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Identification and Avoidance of Potential Artifacts and Misinterpretations in Nanomaterial Ecotoxicity Measurements

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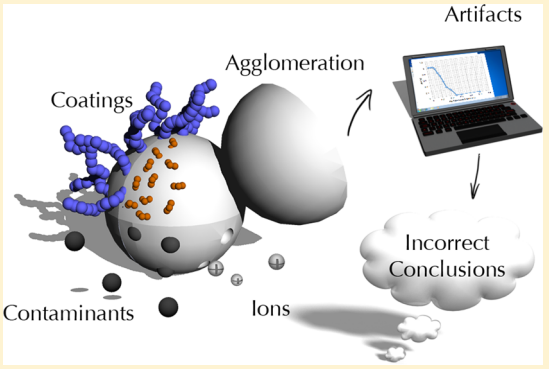
ABSTRACT: Novel physicochemistries of engineered nanomaterials (ENMs) offer considerable commercial potential for new products and processes, but also the possibility of unforeseen and negative consequences upon ENM release into the environment. Investigations of ENM ecotoxicity have revealed that the unique properties of ENMs and a lack of appropriate test methods can lead to results that are inaccurate or not reproducible. The occurrence of spurious results or misinterpretations of results from ENM toxicity tests that are unique to investigations of ENMs (as opposed to traditional toxicants) have been reported, but have not yet been systemically reviewed. Our objective in this manuscript is to highlight artifacts and misinterpretations that can occur at each step of ecotoxicity testing: procurement or synthesis of the ENMs and assessment of potential toxic impurities such as metals or endotoxins, ENM storage, dispersion of the ENMs in the test medium, direct interference with assay reagents and unacknowledged indirect effects such as nutrient depletion during the assay, and assessment of the ENM biodistribution in organisms. We recommend thorough characterization of initial ENMs including measurement of impurities, implementation of steps to minimize changes to the ENMs during storage, inclusion of a set of experimental controls (e.g., to assess impacts of nutrient depletion, ENM specific effects, impurities in ENM formulation, desorbed surface coatings, the dispersion process, and direct interference of ENM with toxicity assays), and use of orthogonal measurement methods when available to assess ENMs fate and distribution in organisms.



INTRODUCTION

The International Organization for Standardization (ISO) defines engineered nanomaterials (ENMs) as materials with any external dimension in the nanoscale or having an internal surface structure at those dimensions (between 1 and 100 nm)[1,2] and that are designed for a specific purpose or function.[2] Within the broader category of ENMs, there are nano-objects, a material with one, two, or three external dimensions in the nanoscale, and nanoparticles (NPs), which contain all three external dimensions in the nanoscale.[2,3] ENMs often have novel or enhanced properties as a result of their nanoscale size, and these properties contribute to unique or enhanced functions for use in commercial products that already impact a wide range of industries. One issue that has limited the commercialization of ENM-containing products is uncertainty regarding the potential human and ecological impacts from exposure to these materials. Given public concern about emerging technologies such as

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nanotechnology, reliable and accurate assessment of the potential toxicological effects of ENMs is critical for scientifically based risk assessments and widespread public acceptance.

The potential toxicity of an ENM (or any substance or material) is a critical consideration for their sustainable production, use, and disposal. Thus, considerable effort has been applied toward development of reliable methods for ENM toxicity assessment. As with any scientific investigation, each step in an experiment to assess toxicity has an associated uncertainty, and the amount and source of uncertainty may be known or unknown. ISO defines uncertainty as a "parameter, associated with the result of a measurement, that characterizes the dispersion of the values that could reasonably be attributed to the measurands".[4] Uncertainty may be expressed for example as a standard deviation or a confidence interval. It is essential that sources of uncertainty are identified, quantified, and then reduced by judicious changes to the experimental method. Uncertainty in toxicity test results of traditional substances (as opposed to ENMs) can result from factors such as impurities in the test material, uncertainty associated with each step of the procedure (pipetting, weighing, etc.), and inherent biological variability of test organisms. In addition to the uncertainties of measuring toxicity of traditional substances, the assessment of ENM toxicity must also consider uncertainties related to dispersion of ENMs in environmental matrices and dynamic changes that can occur to these materials during toxicity tests (e.g., dissolution, agglomeration, and interactions with materials present in test media). Some ENMs may be of minimal toxicity, in which case artifacts are not an issue; however, the conclusion of minimal toxicity could be incorrect if the test method was impacted by an artifact.

Numerous articles have reviewed the literature on the ecotoxicity of ENMs in organisms,[5−19] including effects of carbon nanotubes (CNTs),[20] titanium dioxide,[21,22] fullerenes,[23] silver nanoparticles (AgNPs),[24,25] and zinc oxide nanoparticles.[26] However, while experimental artifacts and misinterpretations that have confounded ecotoxicity tests have been identified in some cases, there has been no systematic review of potential artifacts and misinterpretations associated with ENMs testing or how these confounding factors can be minimized. For example, artifacts may be a result of ENM interference with an assay reagent[27−33] or from an unintended toxic byproduct produced during the ENM dispersion process,[34,35] while misinterpretations may occur as a result of a misattribution of the toxic effect to ENMs when actual effects are a result of particle dissolution to ions. It is important to distinguish between an artifact and measurement uncertainty. Although the magnitude of the bias that artifacts can cause will vary, an artifact is distinct from the uncertainty inherently associated with measurement at each step of a method in that an artifact indicates something fundamentally incorrect. In some cases, identification of and corrections for artifacts has completely changed the perceived ENM toxicity. For example, artifacts related to the formation of byproducts when dispersing fullerenes with tetrahydrofuran (THF) initially caused the perception that fullerenes were of toxicological importance and capable of causing neurological damage to fish.[34,35] When this artifact was recognized and corrected for, subsequent fish studies have shown minimal fullerene toxicity upon dispersal by water mixing.[23] Identification of the artifact caused by the fullerene dispersion in THF differs from the experimental uncertainty related to the inherent biological variability among the individual fish, homogeneity of the nanomaterial concentration in the media, and uncertainty from the steps in the determination of neurological damage.

Given that there are additional and not thoroughly quantified sources of uncertainty in nanoecotoxicology testing as compared to traditional chemicals, it is not yet possible to definitively determine the extent to which differences in the results of nanoecotoxicology studies stem from these sources of uncertainty, differences in the experimental procedures, or artifacts. However, robust experimental design of nanoecotoxicity tests to identify and minimize artifacts and misinterpretations is critical to improve confidence in the results obtained, enable reproducibility among different laboratories, and deliver more reliable results for use in ENM risk assessments.

In this manuscript, we systematically categorize and assess the potential for artifacts and misinterpretations at each stage of the nanoecotoxicology testing process as summarized in Figure 1 and Table 1. These stages include procurement or synthesis

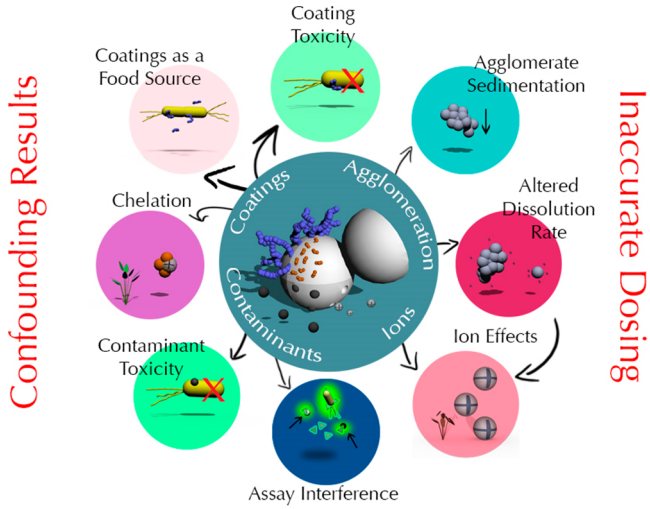

Figure 1. Graphical depiction of potential artifacts and misinterpretations in nanoecotoxicology testing.

of the ENMs (and assessment of potential toxic impurities, such as endotoxins, solvents, or metals in the ENM material); storage of the ENMs; dispersion of the ENMs, if needed, in the test medium (water, soil, or sediment); performance of the ecotoxicological assay; and potentially assessment of the ENM biodistribution in the organism. We discuss artifacts and misinterpretations that can occur when measuring various end points in organisms or ecologically relevant cells (e.g., cells or bacteria) exposed to ENMs. This review will not focus on artifacts that have been identified for toxicological assays related to human health end points or for nanomedicine (interested readers should see, for example, this review article[36]), but insights drawn from these studies concerning artifacts and misinterpretations relevant to ecotoxicological testing will be highlighted. Despite the fact that human health end points are not the focus of this review, ENM-related artifacts and misinterpretations identified with ecologically relevant organisms may also be important for human health studies. Suggestions for robust experimental design to identify and minimize ENM-related artifacts and misinterpretations are also provided and discussed at length.

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Table 1. Summary of Artifacts and Misinterpretations and Test Strategies or Control Experiments to Avoid or Minimize These Counfounding Factors

test stage	artifact or misinterpretation	explanation	tests most likely to be affected	test modification(s) or control experiment(s) to avoid or minimize them
procurement/synthesis	unacknowledged impurity (e.g., metal) causes toxic effect	insufficient ENM characterization leads to overestimation of ENM toxicity when unrecognized impurities had a large effect	organism or cell studies sensitive to contaminants present in ENMs	improved nanomaterial characterization, filtrate-only control experiment
procurement/synthesis	unacknowledged endotoxin impurity causes toxic effect	endotoxin contamination causes an effect which is misattributed to ENMs	organism or cell assays sensitive to endotoxins	improved nanomaterial characterization, filtrate-only control experiment, endotoxin inhibition/enhancement control experiment, testing sensitivity of assay for endotoxins
ENM storage	Unexpected changes to ENM (dissolution, agglomeration, oxidation, etc.)	ENM may change in unexpected ways during storage. This could impact interpretation of toxicity results if changes are not fully accounted for	all tests	careful reporting of storage conditions, recharacterization of material periodically and before experiment, take steps to minimize changes to ENMs during storage, repeat purification steps (e.g., dialysis) shortly before experiment
ENM storage	unexpected changes to ENM coatings	coatings may desorb from ENMs; coatings may also degrade	all tests	filtrate only control to assess impact of desorbed or degraded coating
dispersion	ultrasonication-induced artifacts	ultrasonication may cause multiple undesirable and hard to quantify changes such as degradation of organic molecules, alterations to ENM surfaces, and particle sintering	all tests	optimize sonication protocol to minimize artifacts, avoid sonication of organic chemicals when possible, conduct control experiments by sonicating organic chemicals by themselves or media by itself and testing toxicological effects, minimize sonication duration
dispersion	THF-related artifacts from fullerene dispersion	unexpected toxic byproducts from THF were produced by the dispersion process and observed ecological effects were mistakenly attributed to fullerenes	all tests	filtrate-only control, use a different method such as water mixing to disperse fullerenes
dispersion	ENM mixing	it is challenging to assess the homogeneity of distribution of ENMs in solid media and to characterize changes to the ENMs during mixing	tests with agar, soil, sediments, or bacteria disk diffusion tests	characterize ENMs in media and their homogeneity in the media to the extent possible, include inert markers to assess homogeneity after mixing
conducting ecotoxicity assays	unacknowledged indirect effects (shading)	some indirect effects such as shading can lead to misunderstanding of toxicity mechanism	tests with photosynthetic organisms	quantify reduction in light from ENMs and test impact of this degree of light reduction
conducting ecotoxicity assays	unacknowledged indirect effects (nutrient depletion)	some indirect effects such as nutrient depletion can lead to misunderstanding of toxicity mechanism or overestimation of ENM toxicity	tests with ENMs with high sorption coefficients, tests with media containing micronutrients	conduct control experiment by preincubating ENMs with media and then removing ENMs, sorption experiments for media constituents with ENMs, measure element concentrations in organism tissues to assess nutrient depletion, increase concentration of trace elements in test media and observe extent of changes in end points
conducting ecotoxicity assays	direct interaction between ENMs and test reagents or biomolecules	ENMs may adsorb biomolecules or test reagents or interact with them in an unexpected manner that influences the test result	cellular tests, organism tests that rely upon detection of or effects on biomolecules	test end points using orthogonal methods (i.e., multiple cytotoxicity assays), conduct 0 h controls to assess if ENMs cause an apparent effect, sorption experiments with reagents or biomolecules and ENMs
conducting ecotoxicity assays	ENM produces a signal similar to assay measurand	ENMs may fluoresce, absorb light, or have other behaviors that cannot be distinguished from those of the measurand	cellular tests, organism tests that rely upon detection of or effects on biomolecules	conduct control experiments with ENMs and the analytical method of interest, conduct 0 h control experiments to assess if a toxicity response is observed
conducting ecotoxicity assays	damage to biomolecules or cells occurs after the exposure period	ENMs may cause an effect after the toxicity experiment has ended but during processing of the cells or tissues for end point analysis	tests with sufficient concentrations of ENMs in the tissues or cells after the exposure period, tests with photoactive ENMs	test a 0 h control to assess if there is apparent toxicity, conduct postprocessing using light in a wavelength range that will not excite photoactive ENMs

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Table 1. continued

test stage	artifact or misinterpretation	explanation	tests most likely to be affected	test modification(s) or control experiment(s) to avoid or minimize them
conducting ecotoxicity assays	dynamic changes to ENMs during testing lead to inaccurate dosing	ENMs may undergo a number of changes (settling, dissolution, agglomeration, etc.) that substantially change the dose the organism receives and not accounting for this can lead to inaccurate dosing	All tests	test larger particles (i.e., micrometer-sized particles) and dissolved ions (if relevant) to determine if there are ENM-specific effects, characterize ENMs in the exposure media to reveal the dosage the organism or cells receive across time and changes to the ENMs during the exposure
conducting ecotoxicity assays	changes in cell agglomeration from ENM exposure cause artifactual results	ENMs may cause cells to agglomerate in an unexpected manner which confound typical cell counting methods	bacteria assays and other assays with cells suspended in solution	test number concentrations of cells using orthogonal methods
ENM characterization in organism tissues and cells	mischaracterization of ENMs in cells and tissues	there are possible artifacts related to characterizing ENMs in tissues that could result in an overestimation or underestimation of the ENM concentration	all tests	utilize orthogonal methods when available, assess changes to ENMs in exposure media with and without organism(s), conduct measurements with dissolved ions for metal nanoparticles to assess potential for ENM formation in organism, cell, or media

SYNTHESIS OR PROCUREMENT OF ENMS

Numerous studies in recent years have highlighted the importance of adequate ENM characterization and have noted the frequent discrepancy between ENM characterization information provided by manufacturers and those independently measured in the laboratory.[36−42] Thus, independent characterization is critical. Accurate and traceable ENM characterization of the starting materials is now possible because of the availability of reference materials for some ENMs[43] and standard methods for ENM characterization (e.g., single-wall carbon nanotubes (SWCNTs)).[44−55] Suggested minimum characterization information provided by various reports has recently been compiled[56] and that information will not be repeated here. In addition to characterizing the properties of the ENMs, the purity of the ENM powder or suspension needs to be carefully assessed as impurities in this material may contribute to or be wholly responsible for observed toxic effects. Thus, it may be more accurate to consider ENMs as a complex mixture that may also unintentionally contain impurities from the synthesis process, as well as components intentionally added such as surface coatings and dispersants. One example of an impurity that can potentially cause toxic effects in subsequent toxicological assays is metal impurities, such as yttrium[57] and nickel,[58] from the catalyst particles used to synthesize carbon nanotubes. This could cause a misinterpretation if the toxicity observed is attributed to carbon nanotubes themselves rather than a specific type of carbon nanotubes with a certain residual concentration of impurities. In other words, it is inappropriate to generalize a toxicity result as representative of carbon nanotubes when the toxicity is actually caused by an impurity. In addition, ecotoxicity studies using leachate from as-produced fullerenes and metallofullerenes have shown that the waste byproducts such as metal impurities from nanoparticle synthesis can also cause ecotoxicological impacts.[59] If toxic impurities are associated with ENMs as a result of industrial synthesis of ENMs, these impurities will also be present during their use in commercial applications and are relevant in the context of ENM release into the environment and their ecological effects. However, correctly attributing toxicity to ENMs or the impurities is important.

Endotoxins are another potential impurity of ENMs that have led to artifacts and misinterpretations in some cytotoxicity studies.[60,68−70] For example, one cytotoxicity study with AuNPs showed that the biological effect was solely a result of endotoxin contamination in the initial AuNP formulation.[60] Moreover, some recent studies demonstrate that certain ENMs may synergistically enhance the inflammatory properties of endotoxin.[61−66] The potential that endotoxins have created artifacts in ecotoxicity tests with whole organisms is unknown and must be considered further. In a recent study on the effects of commercially purchased AgNPs with *C. elegans*, endotoxin contamination of the AgNPs had an important effect on growth inhibition.[67] Samples of 10 nm AgNPs from the same manufacturer but different lots had significantly different impacts on organism growth inhibition, a difference consistent with the observation of high concentration of endotoxins in one lot of the AgNPs.[67] Because of the large surface-to-volume ratios of ENMs and conditions commonly employed for synthesis of custom-made and commercial research grade ENMs, these nanomaterials are thought to be of particular risk of endotoxin contamination.[68−70] Thus, the potential for

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Table 2. Summary of Potential Control Experiments to Minimize Artifacts and Misinterpretations

potential control experiments	purpose(s)	references
0 h control	test if ENMs causes a toxicological effect (e.g., DNA damage) during processing steps after conclusion of exposure period test if ENMs would interact with test reagents or biomolecules and cause a false negative or false positive result	191,195
coating control	test if coating has toxicological or stimulatory effects on organisms or cells	92
direct interference control (production of a signal similar to measurand)	assess if ENMs produce a signal (e.g., absorbance, fluorescence) that could impact the analytical method	191
dispersant control	test if dispersant has toxicological or stimulatory effects on organisms or cells	137,138
dissolved ion control	allows for comparison of end points between ENM and constituent dissolved ions assess if NP formation could occur from ions in test media or in organism or cells	145,216
endotoxin inhibition/enhancement control	assess if there is an impact of ENMs on the effects of endotoxins on a specific end point	68
filtrate only control	assess potential toxicity of contaminants on and dissolution from ENMs from the synthesis, storage, and dispersion processes	92
larger/bulk particle control	allows for comparison of end points with ENMs and if nanospecific effects are observed	145
mixing control	assess extent of mixing using inert markers	141
nutrient depletion control	assess extent to which adsorption of media constituents by ENMs could have an indirect toxicity effect on end points	233
shading control	assess light intensity reduction caused by ENMs and if that could impact the end points being studied	147,148
sonication control with media and organic chemicals/coatings	investigate possible changes to media constituents or toxicological properties of organic chemicals from sonication	120

endotoxin contamination to impact nanoecotoxicology results in eukaryotic systems will be discussed in depth.

An endotoxin is a molecule of lipopolysacharide (LPS) that is an essential part of the outer membrane of gram-negative bacteria.[71] While various bacterial strains express LPS with different compositions, the principal structure is the same in all bacteria in that it contains two main components: (1) a lipid A structure, composed of a disaccharide backbone, negatively charged phosphates, and fatty acids, and (2) a polysaccharide structure of various length composed of either an inner core; an inner core and an outer core; or an inner core, outer core, and O-antigen.[68,71] These two LPS components have different biological functions: the lipid A molecule is the biologically active, immunotoxic part of LPS, while the polysaccharide structure has antigenic properties. Although the terms "endotoxin" and "lipopolysaccharide" (LPS) are often used interchangeably, endotoxin specifically refers to a less pure, crude form of LPS.[72] The biological activity of LPS is often described in endotoxin units (EU) which depend on the number of fatty acids and negatively charged phosphates on the lipid A structure, and may vary among bacterial strains.[71] The biological activity or potency of endotoxin is commonly determined by analysis of individual endotoxins in a bioassay, such as the Limulus Amoebocyte Lysate (LAL) assay. One can approximate the biological activity of endotoxin by converting its mass into endotoxin units (EU) using the following equation: 100 pg of endotoxin = 1 EU.[71]

Endotoxins are very common in water and air, and are often found in or on many common laboratory reagents and tools.[68] Water purification systems, reverse osmosis membranes, deionized resins, glass surfaces and chromatography columns have been reported as major endotoxin sources in research materials synthesized in laboratories.[72] Unlike bacteria themselves, endotoxins are remarkably stable—they tolerate high temperatures (up to 200 °C) and are resistant to boiling and even autoclaving.[73] When ENMs are synthesized under nonaseptic conditions and using traditional, nonsterile, non-depyrogenated reagents, which is often the case for commercial nonbiomedical grade ENMs, they may be contaminated with endotoxins.[60] Even ENMs intended for biomedical applications often fail in preclinical development due to endotoxin. For example, >30% of research-grade EMNs intended for biomedical applications contained endotoxin levels per dose above that mandated as acceptable by the U.S. Pharmacopoiea.[36,74] Endotoxin can also be introduced to sterile, pyrogen-free ENM during storage and handling,[68,75] and removing them from formulations may prove challenging due to their pH and temperature stability. They can be removed from an aqueous solution using a 10 000 MWCO or smaller ultrafiltration device or by anion exchange.[76] However it is more challenging and often impossible to remove endotoxin from ENMs because of adsorption to ENM surfaces.[68,75] Methods for endotoxin removal are specific to the type of nanoparticles because many ENMs do not tolerate traditional sterilization and depyrogenation methods.[41,68,75]

It is not possible to provide general guidelines about endotoxin doses that would impact results for a given ecotoxicology end point as a result of the substantial variability

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in endotoxin sensitivity among organisms.[71,77,78] Numerous ecotoxicology studies have reported minimal toxicity for various ENMs (e.g., C_{60}, TiO_2−NPs), and the minimal toxicity suggests that endotoxins were not an issue in those tests, or that end points influenced by endotoxins were not assessed. It is also important to recognize that the potential presence of endotoxins on common laboratory reagents and tools (e.g., glassware and organism exposure containers) are not issues unique to nanotoxicology (i.e., these issues are the same for any type of ecotoxicity test) and concern must not be overstated or receive undue speculation. Given the broad range of endotoxin concentrations present in some ENM formulations, it is also not possible to predict whether endotoxin contamination in these formulations would be substantially higher than those in other chemicals typically used in ecotoxicology tests or those present in typical laboratory reagents (i.e., distilled deionized water). However, the results found in studies thus far suggest that endotoxin contamination may lead to overestimations of ENM toxicity for some end points.[60−67] Taking this factor into account may help account for differences among some nanoecotoxicology studies.

Sufficient ENM characterization can help minimize overestimations of ENM toxicity and incorrect determination of toxicity mechanisms from the unacknowledged presence of toxic impurities (e.g., metals, organic compounds, and endotoxins). As will be discussed in a later section, a filtrate-only control may be helpful for assessing the toxicity of released compounds from ENMs; a summary of all potential control experiments is provided in Table 2. In addition, the concentration of endotoxins in ENM formulations of purchased or synthesized materials is rarely reported in nanoecotoxicology studies and ecotoxicology related laboratories typically do not have experience with making these measurements. It is important to consider the amount of endotoxin in ENM formulations with respect to the dose used in nanoecotoxicology studies so as to understand potential effects on the data. For example, if 1 mg of specific ENM contains 1 EU of endotoxin, the test model is sensitive to 10 EU/mL of endotoxin, and the ENM does not exaggerate endotoxin responses, then endotoxin presence in the ENM will not confound the results of the study provided the highest ENM concentration tested in vitro is less than 10 mg/mL. However, if the sensitivity of the test model is 0.1 EU/mL or if the ENM exaggerate endotoxin responses, then this level of endotoxin will be problematic. Consequently, the following steps are important considerations prior to ecotoxicological investigations in order to avoid endotoxin-mediated artifacts: (1) amount of endotoxin per mg of ENM; (2) sensitivity of test-system or test-species to endotoxin;[77,78] (3) relevance between the endotoxin amount in ENM and model/species sensitivity; and (4) ability of the ENM to exaggerate endotoxin-mediated responses. Validation experiments including inhibition/enhancement controls[68] and assessment of the test system sensitivity to endotoxin with and without the ENM are important prerequisites to a sound toxicological study. Test guidelines for quantifying endotoxin concentrations have been recently described,[74,79] as has the potential for ENMs to interfere with the endotoxin assays.[80]

■ ENMS STORAGE

While careful characterization of initial ENMs is critical, the definition of true "initial" conditions may be ambiguous,[81] and substantial changes to the ENMs can occur during storage that could significantly impact their toxicity. ENMs stored in a suspension are particularly susceptible to physical and chemical changes during storage. Some of the changes that can occur include particle dissolution (resulting in smaller particles and the release of ions that themselves may be toxic),[82−84] particle agglomeration,[85] particle oxidation,[86] or changes to the surface coating.[87] In addition, evaporation of solvents could affect ENM concentrations. In this review, we use ISO definitions to describe particle assemblages: the term agglomerate refers to assemblages of particles held together by relatively weak forces (e.g., van der Waals, capillary, or electrostatic) while aggregates are discrete assemblages of primary particles that are strongly bonded (i.e., fused, sintered, or metallically bonded).[3] A specific example of storage induced artifacts in particle suspensions is the storage of nanoscale zerovalent iron (nZVI) in solution for an extended period inducing "aging" (oxidation) and decreasing its toxicity to a mammalian cell line.[86] Aging depends upon the materials and storage conditions, and the demands of the application. For example, AgNP suspensions with low dissolved oxygen content, infrequent opening and closing of the container, and storage in the dark at 4 °C may be suitable for up to 6 months; however, a shelf life study measuring key parameters to determine material quality for that specific application is always recommended. It is also possible that ENMs could interact with endotoxins which could impact their physicochemical properties.[88] Storage of powders in air could also lead to oxidation (e.g., AgNP, nanoscale zerovalent iron(nZVI), or CuO)[89] or adsorption of small molecules. Adsorption of water vapor by powders could lead to inaccurate ENM mass measurements.[90]

One potentially important change that can occur is the release of the surface coating from the ENM, which could cause an inaccurate measurement of ENM toxicity if the coating itself can exert a toxic or stimulatory effect. Many macromolecular coatings are physisorbed to particle surfaces, and these coatings can partially desorb over time, with less than 30% (by mass) typically desorbed after four months.[91] For example, a recent study found that release of a polyethyleneimine (PEI) coatings from multiwall carbon nanotubes (MWCNTs) during storage increased *Daphnia magna* immobilization.[92] Even though the PEI coated MWCNTs had initially been dialyzed to remove synthesis byproducts, filtrate from these MWCNTs after storage in a refrigerator for several months caused 18% immobilization. When the PEI-MWCNTs were dialyzed a second time prior to the immobilization assay, the calculated 24 h EC50 value increased by 69%. In addition, it is possible that surface coatings that desorb from the ENMs during storage or during an ecotoxicity test could indirectly influence toxicity results if they act as chelators and bind dissolved ions from the ENMs. For example, free citrate was postulated to mitigate the toxicity of silver ions when *C. elegans* were exposed to citrate stabilized AgNPs.[93]

Another example of a nanomaterial subject to storage considerations influencing environmental study end points is ceria nanoparticles.[94] Both the synthesis conditions and thermal history of ceria nanoparticles during storage have been suggested as impacting observed physicochemical properties and biological effects. Additionally, storage time from freshly prepared to 1 day to 3 weeks old showed widely varied oxidation state distributions between Ce^{3+} and Ce^{4+}.[95] Thus, reporting characterization data from the materials just prior to introduction can mitigate the risk of unknown or unexpected changes to the ENMs during exposure. Nevertheless, it is

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critical to report seemingly trivial details of the times, storage conditions and processing history between the initial synthesis or characterization of as-received nanoparticles and when they are actually used in experiments.

Changes over time under seemingly appropriate storage conditions have also been well-documented with AgNPs. While many reviews on AgNPs have been published,[25,96,97] most have focused on other topics, leaving a knowledge gap on the role storage conditions could play on the comparability of results across studies. Kittler et al.[98] were among the first to systematically demonstrate that AgNP storage conditions can influence results of antibacterial activity. Storage of AgNPs was also shown to increase particle toxicity to freshwater zooplankton *Ceriodaphnia dubia*.[99] If Ag-NP storage conditions, incorrect or otherwise, are not taken into account, changes that can occur can lead to inaccuracies in subsequent nano-ecotoxicological testing. A nonexhaustive illustration of potential transformations AgNPs can undergo during storage and/or during experimentation with aquatic organisms is shown in Figure 2. A recent study has shown that degradation

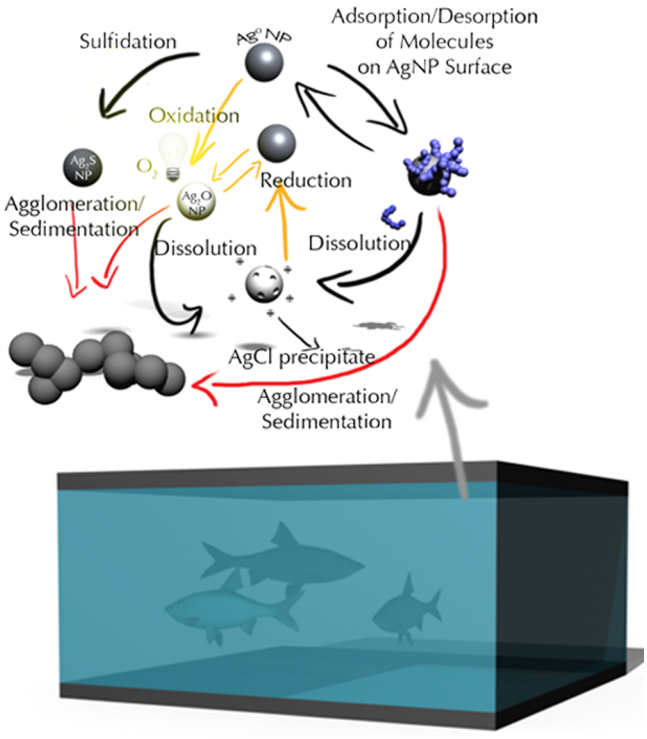

Figure 2. Possible physicochemical transformations of silver nanoparticles during storage or ecotoxicology testing with aquatic organisms. Red lines indicate transformations that remove the AgNPs from the aqueous phase. Yellow lines indicate transformations that can occur as a result of laboratory light. Black lines describe transformations that can occur in the aqueous phase in the dark.

products from the citrate capping agent on AgNPs lead to colloidal instability.[87] Additional studies have shown that AgNPs exposed to UV-light close to the solar spectrum intensities can cause AgNP oxidation and subsequent ion dissolution over a period of days, with a release rates increasing as the AgNP diameter decreases.[100] Sample preparation and storage was also shown to introduce characterization artifacts, such as the formation of new AgNPs in the vicinity of the parent AgNPs when stored in ambient conditions with greater than 50% relative humidity,[101] further complicating how environmental responses are attributed to the original suspensions. Storage conditions of fullerenes could also lead to artifacts, because fullerenes may also be photodegraded in water under sunlight, visible light, or UV irradiation leading to the formation of byproducts that caused *E. coli* inactivation.[102−106]

One of the most critical changes that can occur during ENM storage for metal and metal oxide nanoparticles is particle dissolution. However, measuring nanoparticle dissolution has proven particularly challenging. Separation by centrifugation does not always remove the smallest nanoparticles (for example AgNPs < 4 nm), while ultrafiltration membranes may lead to significant silver ion losses.[107] However, advanced measurement techniques are currently under development. For example, single nanoparticle inductively coupled plasma mass spectrometry (spICP-MS) enables size distribution, particle concentration, and dissolved fraction measurement simultaneously, albeit with minimum particle size requirements often 20 nm or larger.[108,109] Metal and metal oxide nanoparticles and nanowires, such as silver nanowires, titanium dioxide, and cerium oxide, that react or dissolve slowly have been shown to be amenable to spICP-MS; however, rapidly dissolving NPs such as some forms of zinc oxide NPs may challenge this technique if dissolution proceeds more quickly than analytical capability.[110] These advances in metrology will enable more sophisticated and accurate interpretation of environmental end points where observations must disentangle dissolved ion effects from particle-specific effects.

Steps also should be taken to minimize unexpected changes to ENMs during storage, although the appropriate steps vary among different ENMs. Recently, a list of best practices for storing AgNPs was published,[87] which can be summarized as the following: limit light exposure; bubble nitrogen gas through suspensions before recapping the bottle; higher concentrations of both silver and citrate are better; and colder storage yields slower degradation. Understanding the conditions that can lead to the most serious changes in the ENM dispersion (aggregation, dissolution, or photolysis) can help prevent these confounding factors. If a purification step was taken to remove impurities from the ENM dispersion, it may be helpful to repeat this step shortly before ecotoxicological testing. To enable the most reliable comparison of studies, detailed reporting of the storage conditions and measurement protocols used should accompany every manuscript. These steps are intended to keep the ENM from changing during storage conditions, not to represent what would occur to ENMs after environmental release, which is also an important but different research focus.

■ DISPERSION OF ENMS IN TEST MEDIA

Artifacts and misinterpretations can occur both during the preparation of an aqueous suspension of ENMs, and mixing the ENMs in the test medium for experiments conducted with soils and sediments. There are different potential artifacts that can occur during the preparation of ENM dispersion depending upon sonication intensity (i.e., probe vs bath) or if an alternative approach (such as the use of organic solvents with fullerenes (nC_{60})) is used. These topics will be discussed separately, as will issues related to characterization of the ENMs in the dispersion. Potential artifacts and misinterpretations related to mixing ENMs in soils and sediments will then be described.

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Production of ENM Dispersions Using Ultrasonication. The state of agglomeration/aggregation has been implicated as a mitigating factor in the transport, cellular level interactions, and fate of ENMs in the environment.[111−113] In order to assess the ecotoxicity and fate of ENMs that exist in dry form, the ENM must first be dispersed into an appropriate test medium, accompanied by disruption of agglomerates to achieve a particle state that ideally represents the smallest component size, in a sufficiently stable condition, to permit sample introduction into the assay.[114−116] Ultrasonication, or simply sonication, the application of high frequency sound waves, is the method of choice to enable dispersion in many experimental scenarios, since substantial energy is required to break apart agglomerates that contain nanoscale particles.[117] The dispersion process is difficult to replicate for many materials due to variations in ultrasonic equipment, poor control of the delivered energy, variations in sample volume or container dimensions, variations in ENM concentration, and the dynamic processes that accompany the interaction of ultrasonic waves with matter.[117] Failure to control the state of agglomeration or to achieve full dispersion can contribute to uncertainties in the determination of physicochemical and toxicological end points, and may produce artifacts that lead to incorrect conclusions or misinterpretation of results.

Artifacts due to the ultrasonic dispersion process can arise from several factors. For instance, ultrasonication can induce changes at the molecular level.[118] The extreme localized temperatures and pressures generated by the cavitation process can yield highly reactive species within the medium.[119] The presence of even short-lived reactive species can cause molecular changes that may degrade molecular species that are necessary for the chemical and/or physical stability of the system, or can cause the production of toxic byproducts from dispersants.[120] Sonication-induced dissolution or leaching may also result in significant artifacts. Therefore, it is critical that controls are used in such studies to account for potential artifacts. It is also advisable to minimize the energy input necessary to achieve the desired level of dispersion. In the case of TiO_2, studies have demonstrated that it is possible to effectively disperse the nanomaterial in the absence of chemical additives, which might otherwise be subject to degradation by sonolysis.[121] This is achieved by pH control and the use of a device-independent calibration procedure. The resulting stock suspension can then be modified using biocompatible dispersing agents and an appropriate protocol for introduction into the test medium of choice.[122,123] This approach should be viable for a wide range of metal oxides, but may not work as well for intrinsically hydrophobic ENMs such as fullerenes.

The geometry of ultrasonic devices can vary widely. The energy output in ultrasonic baths is highly variable and is mitigated by the intervening sample container. The inverted cup geometry offers higher power output, relative to baths, but the energy is still reduced by interaction with the sample container suspended in the cup liquid. Direct sonication, in which the transducer probe itself is immersed in the sample, achieves the highest delivered power, but the probe surface (typically titanium) can generate microscopic metallic contaminants that could potentially lead to measurement artifacts.[124]

Sonication can also potentially cause several undesirable effects, including reagglomeration, particle sintering and physicochemical alterations to the ENM surface or to other constituents of the medium. Direct sonication at moderate to high output power settings can also result in an appreciable temperature increase in the sample, which could impact sample integrity; an ice bath can be used to mitigate this effect. The temperature increase associated with direct ultrasonciation can also be exploited in a device-independent calorimetric calibration procedure that has been described in detail elsewhere.[105] This calibration relates the temperature increase to the total power delivered to the sample, and allows one to provide and report consistent power levels using any direct ultrasonication device. Other laboratories should thereby be able to replicate the power delivery, even if they use a different device or probe configuration.

To minimize artifacts resulting from dispersion of ENMs via ultrasonication, the presence of organic chemicals during sonication should be limited to the extent possible, and proper controls used when not possible. When practical, researchers are encouraged to mix organic molecules (surface coatings, natural organic matter, etc.) with ENMs after sonication. Organic molecules may undergo substantial changes from the sonication process that are challenging to characterize and may cause artifactual results. However, the presence of ENMs may limit the damage to macromolecules as was recently shown with SWCNTs and DNA oligomers.[125] A general suggestion is to limit the intensity and time of sonication to levels necessary to produce the desired ENM dispersion; this may require an iterative process and repeated characterization of ENM size. An example of this process was previously described for TiO_2 NPs.[121] It may also be valuable to conduct a media control with the media sonicated identically to the ENM dispersion, but without the ENMs.

Artifacts Related to the Synthesis of Fullerene Dispersions Using Organic Solvents. Perhaps the clearest example of ENM dispersion methods that have generated artifacts in toxicity tests was the preparation of aqueous dispersions of C_{60} fullerenes with organic solvents. Because C_{60} is essentially insoluble in water[126,127] but is soluble in various organic solvents, methods were developed whereby the fullerene was first dissolved in an organic solvent then the solvent containing C_{60} was added to water with subsequent solvent evaporation.[128] Solvents used for preparation of aqueous nC_{60} have included toluene[129] and most notably tetrahydrofuran (THF).[128,130] Biomarkers of oxidative stress that were misinterpreted as toxicity from C_{60} after they were dispersed using THF [34] were widely reported, and resolution of these artifacts and reorientation of the scientific literature has taken considerable time and effort from various independent groups.[23,131−135] In THF-prepared aqueous C_{60}, alterations in the solvent that occurred during preparation led to formation of THF decomposition products that were ultimately linked to artifacts of toxicity,[35] a result that has been confirmed repeatedly.[131−133] This example of artifact-based toxicity was in part a consequence of the lack of established methods for ENM assessment toxicity and unknown issues that arose within the test methods that were used.

One important control to consider testing is the filtrate only control: the ENM solution used in the ecotoxicity experiment is filtered using a sufficiently small filter size to remove the ENMs, and then the toxicity of the filtrate is tested.[136] This can provide an estimate of the impact of dissolved or desorbed molecules on the toxicological end points being studied, although there may be some adsorption of impurities to the filtration system which could lead to an underestimation of their impact. This approach can assess the impact of dissolved metals that leached

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Table 3. Nanotoxicity Studies in Which Nutrient Depletion Was Considered

nutrients	NPs	organisms	inhibition/alteration	reference
metal components such as Mn, Mg, Ca in medium	MWCNT	Green alga (*Chlorella* sp.)	MWCNT could adsorb metal components such as Mn, Mg, Ca in medium. However, nutrient depletion due to MWCNT adsorption did not induce algal toxicity	147
NH_4^+ and PO_4^{3-}	CeO_2	Green alga (*P. Subcapitata*)	CeO_2 NPs showed no adsorption of ammonium, but adsorption of phosphate to the particle surface was observed to a large extent; e.g. around 50% PO43 in the 32 mg/L CeO_2 suspensions however, the reduction of phosphate in the medium had no significant effect on the algal growth rate because the phosphate concentration in medium was still sufficient for algal growth	164
macronutrients (N, P, K) and micronutrients (Fe, Zn, Mn)	silica	*Arabidopsis thaliana*	negatively charged SiNPs showed phytotoxicity, which was partly attributed to the adsorption of macro- and micronutrients on Si NPs; after calcination or removal of surface silanols, neutral SiNPs were no longer toxic to the plants	163
Ca in DMEM-FBS medium	metal oxides such as CeO_2 and TiO_2	human keratinocyte HaCaT cells and A549 cells	metal oxide ENMs, in particular CeO_2 and TiO_2, had strong adsorption abilities for Ca^{2+} in medium. Ca^{2+} deficiency in the culture medium did not influence the viability of cells. But if calcium is not included in the medium, cell growth will be affected	165
Ca^{2+} and PO_4^{3-}	hydroxyapatite	catfish cells and Zebrafish embryos	hydroxyapatite ENMs obviously adsorbed Ca^{2+} and PO_4^{3-} ions in both medium and tap water, but the authors did not investigate the contribution of Ca^{2+} and PO_4^{3-} depletion to the overall ENM toxicity	233
proteins in DMEM-FBS medium	CeO_2 and TiO_2	human keratinocyte HaCaT cells and A549 cells	metal oxide ENMs, in particular CeO_2 and TiO_2, had strong adsorption abilities for proteins in the medium; cell proliferations of both cell lines were strongly inhibited by the supernatants after adsorption on TiO_2 and CeO_2 ENMs because of serum protein depletion	165
nutrients in medium	SWCNT	A549 cells	SWCNTs can induce an indirect cytotoxicity by alteration of medium composition	179
micronutrients such as vitamins and amino acids	SWCNT	human hepatoma cell line (HepG2)	SWCNTs altered the micronutrient content of cell culture medium through adsorption the depletion of folate, as well as other essential micronutrients significantly reduced cell viability	180
folic acid (vitamin B9), pyridoxine HCl (a form of Vitamin B6), niacinamide (the amide form of Vitamin B3)	graphene, graphene oxide	HepG2	for all tested ENMs, few-layer graphene (FLG) had the highest adsorption capacity to all micronutrients folic acid depletion of cell culture medium was observed for FLG 10 ug/mL or less folic acid depletion led to growth inhibition in HepG2 cells, causing a 'starvation' toxicity mechanism	181
nutrients in F12K medium	graphene oxide	A549 cells	the supernatant after adsorption by graphene oxide showed no toxicity to A549 cells, indicating that the absorption of nutrients from the culture medium did not influence A549 cells (graphene oxide had no obvious toxicity in this study)	234

from ENMs (such as metals from CNTs) or from ENM dissolution, endotoxins released into the test media, surface coatings that have desorbed from the ENMs, and chemicals produced from the dispersion process (such as THF related byproducts upon fullerene dispersal). Additionally, this approach could test for the potential impact of dispersants which have been shown to impact the ecotoxicity of SWCNTs.[137,138] Potential toxic or stimulatory effects of coatings or dispersants can also be tested independently through well designed control experiments.

Potential Artifacts Related to ENM Mixing. Given the substantial challenges associated with characterizing and quantifying ENMs in soils and sediments, little research has been conducted to date on the homogeneity of ENMs in these media. Additional research is needed to assess the extent to which the ENM properties (e.g., size and surface charge) change during different mixing procedures and to what extent the ENMs can be added homogeneously to natural solids. Some studies with radioactively labeled CNTs have shown heterogeneous CNT distributions in soils and sediments after mixing.[139,140] There may be artifacts (e.g., different ENM characteristics such as size in the media at different ENM concentrations) that could unexpectedly impact subsequent ecotoxicology assays and interpretations of results from those tests. Similarly, the fate of ENMs in solid media such as agar is unclear. The ENMs may be well dispersed initially in the hot liquid but changes may occur during the cooling and solidification process. Performing characterization of the ENM after mixing to the extent possible is desirable. In the absence of an analytical method to assess ENM homogeneity, an inert marker such as chromic oxide can be added and tracked, but there may be differences in mixing for these markers and ENMs.[141]

Differences in ENM transport properties, relative to those of traditional chemicals, can also cause artifacts in standard protocols for antimicrobial testing such as the disk diffusion test.[142,143] In this test, the antimicrobial is placed on a filter paper disk which is incubated on a plate which has been streaked to grow a lawn of bacteria. Once incubated, a zone of inhibition around the disk is measured. The zone of inhibition test is likely to demonstrate artifacts when the test agent is an ENM suspension, because of adsorption of particles to the filter paper and the lower diffusion coefficients of the particles relative to traditional small molecule antibiotics.[144] Therefore, unless the particle concentration can be quantified in the surrounding agar, it is important to consider that the disk diffusion assay may not be an accurate assessment of particle toxicity when the mechanism of toxicity requires cell-particle contact.

POTENTIAL ARTIFACTS WHEN CONDUCTING ECOTOXICOLOGY ASSAYS

Even if the ENMs are carefully dispersed and characterized prior to the ecotoxicological assay, numerous artifacts and misinterpretations may occur during the assay. The unique behaviors of ENMs and the potential changes to them that may occur during the ecotoxicological assay may produce artifacts and misinterpretations in the absence of careful experimental design. For example, there may be incorrect interpretations of the observed toxicity results if the contributions of indirect toxicity mechanisms, such as shading during studies with photosynthetic organisms like algae or adsorption of nutrients in the test media by ENMs, are not taken into account. In addition, ENMs may directly interfere with the assay by adsorbing test reagents, producing a reporter signal (e.g., absorbance or fluorescence) similar to the assay's measurand, or interacting with biomolecules extracted from the organism after the conclusion of the assay (e.g., ENM binding to extracted DNA). An additional challenge for testing the potential ecological effects of ENMs is that the particles often undergo significant changes during the exposure period (settling, dissolution, changes to the surface coatings, etc.) that may be challenging to measure. Thus, there is a potential for misinterpretations of toxicity results as a result of the complex, dynamic set of changes that can occur during nanoecotoxicological assays.

Potential Artifacts and Misinterpretations Related to Indirect Toxicity Mechanisms. The importance of indirect effects with regards to artifacts and misinterpretations of nanotoxicity testing is significant; failure to investigate the impact of these factors can lead to an incorrect interpretation of the toxicity mechanism and thus an overestimation of the impact of other direct mechanisms such as membrane leakage, oxidative stress, and DNA damage.[42,145,146] One important indirect toxicity mechanism that has received recent attention is the potential for shading to impact carbon nanotube toxicity to algae.[147,148] In one study, a substantial fraction of the observed toxicity was concluded to be a result of shading.[148] In addition, ENMs often have high adsorption capacity for organic molecules and inorganic ions[149−151] due to their high surface area and unique surface properties, which can lead to nutrient depletion in a culture medium, and thus cause an indirect toxic effect. This indirect toxicity effect may be referred to as a "nutrient depletion effect". While this effect has been previously observed during ecotoxicology tests with chelating agents (e.g., ref 152) and sorption of chemicals to solid media such as hard carbons has been extensively studied,[153−162] this mechanism has not been frequently considered in nanoecotoxicology tests.

Adsorption of Mineral Nutrients during Toxicity Tests. The main components of the media used to culture organisms such as plants, algae, and bacteria are inorganic elements, including macronutrients (N, Ca, K, Mg, P, N, and S) and micronutrients (Fe, Mn, Cu, Zn, Mo, B, and Co). The toxicity studies which considered mineral nutrient depletion are presented in Table 3. Only one ecotoxicity study observed a toxic effect from nutrient depletion, in which toxicity of negatively charged SiNPs to *Arabidopsis thaliana* was attributed to depletion of macronutrients and micronutrients by mineral adsorption on the ENM surface.[163] Interestingly, calcining the neutral SiNPs eliminated their toxicity to the plants, probably because the sorption of metal ions is surface-charge dependent. A detailed schematic illustration for the relationship between nutrient adsorption and observed growth inhibition by ENMs is shown in Figure 3. Other studies in Table 3 observed the adsorption of inorganic components (e.g., PO_4^{3-}, Ca^{2+}, Mg^{2+}) on the surface of the tested ENMs (e.g., TiO_2, CNTs) but no toxic effect was observed in these studies due to sufficient nutrients even after adsorption. For example, CeO_2 NPs adsorbed around 50% of PO_4^{3-} in the medium during exposure of green alga (*P. Subcapitata*), but the remaining PO_4^{3-} in the medium was sufficient for algal growth.[164] In another study, CeO_2 and TiO_2 strongly adsorbed Ca^{2+} ions, but the Ca^{2+} deficiency did not alter cell viability of HaCaT and A549 cells, although cell growth was affected when Ca^{2+} ions were not included in the medium.[165] Therefore, nutrient depletion and related growth inhibition of organism/cells depend on the

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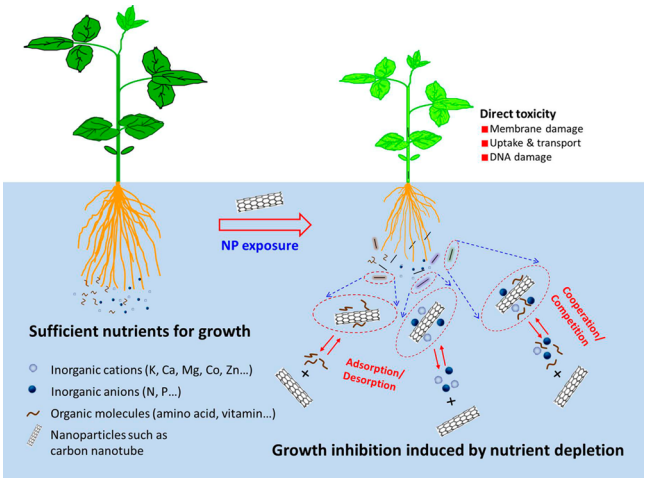

Figure 3. Schematic illustration of indirect toxicity of NPs to plants caused by nutrient depletion.

nutrient concentration in the media, the NPs concentration and type, as well as the organism/cell types and density. As a result, it is difficult to generalize what exact assays are most likely to have the nutrient depletion effect due to the limited available data and complexity of various test media and organism species.

Nutrient depletion by ENMs is not commonly reported in nanotoxicology tests. For example, the leaves of red spinach (*Amaranthus tricolor*) became chlorotic (in particular, blade tip), wilted and curled upon MWCNT exposure, but these symptoms were attributed to oxidative damage directly induced by MWCNTs.[166] However, at the test MWCNT concentration (as high as 1000 mg/L), nutrients, particularly metal ions in the medium could be largely adsorbed on MWCNTs. Moreover, chlorosis is a common symptom of macronutrient (e.g., K, Ca, Mg) and/or micronutrient (e.g., Zn, Mo) deficiency. The observed oxidative stress was diminished by supplementing with the antioxidant ascorbic acid. One likely explanation for this result is that synthesis of ascorbic acid by the plants was hindered by the depletion of micronutrients such as Cu and Mo, which participate in ascorbic acid synthetic pathway. The same explanation may be applicable to the observed toxic effect by graphene,[167] which has a similar graphitized structure to MWCNTs. For metal and metal oxide ENMs, the nutrient depletion may be not as severe as during exposures with carbon ENMs such as CNTs at similar exposure concentrations because of their comparatively low surface areas.[168] Nevertheless, adsorption and precipitation of metal and phosphate ions on metal and metal oxide ENMs need to be accounted for as part of sound nanotoxicity test design.

Adsorption capacities of ENMs for inorganic ions in aqueous solutions have been thoroughly investigated. Graphene exhibited the highest adsorption capacity for phosphate at pH values of 6–8, with an adsorption amount of 89 mg/g at an initial phosphate concentration of 100 mg/L.[169] High sorption of metal ions (Cu, Co, Cd, Zn, Mn, and Pb) on MWCNTs[170] and Hg^{2+} on nC_{60}[171] have also been reported; mechanisms included a combination of chemical complexation, electrostatic attraction, and/or cation-π interaction.[172] Metal oxide ENMs could also adsorb metal ions and have potential to be used as adsorbents for heavy metal removal from wastewater.[173] The mechanisms of metal adsorption by metal oxide ENMs relate to electrostatic attraction, ion exchange,[174] and covalent bond/inner-sphere complexaton as well.[175] In addition, metal ion adsorption on ENM surfaces is influenced by solution pH and ionic strength. Competitive sorption among different metal ions could occur on the ENM surface,[176] thus suppressing nutrient depletion.

Adsorption of Organic Nutrients during Toxicity Tests. Organic molecules and mineral ions coexist in almost all culture media, especially for cell media, and their coadsorption on NPs surface warrants consideration in nanotoxicity tests. EDTA salts (Hoagland solution) and citric acid (BG11 medium) are two of the main organic compounds in media for culturing plants[177] and algae,[178] respectively. These compounds are used for solution buffering and chelating, not for nutrient supply. Therefore, nutrient depletion is not expected if they are adsorbed by ENMs unless their adsorption reduces the availability of inorganic nutrients. However, adsorption of these organic compounds could change the surface properties of ENMs, including surface charge and the ENM suspension stability.

During in vitro assays, cell culture media are rich in organic nutrients such as proteins, amino acids, glucose, and vitamins. The adsorption of these organic nutrients by ENMs has been observed in cytotoxicity studies (Table 3). For example, CeO_2 and TiO_2 were reported to inhibit cell proliferation because of protein depletion.[165] For carbon nanomaterials (CNTs and graphene) at exposure concentrations of 10 mg/L to 25 mg/L, organic nutrient depletion and obvious growth inhibition of human cells were observed by different research groups.[179−181] In addition to the organic nutrients, antibiotics (e.g., streptomycin, amphotericin B) and phenol red (pH indicator) are contained in most mammalian cell culture media. Guo et al. showed that 90% of the phenol red in a medium (RPMI medium 1640) was removed by a SWCNT at a concentration of only 90 mg/L.[180] Adsorption of antibiotics by ENMs in culture media has not been reported. High adsorption capacities, however, could be anticipated based on previous studies of antibiotic adsorption from water by CNTs.[182,183] Therefore, cytotoxicity tests may be impacted by the depletion of these organic compounds in addition to nutrient depletion. Adsorption mechanisms toward organic components by metal oxide ENMs are a function of hydrogen bonding and electrostatic attraction. Interactions between carbon nanomaterials and organic components (e.g., proteins, amino acids, antibiotics) are more complex. In addition to hydrogen bonding and electrostatic attraction, hydrophobic interactions and $\pi-\pi$ stacking could be important mechanisms in some cases. A relatively high possibility of nutrient depletion is expected for SWCNT and graphene, which have larger surface areas and higher adsorption capacities for organic molecules.[184,185]

Almost all media in nanotoxicity tests contain both organic molecules and mineral ions, and their coadsorption on NP surface warrants discussion (Figure 3). In neutral cell culture medium, negatively charged metal oxide ENMs (CuO, ZnO) were reported to form complexes by binding mineral ions (Ca^{2+}, Na^{+}).[186] This interaction occurred independent of protein molecules. In another study, negatively charged TiO_2 ENMs adsorbed proteins by bridging divalent ions such as Ca^{2+} and Mg^{2+}.[165] For positively charged ENMs (e.g., Fe_3O_4), organic molecules could act as a bridge between the ENM surface and cations, thus enhancing the adsorption of cations.[187] The same cooperative adsorption was observed between humic acid and metal cations (Pb^{2+} and Cd^{2+}) on MWCNTs in aqueous solution,[172,188] suggesting that amino

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acids and fetal bovine serums (FBS) could enhance the adsorption of cations on CNTs in cell culture media. On the other hand, competitive adsorption may also occur on ENM surfaces between organic molecules and mineral ions of the same charge; both are available to be adsorbed onto ENMs surface via electrostatic attraction. However, to further evaluate and predict the influence of these interactions on observed cytotoxicity, systematic investigation on the adsorption of medium components by the test ENMs is required.

To date, there are no reports on nutrient depletion in toxicological studies toward other organisms such as fish or bacteria. Fish are commonly cultured in tap water during ENM exposure. In short-term tests, the influence of nutrient depletion in tap water is expected to be negligible. However, in long-term tests with food amendment, fish food (commercial fish-food flakes) was observed to adsorb CuO ENMs.[189] Such interactions may result in settling of food together with ENMs, thus causing a reduced amount of food available for fish consumption. Media for bacterial culture contain mineral components and organic components such as glucose and peptone for carbon sources. For some studies in which the test bacteria were suspended in physiological saline during the test,[190] nutrient depletion can be neglected. For other studies conducted in other types of minimal media, nutrient depletion should be considered as a source of indirect toxicity even in short-term tests because substantial adsorption can occur during the first few hours.

The following factors during the toxicological tests can influence nutrient depletion: (1) nutrient-ENMs ratio. If the nutrients in a medium are designed for optimal organism/cell growth, the nutrients are likely to be deficient when ENMs are added to the medium. Engineered nanomaterials with high concentration (low-toxic materials) and strong adsorption affinity (carbon based ENMs) are most likely to cause toxic effects from nutrient depletion; (2) Exposure time. Depletion of nutrients is more obvious in long-term tests. Some symptoms (e.g., reduction of flowering, prevention of fruit ripening) could exhibit only after long-term deficiency of certain nutrients; (3) Desorption processes. Nutrients adsorbed on ENMs can be available for organisms/cells after ENM uptake if the adsorption process is reversible. Hence, determining the contribution of nutrient depletion by allowing ENM to interact with the media and then testing the impact of decreased nutrient concentrations in the media after ENM removal may overstate the impact if substantial desorption of nutrients from the ENM occurs in the organism gut tract.[165] By controlling these factors, we may be able to evaluate the contribution of nutrient depletion during the toxicity tests and the related misinterpretation of observed toxic phenomena can be possibly avoided.

The impact of nutrient depletion can be evaluated via control experiments with media that had been incubated with the ENM test material, as was recently included in a study on CNT toxicity to algae.[147] The test media is incubated with the ENM dispersion at the highest ENM concentration for the duration of the assay, the ENMs removed (i.e., by centrifugation or filtration), and the organisms are then exposed to the depleted test media. However, this solution would also assess the toxicity of impurities or ions released into the test media similarly to the filtrate only control described earlier. A different approach to assess indirect toxicity is through conducting a sorption experiment where the extent of sorption is quantified by measuring the decrease in the freely dissolved concentration of the test media component of interest. One important limitation of these approaches is that nutrients sorbed to the NPs may be desorbed after uptake into the organism such as passage through the gut tract and thus this approach could overestimate the extent of nutrient depletion. A third approach to quantify nutrient depletion is to assess the concentration of different critical elements in the organism tissues. If substantial toxicity occurs as a result of nutrient depletion, it may be prudent to modify the test medium to include higher concentrations of critical nutrients or to simply use a different test medium if one exists for the organism being evaluated. However, modifying the test medium could also influence the agglomeration and dissolution behaviors of the ENMs being tested.

Examples of Direct Interference by ENMs during Ecotoxicity Assays. Frequently observed artifacts during ENM toxicity testing include direct interaction between ENMs and biomolecules or test reagents, ENM production of a signal similar to the assay's measurand by the ENM, or damage to cells or biomolecules caused by ENMs after the exposure period but during subsequent sample processing steps. These artifacts have been observed in numerous cytotoxicity tests such as the MTT,[27−31] lactate dehydrogenase (LDH),[28,32] MTS,[33] and neutral red assays.[29,30] One study also showed artifacts when assessing ENM bacterial toxicity using an electron transport assay, a membrane potential assay, a membrane integrity assay, and a superoxide production assay.[191] In addition, a study assessing lipid peroxidation in fish (*Cyprinus carpio*) brains showed that fullerenes may cause lipid peroxidation if the assay is conducted under light conditions (600 lx for 30 min). Thus, assays with photoactive ENMs such as TiO_2, ZnO, and fullerenes may lead to artifacts if photoactive damage to the biomolecule occurs during the assay. While many of these artifacts were measured during cytotoxicity assays, the potential for artifacts is relevant for larger organisms if the tissues being tested have sufficiently high ENM concentrations. In addition, ENMs may interfere with quantitative polymerase chain reaction (qPCR) assays if the addition of particles (particularly at high concentrations) changes the PCR amplification efficiency.[192] qPCR and other DNA based assays require that DNA be extracted from the environmental matrix. DNA extraction can introduce biases into ecotoxicity experiments as PCR inhibitors can be carried through the extraction process and polymer nanoparticle coatings can compete with DNA for adsorption sites onto surfaces in the environmental matrix, changing the extraction efficiency. Titanium dioxide NPs were shown to hinder measurements using a Coulter Counter to analyze algae biomass.[193] The TiO_2 NPs provided a signal when analyzed without algae cells, and subtracting a background signal caused a "negative" cell density, likely the result of NP interaction with the algae to form agglomerates of larger sizes. Similar challenges were observed for making measurements with AuNPs.

One assay in the literature that has shown the potential to produce artifactual results on multiple occasions as a result of direct interferences from ENMs is the Comet assay.[42,194−197] In this assay, gel electrophoresis is performed on encapsulated cells to assess the extent of DNA damage in cells. A longer comet (wider distribution of DNA migration distances) indicates increased DNA damage. In one study with Caco 2 cells, a statistically significant increase in damage after NP TiO_2 exposure was observed when the gel electrophoresis was performed under ambient light conditions, but not under dark conditions.[197] This artifact likely resulted from damage to DNA

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caused by the TiO_2 associated with the cell during the gel electrophoresis step. In addition, CuO and TiO_2 ENMs have been identified in the heads of comets after cells were exposed to these particles.[194] Importantly, germanium nanoparticles were shown to cause a toxic effect even when the cells were harvested immediately after NP addition, suggesting again that DNA damage occurred during the processing steps.[195] False positive results were recently demonstrated for the Comet assay after the eukaryotic organism *Tetrahymena thermophila* had been exposed to TiO_2 ENMs.[196] While elevated reactive oxygen species, lipid peroxidation, and changes to the cell membrane composition were not observed, elevated DNA damage was apparently measured. When a *post fetum* exposure was conducted with TiO_2 ENMs and nuclei embedded directed in the gel, a positive effect was still observed. These data indicate that interactions between the DNA in the cells and TiO_2 ENMs were the cause of artifactual results. These results raise questions about the applicability of the Comet assay for use with ENMs and that additional modifications are needed to ensure that reliable results can be obtained.

Control experiments are thus critical to assess the potential for direct interference of ENMs with toxicity assays. For example, Horst et al.[191] systematically assessed the potential for the ENMs to cause artifacts during bacterial toxicity studies by assessing the interactions between the ENMs and the test reagents and by testing the bacteria using a 0 h time point for which the ENMs were added and then the assay immediately performed. During studies with more complex biota, researchers should quantify the highest potential concentration of ENMs in the tissues of interest and then conduct experiments to assess whether the presence of the ENMs at that concentration could impact the assay. This could be conducted by assessing whether the presence of ENMs spiked to the tissue at the relevant concentration would impact control tissues (i.e., tissue without an expected change in the end point of interest) and also positive control (i.e., tissue with an expected change in the end point of interest). Data demonstrating that ENMs at the concentrations being tested do not cause an artifact with the assays utilized should be included in all nanecotoxicology manuscripts.

Dynamic Changes That Can Occur to the ENMs during Testing May Lead to Inaccurate Dosing. Traditional toxicity tests of dissolved substances assume a relatively homogeneous exposure scenario (i.e., in the aqueous phase) during the exposure period, although it is well-recognized that nominal concentrations can change during tests (e.g., as test substances are hydrolyzed or partition onto surfaces of the container). The situation can be much different with ENMs as exposure conditions can depart rapidly from initial conditions and may not represent a homogeneous exposure that can be readily quantified using existing analytic methods. These changes to ENMs during ecotoxicity testing can not only hinder establishing mechanisms of toxicity but also can complicate efforts to merely obtain reproducible results. Not accounting for the dynamic changes that can occur during a nanoecotoxicity test can lead to misinterpretations as a result of inaccurate dosing.

One example of an ENM that undergoes a broad range of changes in environmental systems is AgNPs (see Figure 2 for a schematic). For example, AgNPs can undergo significant changes/transformations in environmental waters,[24] including agglomeration.[198] Silver ions have also been shown to form AgNPs when reduced by humic acids,[199] fulvic acids,[200] and sunlight.[201] To avoid additional confounding factors, one must also recognize that the stability and physical characteristics of aqueous ENM dispersions can vary as a function of solution type (e.g., distilled water, EPA moderately hard water, or a media specifically designed for a certain test organism).

The following is an example of when the dynamic changes of ENMs in the test media can substantially alter the results obtained. Musante and White noted the highly counterintuitive phenomenon of decreasing Cu ion in solution with increasing initial CuNP concentration.[202] A more detailed analysis showed that Cu oxidation in solution was reducing oxygen and subsequently consuming protons, which then increased pH. As the pH rose, ionic Cu in solution precipitated as Cu phosphates, carbonates, and hydroxides with constituents of the Hoagland's solution, a media commonly used in hydroponic phytotoxicity studies. At higher initial Cu concentrations, the reaction proceeded more quickly, resulting in higher pH values, greater rates of Cu precipitation, and ultimately lower Cu ion levels in solution. It is also noteworthy that this reaction was much greater for the CuNPs than for the bulk Cu; clearly a function of the increased surface-to-volume ratio and reactivity of ENMs. Also notable is the fact that humic acid partially minimized this phenomenon. Although this is a rather interesting series of chemical reactions, the practical significance should not be underestimated; actual exposure levels ended up being nearly ten times less than those initially calculated. One could predict similar reactions with other metals, both in nanoscopic and bulk form and care should be taken to accommodate this phenomenon. Thus, careful characterization of the ENMs during the ecotoxicology test such as settling, agglomeration, and dissolution is needed for an accurate measurement of the exposure dosage. Changes to ENMs will likely occur in environmental systems and are not inherently problematic but they can make accurate measurement of the exposure dosage more complicated.

There are also several unique confounding factors one must consider when attributing observed phytotoxicity to ENM exposure. Often ignored is the highly dynamic and bidirectional process of ENM dissolution and reaggregation under reducing conditions within, on, or in the vicinity of the plant surface. For example, Gardea-Torresdey et al[203,204] noted in planta formation of Au and Ag NPs after exposure to media containing ionic forms of the elements. Conversely, it is just as likely that some or all observed phytotoxicity may result from enhanced ion dissolution from metallic NPs as a function of increased surface area and not from the actual elemental NP itself.[145,202,205] To accurately identify the precise mechanism of toxicity, one may need to follow particle uptake over time while simultaneously providing a real-time determination of particle type and characteristics. Given the current limitations in NP detection techniques in complex matrices and the great effort associated with such an experimental design, one should proceed with great caution when attributing mechanisms of phytotoxicity to ENM exposure. In hydroponic and soil based experiments, plant root exudation during exposure can alter media conditions, although there is little data yet to quantify the impact of the exudates. For example, exuded organic acids can lower pH,[206] changing nutrient availability and potentially altered ENM physical characteristics that could impact exposure. In addition, exudation will stimulate microbial growth (bacteria, fungi, protozoa), which could theoretically impact ENM activity in a number of ways, particularly those particles with degradable coatings or capping agents (citrate, PVP).

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However, we could find no published reports on this phenomenon.

Additional controls are necessary to test for ENM-specific effects.[205] Adequate design requires not only the use of the ENMs of interest but also the appropriate bulk material and ion controls (if relevant). Without appropriate non-ENM controls, accurately attributing any observed toxicity to ENMs themselves may be extremely difficult. Testing the toxicity of released ions provides information about the effects from dissolution of the ENMs and the extent to which the toxicity observed can be attributable to particle dissolution. The broad range of changes that ENMs can undergo during test exposures as described earlier highlight the importance of measuring the changes occurring in the test system. Testing larger materials of the same elemental composition provides information about whether there are any specific toxicological effects unique to the ENM size, although there may be stability issues for some larger particles. In addition, changes to the ENMs (and larger particles and ions) during test exposures provide critical information for understanding the toxicity mechanism and the potential for ENM-specific toxic effects. For example, initial reports of toxicity indicated by changes in tissue biochemistry in rainbow trout exposed to aqueous TiO_2−NPs[207] and SWCNTs[208] appear now to have now been related to occlusion of fish gills and respiratory distress rather than NP absorption and direct toxicity in tissues.[22,209] Inclusion of bulk material controls in the initial studies might have enabled the authors to identify this toxicity mechanism and that the effects do not appear to have been specifically related to ENMs.

Potential Artifacts Related to Changes to Cell Agglomeration after ENM Exposure. In addition to the dynamic changes that can occur during toxicity tests, interactions between ENMs and test organisms may lead to unexpected changes to the test organisms that could result in artifacts or misinterpretation of results if they are not appropriately considered. ENM properties may impact tests using CFU (colony forming unit) counts to quantify the effects of ENM exposure to bacterial cells. These tests can either be performed by plating planktonic cells that have been exposed to nanoparticles or by plating cultures directly onto nanoparticle containing plates to assess the minimum inhibitory particle concentration. Planktonic exposures are prone to varying concentrations based on the agglomeration state of the particle. In addition to considering the artifacts caused by particle agglomeration and changes in suspension, the researcher must also ensure that the particles do not change how the cells agglomerate[210] as this will also change the plate count results. Other cells are susceptible to these artifacts as well. For example, exposure to TiO_2 NPs was shown to cause agglomeration of algae cells.[193] This hindered algae biomass measurements using a hemocytomer, because it was challenging to count the algae cells that were attached to or inside of TiO_2 NP agglomerates. Challenges related to making Coulter Counter measurements of algae biomass after exposure to TiO_2 NPs or AuNPs were described in an earlier section. For these assays, the potential for unexpected cell agglomeration behaviors as a result of the ENMs must be assessed through use of orthogonal methods to verify results. If the assays provide substantially different answers, additional research is needed to determine which approaches are reliable and which are biased by the presence of ENMs or some other unknown confounding factor.

POTENTIAL ARTIFACTS RELATED TO ENM CHARACTERIZATION IN ORGANISM TISSUES AND CELLS

There is also the potential for artifacts related to ENM characterization and quantification in organism tissues and cells after an experiment. One of the substantial challenges with characterization in tissues is the lack of orthogonal and standardized methods. While we will not cover all procedures for ENM characterization in tissues as other reviews have been published on this topic,[20,211−213] a few examples will be provided to highlight some of the challenges that may be encountered. For example, a number of potential artifacts have been highlighted during electron microscopy analysis of organism tissues. In a cytotoxicity study, electron energy loss spectroscopy was used to show that a number of the apparent quantum dot NPs identified using transmission electron microscopy (TEM) actually had a different chemical composition suggesting that they were not quantum dot NPs.[214] While TEM initially had suggested substantial absorption of SWCNTs by *Daphnia magna*, high-resolution TEM and other analytical TEM techniques showed that these apparent nanotube bundles were actually amorphous carbon.[215] Another substantial challenge for assessing the biodistribution of NPs is that some organisms can cause the formation of NPs from dissolved ions. As stated earlier, in planta formation of nanoparticles such as AgNPs and AuNPs has been confirmed.[203,204] In addition, AgNPs have been observed in the hemolymph of *Daphnia magna* after exposure to silver ions.[216] Determination of ENM absorption into organisms may be complicated by the potential for ENMs to substantially change in the culture media and by the organism such as passage through the gut tract.[216−219] Thus, detection of ENMs that have a different size or chemical properties may not definitively indicate a lack of uptake.

It is important to use orthogonal methods whenever possible to characterize ENM concentrations in biota. When the potential artifacts for one method are not well-known, it is important to use a complementary method for identification and quantification, especially when there is not a clear route for uptake into the tissue (i.e., ENMs in fish brains). Furthermore, additional research is needed to continue developing robust and reliable analytical methods to quantify ENMs in organism tissues at environmentally relevant concentrations. For example, one promising option for laboratory experiments is to use radioactively labeled ENMs.[92,139,140,220−232] When testing for potential uptake of metal and metal oxide ENMs, it is important to also test uptake of dissolved ions and their potential formation into ENMs in the organism. Measuring changes to ENMs that may occur in the culture media may also help the determination of whether ENMs observed in tissues are from the dosed ENMs.

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Notes

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