle stability

55. The discretionary stability pre-tests (Section 5.2) may be used to determine if water changes are necessary and feasible to maintain the MN exposure. Depending on particle stability, impact of test media renewal frequency on test organisms, and available resources, water exchanges may be conducted as static non-renewal, static renewal, intermittent flow, or continuous flow methods, with test dispersion exchanges performed at frequencies that are practicable. It may be desirable to increase the frequency of volume replacements per test day to increase the consistency of exposure for MNs that are partially stable and settle, prior to considering test media pH or ionic strength manipulations. However, the logistics of more frequent water exchanges needs to be weighed against the benefits of improved consistency in the exposure concentration during the test period for more rapidly-settling MNs.

6.2.2. Use of limit tests

56. The intended purpose of limit testing for conventional (dissolved) substances (100 mg/L in water, 1000 mg/kg in sediment) is to assess the potential toxicity of a compound at a single high concentration to determine if there is any potential for toxicity that needs further investigation in more intensive testing that includes dilutions. If there is substantial evidence (from previous documented testing, pre-testing of MN stability in terms of concentration, agglomeration, dissolution), limit testing might be considered to reduce testing on animals. However, limit tests for pelagic tests are not recommended as a result of two issues related to differences between nominal and actual exposure concentrations as a result of settled MNs during the toxicity test and how that is interpreted relative to the

observed effects: (1) potential for lower exposure in the suspended-water phase at higher concentrations, since MNs may agglomerate and settle at faster rates relative to lower concentrations; and (2) unclear effect mechanisms, since greater agglomeration rates at higher concentrations may reduce exposure to suspended nano-scale material. Thus, the applicability of limit testing for MNs is unclear for pelagic testing mainly because processes such as agglomeration (and settling), MN transformations, ionic release and re-adsorption to suspended particles are concentration-dependent. It is possible that lower initial concentrations in the water phase could produce a higher time-weighted exposure in the test media, while, higher limit test concentrations might result in lower exposure concentrations (Petersen, 2015a; and Kennedy, 2017). Therefore, limit tests for pelagic organisms are generally not recommended if there is no information regarding whether there is a monotonic (or linear) exposure response for a given MNs and a range-finding test involving a concentration gradient (e.g., 100, 10, 1 mg/L) may be more comprehensive. These limitations for pelagic testing would not necessarily impact limit testing for sediment tests.

6.3. Modifications of test procedures and systems

57. The purpose of this section is to describe modifications including selection of test vessel material, dimensions and volume, water renewal frequency, and use of interpretative controls; this section supplements previous sections outlining general recommendations, such as MN-specific characterization (Section 4), stock dispersion, test dispersion preparation and monitoring frequency (Section 5).

6.3.1. Test vessel type and material considerations

58. Test vessel material is known to affect hazard testing results (e.g., Sakka, 2016; Sekine, 2015). While impacts from test vessels and the material they are made from are not unique to testing of MNs, this may be exacerbated by MN-unique behaviours (e.g., rates of agglomeration). Specialized exposure vessels have been designed to maintain more consistent MN exposures (e.g., Boyle, 2015), reduce contact with settled particles (Skjolding, 2016), and distinguish between shading effects for algal toxicity testing (Verneuil, 2014). Feasibility for using specialized exposure vessels with meshes to limit organism exposure to settled MNs should be determined on a case specific basis taking into account: 1) the purpose of the testing; 2) considering that their use limits organism exposure to MNs that have settled out of the test media; and 3) costs of using non-standard vessels not described in TGs. Common options for test vessel materials include glass, plastic (e.g., polypropylene, polycarbonate, etc.) and stainless steel. Some plastics may not be ideal due to charge interactions and loss of MNs to the surface. Polycarbonate vessels are preferred over other plastics. Glass is a relatively inert material but can result in loss of test material from adsorption during the ecotoxicological test. Silanization of the glass is an option to reduce attraction of MNs, but such coating must be applied with caution as silanol is toxic to many test organisms if it leaches into the water. Overall, glass and polycarbonate are recommended materials for testing; pre-tests can be performed to determine which surface has less affinity to adsorb MNs.

6.3.2. Water renewal and delivery

59. The frequency of test dispersion renewal required for pelagic organism testing to maintain consistent exposure and water quality are specific to type and duration (acute/chronic) of the test and the stability of the MN in dispersion and rate of dissolution. As described in Section 5.2, pre-testing should be conducted to characterize MN stability in the test media to be used, and to determine renewal frequency. Section 5.5 suggests sampling point frequency to characterize or monitor changes in the exposure. Generally, there are three different options for conducting water exchanges to maintain MN stability and water quality.

1) Selection of static non-renewal: this is adequate for stable MNs, acute (short term) testing durations, or for unstable particles when MN characteristics can be frequently monitored (see Section 5.5.3; Figure 7).

2) Selection of static renewal (e.g., daily): for partially stable MNs, chronic (longer term) testing durations, or for unstable particles when MN characteristics cannot be frequently monitored. The suspended concentration should be measured to test for potential redisperion or dissolution of settled particles.

3) Flow-through: for partially stable or unstable MNs, or when MN cannot be monitored frequently. Caution should be applied as MNs may be lost to tubing used for water delivery (Petersen, 2015a). Also, settled particles in flow through systems may also increase the total MN concentration in the wells even if the suspended concentration remains constant. Alternatively, static/static renewal testing according to Option 3 (Section 5.4) may be conducted for unstable MNs. If this is performed, the suspended concentration should be measured to test for potential redispersion or dissolution of settled particles.

6.3.3. Feeding considerations

60. OECD chronic TGs range in duration from 14 to over 100 days and stipulate feeding of test organisms during exposures. The suggested feeding frequencies vary from two to three times per day to three times per week. Depending on the guideline and species being tested, food may include live algae, brine shrimp, and dried commercial fish food. The Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment (OECD TG 225., 2007) stipulates the addition of ground leaves as food to sediment at test initiation, with no additional food added over the 28-day test duration. The addition of food during toxicity testing has implications for both traditionally-tested chemicals and MNs, including alteration of test substance concentration, substance uptake via food, and variable bioavailability (Naddy, 2011; Kolts, 2006). Feeding has additional implications for MN toxicity testing, including potentially influencing MN stability (via coating of particles by food-related substances), changing agglomeration and settling behaviour, and modifying particle surface properties, all of which can alter test organism exposure to the MN (Allen, 2010; Hoheisel, 2012; Conine, 2017; and Stevenson, 2017). When feeding is required, it is recommended that the behaviour and characteristics of the test dispersion be evaluated in the presence of the specific food to be used in the test, and at the expected feeding frequency. It may also be possible, depending on the specific TG and species to be tested, to feed test organisms shortly before test dispersion renewal (Hoheisel, 2012), allowing sufficient time for consumption. While this approach can minimize the period of time that food can interact with the tested MN, it may not be feasible for all tests and test species (e.g., lower reproductive output in cladocerans; (Harmon AR, 2017). Preliminary testing should be conducted to confirm that feeding time is sufficient to maintain organism health and the effects on sublethal endpoints (e.g., reproduction, growth).

6.3.4. Lighting considerations

61. In general, the laboratory lighting used in standardized ecotoxicological tests should be acceptable for testing MNs. However, some MNs, similar to some other photoactive chemicals, have been shown to be phototoxic under ultraviolet (UV; 280-400 nm) radiation, e.g. nano-TiO_2 and nano-ZnO_2 (Ma, 2014; Li, 2015). Standard laboratory lighting typically provides little UV and other light radiation. MNs known to be photoactive under visible light (400-700 nm) should be tested for this effect. Description of specific methods for such testing are outside the scope of this GD but general information can be found in Ma et al. (Ma, 2014; Li, 2015). There is an OECD TG for ROS detection (OECD TG 495., 2019c) for substances, which could be used to test if MNs produce ROS through photocatalytic activity, but there is no MN-specific guidance in that TG.

6.3.5. Comparative control requirements

62. A non-exhaustive list of comparative controls that should be considered in toxicity testing of MN is provided in Table 5. The implementation of these MN-specific comparative controls will be based on specific testing needs such as to better understand the toxicity observed and at the discretion of testing laboratories. Thus, this section is informational and brief; an in-depth discussion of these controls is provided in Petersen, 2015a and Petersen, 2015b. Also see OECD GD 23 (OECD GD 23., 2018), Section 7, for discussion of related control requirements for non-MN testing. The number of comparative controls is to be kept as low as possible to reduce the number of animals in testing.

Table 5. Consideration of controls specific to MN testing

Type of control	Considerations
Indirect effects	The potential for MNs to alter the chemistry of test media can be evaluated. Examples might include changes in pH, adsorption of ions, nutrients, or other media constituents, etc.
Dispersants	The potential biological effects of dispersants used to stabilize test media dispersion can be assessed. These effects may include toxicity, stimulation, nutrition (both supplementary or depletion), alteration of media chemistry or other aspects of the assay environment, etc. Dispersants may also confound chemical analyses of the target MN. This control is only needed when the dispersants are not in the initial MN formulation but are selected instead to achieve dispersion. In the case that dispersants are present in a MN formulation in addition to those used for the MN coating, this could also be tested using this control measurement.
Coating	While coating may be part of the as-delivered MN for testing, its impacts on test organisms may be determined in a coating-only exposure (e.g., at the highest exposure concentration). This is only relevant when the coating is released from the MN during the ecotoxicological test (Petersen, 2011[86]).
Measurement or analytical interference	The potential analytical or measurement interference of the tested MN (including coatings) or dispersants in the test dispersion on the toxicity endpoint to be measured should be evaluated. For example, the accuracy of absorbance or fluorescence analyses may be affected by the presence of particulate MNs which may reflect, absorb, or emit radiation at measurement wavelengths.
Operational	This control includes all manipulations performed on the test media including sonication, shaking, addition of dispersants, surfactants and any other substances or manipulations added to or performed to enhance dispersion or stability.
Soluble material	The potential effect of ions or other substances that dissolve from the tested MN can be assessed. Implementation of this control will require an understanding of rates of dissolution so that dissolved species can be tested at representative levels. The amount of dissolved ion in the test dispersion could be measured and compared to results from this assay with the dissolved ion (e.g., from dissolving a metal salt) if this information is available. This comparative control is particularly relevant as a reference toxicity test for MNs and associated ions that cause ionoregulatory stress (e.g., Ag, Cu) when the test media ionic strength was diluted.

6.4. Additional specific modifications for particular TGs

63. The considerations discussed above are generally applicable to the majority of existing OECD TGs for assessing the aquatic (and sediment) ecotoxicity of MNs in water and sediment. General recommendations on modifications to individual TGs for testing of MNs are available (See Hund-Rinke, 2016; Shaw, 2016; OECD, 2014a; and Kühnel, 2014) and have informed the discussion. In addition to considerations described in Section 6.3, certain TGs require additional unique considerations not previously discussed. These are discussed below. For topics that have been discussed previously (e.g., photocatalytic activity), these topics are not described for specific tests.

6.4.1. TG No. 201 - Freshwater Alga and Cyanobacteria, Growth Inhibition Test

64. The testing of MNs using dispersions of photosynthetic plant or bacterial cells presents unique challenges that derive from the particulate nature of both the test organism and the MN, and by limitations on renewing media over the exposure period. These challenges and possible modifications are discussed below, informed by (Hjorth, 2016) and (Hund-Rinke, 2016)

65. ***Agitation of test dispersion***: This TG stipulates that the test dispersion be agitated during cell incubation to keep algae in dispersion. In some cases, agitation may also keep MNs in dispersion. However, in other cases such energy may fail to keep MNs in dispersion or actually increase agglomeration due to increased frequency of particle collisions. Further, agitation may result in deposition zones or spatially separate MNs from algal cells due to density differences. Pre-test experimentation specific to the test MN may be required to develop mixing methods that minimize MN agglomeration or make agglomeration consistent among replicates at the different MN concentrations, while also maintaining the cell dispersions (Manier, 2015); these processes will likely be dependent on MN concentration. Precise details on agitation during the test: e.g., orbital shaking, stirring, or mixing should be reported.

66. ***Shading effects***: MNs may limit penetration of photosynthetic radiation through the test dispersion, and thus reduce growth indirectly, rather than toxicologically. This is especially true for MNs where effects are observed only at concentrations that result in turbid dispersions. Efforts should be made to reduce or quantify these effects. This includes using smaller and/or thinner test vessels to maximize light transmission (e.g. glass scintillation vials or 24 well plates) or applying overlying shading external to the test dispersion to produce identical light levels among the tested MN concentrations (note that precise details of shading regimes should be reported). If this is performed, a non-shaded control should also be tested to verify that the overlying shading does not influence the overall test results. Test vessels other than those typically employed (i.e., Erlenmeyer flasks) should be validated prior to use in tests, because one study showed that the test container (Erlenmeyer flasks, 24-well microplates, or cylindrical vials) impacted TiO_2 test results in this assay (Manier, 2015).

67. ***Quantification of biomass***: Methods such as flow cytometry, absorbance, and fluorescence detection are confounded by the presence of particles that can reflect, absorb, and/or emit photons. These and other measurement techniques may also be confounded by adherence of MNs to cells, altering their behavior within the analytical system and also making the differentiation between cells and MN problematic. Possible solutions to these problems include: (1) adding MNs to algae and then immediately performing the analysis to assess if the presence of MNs biases the measurement compared to the algae without added MNs; and (2) the use of methods that are not influenced by presence of MNs, e.g. extraction and measurement of chlorophyll A (Hund-Rinke, 2016), or where such influences can be corrected. Possible methodology, including use of optical or electron microscopy and hemocytometers are discussed by (Hartmann, 2012; Handy, 2012a; Handy, 2012b; and Kalman, 2015).

6.4.2. TG No. 221 - Lemna spp. Growth Inhibition Test

68. TG-specific issues for the testing of *Lemna* arise because of the location of the plants on the surface and upper portions of the water column. Possible modifications to address these issues are discussed below, organized by the specific section of the TG to which they apply.

69. ***Test apparatus***: To ensure that the plants are only exposed to suspended MNs, test vessels should be deep enough to prevent root contact with the bottom of the vessel, or with settled material at or near the bottom of the vessel. Agglomeration and settling is typically higher (as a percentage of added material) for higher concentrations of MNs. Root contact with this settled material could result in undocumented and un-quantified variation

in exposure. When this outcome is observed it should be reported, and depending on the consistency of exposure-response may require use of test vessels deep enough so that root tips get no closer than 1 cm from the bottom of the vessels

70. ***Test solutions:*** *Lemna* spp. float on the surface and may be exposed to MNs that collect at the water-air interface (e.g. poorly water-soluble, dispersible, hydrophobic, or surface-active substances). Under such circumstances exposure will involve material that is not in dispersion, resulting in ecologically relevant but undocumented and un-quantified exposure levels. It may be necessary to experiment with application of dispersants or test-specific dispersion approaches as described in Section 5 to avoid these exposure conditions depending upon the goal of the testing.

6.4.3. TG No. 236 - Fish Embryo Acute Toxicity (FET) Test

71. Note that OECD TG 236 may be used within a weight of evidence approach together with other independent, adequate, relevant and reliable sources of information leading to the conclusion that the substance is or is not acutely toxic to fish (ECHA, 2016; Hund-Rinke K., 2017). TG 236 (OECD TG 236., 2013b) describes an acute (96-hour) test using zebrafish embryos exposed in 24-well plates. Most of the issues associated with testing MNs described previously apply to this test.

72. An additional concern is uncertainty and inconsistency in exposure levels due to test materials settling to the bottom of exposure wells, where embryos are located, resulting in exposure levels higher than those represented by initial test dispersion measurements. Since the extent of settling is likely to vary with exposure concentration, this effect may not be consistent across treatment levels. Preliminary testing described in Section 5.2 should include measurements made in the 24-well plates to be used in the assay, with sampling and analyses of both the overlying test dispersion and settled material. It is also suggested that preliminary testing be done in the presence of embryos. The results of such testing will indicate whether test dispersion renewals, or MN measurement intervals should be modified to account for the settling effects on exposure. The sensitivity of the embryos to not being immersed in test media is an important factor to consider with regards to test media renewal.

6.4.4. TG No. 238 - Sediment-Free Myriophyllum spicatum Toxicity Test and TG No. 239 Water-Sediment Myriophyllum spicatum Toxicity Test

73. OECD TGs 238 and 239 are intended as high-tier toxicity tests of substance effects on aquatic plant growth, using the submersed aquatic milfoil, *Myriophyllum spicatum*, maintained under sterile assay conditions. The test can be done without or with sediments (OECD TG 238., 2014b and OECD TG 239., 2014c, respectively). When sediments are included, the test substance can be either added directly to the water phase or mixed in sediments. Specific issues with these tests are described below.

74. ***Requirement for Sterile Conditions*** - The need to maintain sterility in the test system might be a problem given the absence of any guidance on sterilizing MNs and concerns with how sterilization might alter characteristics or properties of the nascent material. The need for sterility also complicates test dispersion renewals, which are discussed in TGs 238 (OECD TG 238., 2014b and OECD TG 239., 2014c) for test substances that cannot be maintained in non-renewal conditions at 80% or more of initial concentrations. The effects of sterilization on the test MN should be fully evaluated on a case-by-case prior to initiating either of these tests.

75. ***Specific test medium (Andrews)*** - While not a unique concern relative to other OECD aquatic TGs, modified Andrews media stipulated here contains 3% sucrose; an additive that is likely untested for its effect on MN stability. This issue should be addressed by preliminary testing and evaluation of material stability in this test medium. However, the TG does not accommodate test media modifications that might increase test material stability.

76. ***When Sediments are Tested*** - TG 239 (OECD TG 239., 2014c)provides for addition of test substances to either the water phase or mixed into test sediment. Key considerations in the latter case are covered in previous discussions of other sediment TGs. However, when the tested MN is to be added to the water phase some consideration should be given to stability, and the potential for MNs to agglomerate and settle on sediment surfaces. TG 239 (OECD TG 239., 2014c) is intended to be used in higher tier testing (e.g. in pesticide risk assessment) and provides for consideration of the environmental fate of the test substance, including possible rapid partitioning of test substances to sediments. However, the particulate nature of MNs and their tendency to attach to sediment particles may limit their diffusion to lower sediment layers, resulting in limited exposure of the *M. spicatum* root system. As with soluble test substances, it may be decided that settling of the tested MNs, and sediment surface-only exposures represent acceptable environmentally-realistic fate and exposure. In other cases, it may be preferred to mix the test MN directly in sediments. These decisions will necessarily be made on a case by case basis, depending on regulatory requirements, and may require some determination of MN fate and behavior in test sediments.

6.4.5. TG No. 242 - Potamopyrgus antipodarum Reproduction Test and TG No. 243 Lymnaea stagnalis Reproduction Test

77. TGs 242 (OECD TG 242, 2016b) and 243 (OECD TG 243, 2016c) are chronic (28-d), aquatic, reproductive toxicity tests using snails. Both test species are surface grazers and feed using a scraping radula. TG 242 (OECD TG 242., 2016b) recommends feeding (fish flakes) once per day but no less than three times per week; TG 243 (OECD TG 243, 2016c) stipulates feeding (lettuce) once per day. The test dispersion is renewed two times per week in TG 242 (OECD TG 242, 2016b) and three times per week in TG 243 (OECD TG 243, 2016c). The surface feeding behavior of these test species and the required frequent addition of food introduces test-specific issues for MNs including agglomeration, settling, routes of material uptake and unquantified variability in exposure level.

78. ***Effects of food***: MNs typically attach to surfaces, in particular organic (or food) surfaces. This suggests that a primary pathway for uptake will be MNs ingested with food. The extent of this effect will vary with the specific MN tested and possibly with treatment concentration. Both of these TGs describe exposure level in terms of concentration in water and do not address substance concentration in ingested food. The relationship between water concentration and amount of MN attachment to food is likely to be inconsistent or unpredictable, resulting in significant uncertainty or error in exposure estimation, and over or underestimation of toxicity; however, it is also possible that MN attachment to food may be more consistent for some MNs. These issues might also complicate comparison of different MNs, or forms of the same MN due to differences in attachment behaviors. These issues apply as to soluble chemicals but are exacerbated by the particulate form and settling behavior of MNs.

79. ***Effects of agglomeration and settling***: Variation in exposure due to agglomeration and settling of particles has been discussed previously in this GD (Section 6.4.5). However, the issue in TG 242 (OECD TG 242., 2016b) and 243 (OECD TG 243., 2016c) is related to increased exposure of test organisms to settled material rather than or in addition to decreased exposure to suspended particles. This material behavior has implications for either exposure estimation or measurement and varying modes of uptake and is exacerbated by the effect of food substances on particle stability and the surface feeding behavior of the test organisms. It is also important to add that TG 242 (OECD TG 242, 2016b) recommends the addition of sea salt at a concentration of 0.3 g/L, increasing ionic strength and, hence the instability and settling rate for many MNs.

80. These are MN-specific issues that have been identified as research problems in the scientific community. Thus, there is little prescriptive guidance that can be recommended. However, it is recommended that material behavior be assessed and reported under conditions very similar to those actually used in organism exposure, including frequency of feeding and test dispersion renewal.

6.4.6. Alternative Hazard Testing

81. There is significant interest in development of new toxicity testing approaches that reduce use of vertebrate animals in testing and may also provide for rapid assessment of potential adverse impacts based on testing for responses in specific biological processes, including gene expression, cell growth, etc. (Hjorth, 2017b; Shatkin, 2016; Thomas, 2011). Many of these alternative tests involve using high throughput screening (HTS) techniques (Hjorth, 2017a; Wang, 2013) that employ bacterial and cell culture-based assays (described by (Tanneberger, 2010; Tanneberger, 2012), gene microchip arrays, etc., and target endpoints including cytotoxicity, alteration of cell growth, activation of transcription factors, gene expression levels, and many others. HTS screening assays, in particular, have been touted as one approach to expedite the hazard assessment of the ever-increasing number and complexity of MNs (Hjorth, 2017a; Shatink, 2016; Damoiseaux, 2011).

82. High-throughput and other alternative testing procedures to animal testing have yet to be formalized in TGs or GDs for MNs or non-MN ecotoxicity testing. They are discussed here because it is reasonably likely they will be formalized in the future and will require novel considerations for hazard testing of MNs. There are several examples from European and US legislation that require, or strongly encourage, the replacement of animal testing (US EPA, 2019; Comission Directive 2010/63/EU; Comission Regulations (EC) 1272/2008; 1223/2009; and 1907/2006) or that minimize animal usage (Nel, 2017). While the focus of this reduction is on vertebrate testing, discretionary efforts may also be made to reduce use of invertebrates.

83. However, to date, alterative hazard testing approaches for MNs ecotoxicity testing remain largely experimental, have yet to be standardized, and often require use of complex test media comprised of salts, proteins (bovine serum albumin; BSA, and fetal bovine serum; FBS), and other biological substances that can dramatically alter the characteristics and behavior of MNs such as through a protein corona on the NMs and thereby impact the toxicity observed.[7] These effects may also be exacerbated by the use of very small exposure volumes (Damoiseaux, 2011). Alternative tests also require use of exposure approaches and systems (e.g. gene arrays, single layer cell cultures, etc.) that differ from those currently used in ecotoxicity TGs. In addition, these assays often require measurement and analytical methods that might be confounded by the presence of MNs, for example, plate reader-based systems that measure luminescence, fluorescence, absorption, etc. However, interlaboratory studies with MNs have been successfully performed on some cellular assays (Xia, 2013; Elliott, 2017; Piret, 2017). In these studies, approaches to minimize or correct for MN interferences were developed and utilized. Currently, alternative testing methods are most effective when used to elucidate fundamental knowledge (e.g., mechanism of toxicity, mode of action) that could be used in developing testing strategies and overall risk interpretation. This is of special importance for MNs and their individual forms. However, their further development may lead to their more robust use in regulatory testing.

84. Given the current state of development of alternative tests for ecotoxicity endpoints, no assay-specific guidance can be offered here. However, the need for characterization of test MNs and development of methods for producing repeatable and quantifiable exposures discussed for current TGs apply equally for alternative tests. In addition, the small test volumes and high volume to surface area ratios present in many alternative tests will present challenges for monitoring and maintaining exposures (Wang, 2013). If alternative tests are standardized as formal TGs, it will be necessary to evaluate their applicability to testing of MNs, and potentially draft MN-specific guidance for their use.

[7]It is relevant to note that animals are used in the production of the BSA and FBS and therefore assays which use these constituents do not entirely replace animal usage during the test method.

7. Data analysis and reporting (Nanomaterial-specific)

85. These reporting elements are supplemental for MN-specific testing to general reporting requirements already prescribed in each toxicity test TG (Table 1). Thus, it is not the intention to list reporting requirements already covered by existing toxicity test TGs (e.g., date, water quality, food, etc.) in this section.

7.1. Test Report

86. The parameters summarized in Table 6 should be reported for each test conducted. It is critical that the basic properties of the MNs, details regarding preparation and testing and a robust exposure characterization be reported.

Table 6. Test reporting checklist

Category	Details	Additional Reporting Considerations
Test substance	○ Test material composition, as produced ○ Shape ○ Appearance ○ Surface chemistry (coating, functionalization) ○ Known unique[8] properties ○ Known enhanced[9] properties ○ Impurities ○ Primary particle size ○ Size distribution ○ Degree of agglomerate size ○ Surface area ○ Crystallinity ○ Storage duration and handling ○ Purity and chemical composition (principle elements, stabilizing agents, dopants, matrix composition) ○ Primary particle mean diameter, size distribution, particle size range, number of particles included in analysis	○ MN identifiers (e.g., CAS, batch, lot number, creation date) ○ Appearance (color, aqueous, powder, etc.) ○ BET surface area or geometric estimate (or other surface area technique) ○ Specific density ○ Storage and handling conditions ○ MN stability characteristics (OECD TG 318., 2017), agglomeration rate, dissolution rate, etc.) ○ Expiration date

[8] Unique, or novel, properties are properties that are not observed in the bulk material and arise as a result of smaller size.

[9] Enhanced properties are properties that are present in the bulk form but are enhanced as a result of smaller size.

Category	Details	Additional Reporting Considerations
Preparation	○ Dispersion method ○ Dispersion energy ○ Stock preparation and concentration ○ Stock stability (concentration, agglomeration, dissolution) ○ Stock dispersion age, storage, equilibration time prior to organism testing, dissolution ○ Addition or spiking method (direct, indirect) ○ Analytical methods used	○ Description of how test substance was added to test system ○ Description of any employed stabilizing agents ○ Description of toxicity control experiments of stabilizing agents if conducted ○ Description of any procedures used to evaluate the suitability of the test system (i.e. visual observations, exposure verification, size distribution confirmation, etc.) ○ Description of any stability test or other control studies done prior to addition of organism
Test conduct	○ TG used ○ Test media ○ Test vessel ○ Duration ○ Water change frequency ○ MN characterization, monitoring frequency (concentration, agglomeration, ratio total vs. dissolved) ○ Analytical methods used	○ Nominal and measured concentrations of test substance in test dispersions and controls, total vs. dissolved fractions ○ Duration, test type (i.e., static), test vessel description, medium, number of organisms per concentration, loading of organisms, light periodicity, aeration and method used for organism introduction ○ Observations, interaction potential with food, vessels, organisms ○ Full description of test medium source, characteristics and composition (water/sediment as appropriate) ○ Description and justification of dosing levels used in limit or range-finding tests ○ Any control conditions that are tested (e.g., stabilizing agent)

Category	Details	Additional Reporting Considerations
Test acceptability criteria	○ TG specific control criteria (e.g., survival) ○ TG specific water quality ○ Stability criterion (e.g., ±20%) during tests (e.g., concentration, agglomeration, dissolution) for summarizing exposure concentration (e.g., arithmetic mean, geometric mean, time weighted averaging) ○ Summary method used in dosimetry (e.g., nominal/measured, arithmetic mean, geometric mean, time weighted average) ○ Exposure metric used (e.g., mass, surface area) ○ MN stability monitoring (i.e., was it sufficient?) ○ Sufficient exposure characterization during test ○ General observations (organisms, MN behavior)	○ Sampling and characterization frequency ○ Characterization methods ○ Volume of sample removed ○ Sample storage ○ Description of test method(s) used for analysis of test substance in test dispersions
Test material stability in test media	○ Agglomeration (initial, intermediate, and final mean MN diameter and geometric standard deviation, agglomeration rates) ○ Settling (change in suspended MN number/mass concentration, observations of MN settling and MN morphology changes) ○ Transformation/degradation (dissolution, change in dissolved ion concentration and/or MN mean diameter)	○ Compared to strictly soluble, single chemicals, MNs suspended in test media have additional potential transformations that need to be evaluated in the stock dispersions, test dispersions and test systems. Additional case-by-case considerations may exist beyond what is listed at left, and the potential influence on toxicological endpoints should be discussed ○ Coating/surface functionalization stability

7.2. Exposure dosimetry

87. Adequate characterization must be performed to suitably express exposure, as recommended throughout this GD. Exposure quantification and its relationship to endpoint response should be expressed at a minimum on a mass concentration basis. When possible, it is best practice to include the mass concentration of dissolved materials (e.g. silver ions from nano-scale silver particles) because this measurement can reveal if the test species are being exposed only to MNs, ions, or a combination of the two which supports the understanding of the mode of action and to compare the toxic profiles of different nanomaterials. In addition, particle number concentration, mean particle diameter and geometric standard deviation, surface area concentration, or other metrics that may be available can be reported. OECD, (OECD, 2012a) recommends measurement of particle

counts, surface area, and mass when feasible to allow calculation of other exposure metrics. In addition, the accuracy and precision of these exposure measurements as determined from the analysis of Standard Reference Materials (SRMs), lab control and/or matrix spike control samples should be included and evaluated. The accuracy and precision of the analytical test methods should be considered during the interpretation of the test results and evaluation of toxicological endpoints.

7.2.1. Mass concentration

88. Exposure quantification is most often expressed as a mass concentration: the mass of MNs suspended in a given volume of aqueous test dispersion. Methods for sampling can be found in Section 5.5.2. This can be expressed as follows:

- The gravimetric mass of MNs added to given volume of a test system.
- The mass concentration that results from the dilution of a known volume of a stock dispersion having a traceable mass concentration.
- The mass concentration of suspended MNs as measured using some gravimetric measurement of suspended MNs in the test system.
- The mass concentration of a given element (i.e., Zn, Ag) as measured using an analytical method such as ICP-AES or ICP-MS. MN dissolution must be considered in such measurements.
- In cases where the mass concentration of only one element of a MN that is comprised of multiple elements or chemical compounds is measured, the mass concentration could be related to total MN mass using molecular formula or chemical composition of the MN. For example, for a test system containing CeO_2, where Ce is measured by ICP-MS, the mass of CeO_2 in the test system could be determined using the formula weight of CeO_2 and the measured mass of CeMN dissolution must also be considered in such measurements.

7.2.2. Metrics other than mass concentration

89. Standardized approaches for expressing exposure-response relationships based on metrics other than mass concentration are not available and research demonstrating the concept is limited (Hull, 2012; Hoheisel, 2012; Kennedy, 2015; and Mottier, 2016). Therefore, mass concentration is currently the most expedient standard dosimetry, especially considering that there are no standard methods for other exposure metrics and no clear procedure for how to use this information in a risk assessment. However, efforts can be made to collect information suitable for other exposure metrics to aid interpretation and prepare for the future when other exposure metrics can be used in risk assessments or to support understanding the mode of action and to compare the toxic profiles of different MNs. For example, the methods in Table 3 may enable an incremental improvement in measurement (or calculation) of alternative metrics to express exposure and relate it to organism response in the various test systems and controls. Other suggested metrics include:

- Particle, agglomerate number concentration or surface area concentration.
- In the case that particle size distribution data are available, the data could be used as follows:
  - combined with specific density and assuming a geometry of the MN to estimate mass concentration and surface area concentration.
  - should the resolution of the particle size distribution measurement be sufficient, the data can be parsed and the number concentration and possibly mass concentration reported over a

given size range. For example, the number concentration of MNs having diameters between 1 to 100 nm.

7.2.3. Expression of exposure-response relationships

90. Exposure-response for all metrics (e.g. material mass, particle number, surface area) should be expressed as concentration (e.g. LC/EC50, LC/EC10, LOEC, NOEC, etc.). When reporting effect levels, the metric must always be identified; e.g. mg/L or mg/kg, particle number/L or particle number/kg, surface area/L or surface area/kg.

7.2.4. Calculation of exposure-response relationships when exposure variability exceeds ± 20%

91. For tests where exposure does not remain within 20% of the initial measured values, exposure may be calculated using geometric mean or time-weighted averaging methods. The specific method to be used will be determined, in part, by the kinetics of exposure variation and on available data as described below. Additional guidance on this issue is provided in OECD GD 23 (OECD GD 23., 2018): "where a measured concentration at the end of the exposure period is absent or where it indicates that the test chemical is not detected, the validity of the test to meet regulatory requirements should be reconfirmed with the regulatory authority. In order to calculate a mean exposure concentration when the test chemical is detected but not quantified in a sample, one possible method is to use a value of half of the limit of quantification. Since there may be various methods for determining the exposure concentration, particularly when concentrations are below the limit of quantification, the method selected should be made explicit in the reporting of test results. It is also advisable to seek guidance from the regulatory authority to ensure that the method meets regulatory requirements."

- *Exposure variation is linear*: e.g., a linear decay in concentration. Exposure levels at the start and end of the test or renewal period are sufficient and exposure can be expressed using the geometric mean of the starting and ending values.
- *Exposure variation is non-linear*: e.g., a non-linear decay in concentration. Exposure level should be measured, at a minimum at the start, midpoint, and end of the test or renewal period, and preferably at three time points between the start and end of the test or renewal period. These values should then be used to calculate the time-weighted average exposure (Simpson, 2003).
- *Exposure data are limited to initial and final values*: When concentration data are limited to initial and final values, exposure can be quantified using the geometric mean of those values. However, there may be cases where the kinetics of varying exposure are well-defined and have been demonstrated to be consistent across tests. In those cases, the kinetic information may be used to calculate time-weighted average exposure.
- *Dose-response curve extends to below the mass concentration detection limit:* In cases where the higher concentrations can be quantified but the lower concentrations cannot and when there are toxicological effects observed in those concentrations below the mass detection limit, it must be reported that the actual exposure concentrations are unknown and what exhaustive analytical approaches were attempted. For tests with MNs that cannot be quantified by analytical methods at the lower exposure levels causing effects in complex matrices such as sediment, it is possible to use the MN mass loading. For pelagic testing, the approach taken will vary based upon which of the hazard testing options described in Table 4 is used but the approach must be scientifically defensible. Consider a hypothetical scenario where ecotoxicological effects are observed in a pelagic assay at nominal initial MN mass concentrations of 10 mg/L, 5 mg/L, and 0.5 mg/L; the detection limit is 2.5 mg/L; and there is a 30 % loss in the suspended MN mass concentration

for the nominal 10 mg/L and 5 mg/L concentrations during the assay despite efforts to minimize losses. If option 1 from Table 4 was used (exposure is based on settled MNs, suspended MNs, and dissolved substances), it could be reasonable to use the total MN mass added to the test system for the 0.5 mg/L concentration. If option 2 from Table 4 was used (exposure is based on the concentration of suspended MNs and dissolved substances), it could be reasonable for the 0.5 mg/L concentration to base the exposure concentration on a dilution from the lowest concentration that can be measured and a loss of 30 % during the exposure period.

7.3. Implementation

92. The general scope of this document is to provide guidance for improving MN exposure consistency, monitoring and characterization using existing OECD TGs to enable more repeatable, reliable and usable ecotoxicological results. Robust prescriptive and interpretative guidance is beyond scope of this GD due to lack of precedent for how MN-specific attributes previously discussed (e.g., agglomeration vs. single particle exposures, suspended particles vs. settled particles, physical vs. chemical toxicity) will be handled in an ecological hazard assessment and risk assessment and if the mass-only exposure paradigm will continue to be the standard for MNs. If a distrinction between chemical and physical effects can be made, this should be reported.

93. Use of this document should facilitate more robust exposure monitoring and reporting of the dynamic changes that may occur in a MN exposure. Reporting additional supplementary information may be considered, such as using comparative controls (Table 5) and complementary exposure scenarios (Table 4) to generate and document lines of evidence regarding whether any observed ecotoxicological effects resulting from the TGs are most likely due to exposure to dissolved (aqueous) chemical, suspended particles or settled particles.

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