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Strategies for robust and accurate experimental approaches to quantify nanomaterial bioaccumulation across a broad range of organisms

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Abstract

One of the key components for environmental risk assessment of engineered nanomaterials (ENMs) is data on bioaccumulation potential. Accurately measuring bioaccumulation can be critical for regulatory decision making regarding material hazard and risk, and for understanding the mechanism of toxicity. This perspective provides expert guidance for performing ENM bioaccumulation measurements across a broad range of test organisms and species. To accomplish this aim, we critically evaluated ENM bioaccumulation within three categories of organisms: single-celled species, multicellular species excluding plants, and multicellular plants. For aqueous exposures of suspended single-celled and small multicellular species, it is critical to perform a robust procedure to separate suspended ENMs and small organisms to avoid overestimating bioaccumulation. For many multicellular organisms, it is essential to differentiate between the ENMs adsorbed to external surfaces or in the digestive tract and the amount absorbed across epithelial tissues. For multicellular plants, key considerations include how exposure route and the role of the rhizosphere may affect the quantitative measurement of uptake, and that the efficiency of washing procedures to remove loosely attached ENMs to the roots is not well understood. Within each organism category, case studies are provided to illustrate key methodological considerations for conducting robust bioaccumulation experiments for different species within each major group. The full scope of ENM bioaccumulation measurements and interpretations are discussed including conducting the organism exposure, separating organisms from the ENMs in the test media after exposure, analytical methods to quantify ENMs in the tissues or cells, and modeling the ENM bioaccumulation results. One key finding to improve bioaccumulation measurements was the critical need for further analytical method development to identify and quantify ENMs in complex matrices. Overall, the discussion, suggestions, and case studies described herein will help improve the robustness of ENM bioaccumulation studies.

Environmental Significance Statement

While the potential for engineered nanomaterials (ENMs) to bioaccumulate has been the focus of substantial research attention, how best to conduct needed measurements has yet to be comprehensively evaluated for the broad range of organisms present in the environment. This analysis develops key recommendations for improving the quality of ENM bioaccumulation measurements during different steps of the measurement procedure, such as how to avoid artifacts in the analytical measurements in the organism tissue and environmental media, and unique considerations for different types of test organisms. The suggested strategies and discussion described herein will help to improve the robustness of ENM bioaccumulation measurements and promote the sustainable development of products utilizing ENMs.

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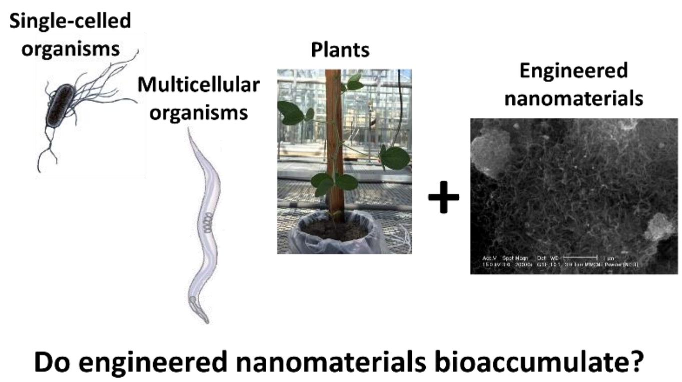

Strategies, discussion, and case studies are provided for making robust and accurate measurements of engineered nanomaterial bioaccumulation by single-cell organisms, multicellular organisms, and plants.

Introduction

There is a broad range of potential applications of engineered nanomaterials (ENMs), materials with at least one dimension between 1 nm and 100 nm,[1, 2] stemming from their novel or enhanced properties as compared to equivalent materials of larger sizes or conventional chemical form. Thus, it is anticipated that ENMs will be increasingly used in consumer products and for commercial applications in the future.[3-5] To responsibly develop ENM-enabled products, it is critical to develop a comprehensive understanding of the potential environmental and human health risks that ENMs may pose during a product's life cycle (i.e., manufacturing, usage, and disposal).[6-9]

Regulatory decision making on potential environmental risks focus on the extent to which substances such as ENMs exhibit persistent, bioaccumulative, and toxic (PBT) behaviors. This highlights the importance of understanding the capacity for ENMs to bioaccumulate in organisms and subsequently transfer through and biomagnify within food chains. In addition, fundamentally understanding the target organs and absorption, distribution, metabolism and excretion (ADME) processes that together determine bioaccumulation extent and dynamics are important to identifying the hazards of ENMs to whole organisms, as well as to specific target organs, systems (e.g., digestive system), or organelles.

As for conventional chemicals, it is recognized that an understanding of the toxicokinetics of ENM uptake is important for determining their behavior and risk. There is a broad range of studies in the nanotoxicological literature evaluating the bioaccumulation and biomagnification of various ENMs including carbon nanotubes (CNTs),[10, 11] fullerenes,[12, 13] graphene family nanomaterials (GFNs),[14, 15] Au ENMs,[16-18] Ag ENMs,[19, 20] CuO ENMs[21] and cadmium selenide quantum dots.[22, 23] Results from these studies have often shown that ENMs behave differently from conventional bioaccumulative substances such as hydrophobic organic chemicals. For example, ingested ENMs may accumulate on or in gut tissues of organisms and are often not readily absorbed across epithelial surfaces for systemic circulation.[11, 15, 24] Further, ENMs are likely absorbed by vesicular transport across cell membranes, rather than passive diffusion or facilitated uptake on solute transporters. Thus, the typical assumption for organic chemicals and metals of rapid absorption across the tissues and distribution into specific tissues or organelles (e.g., lipids for hydrophobic organic substances; inorganic biominerals for some metals) may not generally be applicable for ENMs. While it is possible for terrestrial wildlife to be exposed through inhalation, there have not been studies on this topic to our knowledge relating to environmental exposure, except for the extensive literature in which rodents are exposed through inhalation to assess potential worker safety or consumer health risks.[24-27] Therefore, this paper will mainly focus on ENM exposure in soil, sediments, or water. Further complicating our understanding of ENM bioaccumulation is the dynamic nature of ENM fate, with some ENMs releasing dissolved constituents[21, 28, 29] and with some biota capable of reducing dissolved elements to an ENM form.

While a large number of ENM bioaccumulation studies have been conducted, differences in the experimental methods used such as quantification method, exposure time, ENM physicochemical characteristics and associated transformation during exposure, and ENM dispersion methods, make comparisons difficult, even when the same taxa and same type of ENM were tested. In addition, the terminology used among studies to describe bioaccumulation-related results is neither consistent nor standardized, which can lead to confusion when comparing the results of different studies. There may also be artifacts or biases when quantifying concentrations in organisms such as different gut voidance approaches or methods to remove gut contents from consideration, incomplete separation of the test species from suspended ENMs, and variations in methods for the removal of loosely attached ENMs from the outer surface by washing. Therefore, the value of many studies is to demonstrate the potential for bioaccumulation or biomagnification based on individual study conditions; extrapolating to real-world conditions outside of the laboratory depends on environmental measurements that can confirm that such potentials manifest in field conditions.

In this perspective, the overall aim is to assess the current literature on ENM bioaccumulation methods and describe best practices for making measurements to support comparability across ENM bioaccumulation studies. To accomplish this aim, we propose bioaccumulation terminology, describe relevant analytical methods, and offer guidance for conducting bioaccumulation studies for a number of different groups of test organisms. In addition, we describe key considerations for associated measurements, such as approaches to differentiate between ENMs remaining in the gut tracts of organisms and those absorbed by multicellular organisms after oral exposure. When available, we also describe strategies using the unique physiologies and behaviors of the organisms to provide additional insights into ENM bioaccumulation quantification.

Bioaccumulation terminology, metrics, and considerations for ENM bioaccumulation test design

There are several issues to be considered in the vocabulary and quantification of ENM bioaccumulation. First, terminology from studying the bioaccumulation of other chemicals should be scrutinized for applicability, as common terms relating to physicochemical characteristics and transport processes differ for ENMs. Second, testing guidelines[30-32] may recommend modeling approaches and bioaccumulation metrics without stating modeling assumptions. Before use, models should be evaluated to identify assumptions and their validity for ENMs. Issues related to ENM bioaccumulation measurements and metrics have been addressed before in the context of a specific type of ENMs[10] and a specific organism[33] but are discussed more generally here covering all types of ENMs and several organism groups.

A non-exhaustive list of common terms used in the general subject of bioavailability and bioaccumulation is provided, and critically adapted for application to ENMs (Box 1). There are many other terms that are potentially of interest but not listed herein, including “bioaccessibility” and “bioactivity” which have been used in discussing ENMs in soils although they can also be applied to all environmental organisms and humans.[34] In our listing of terms, we do not aim to be exhaustive, but rather to make suggestions based on synthesis across relevant sources, when and how common terms can apply to ENM bioaccumulation considerations.

In general, bioaccumulation is defined as the accumulation of a chemical in, or on, an organism from all sources including water, air, soil, sediment and food (Box 1).[35] Bioconcentration (i.e., chemical accumulation in an organism from water only) is a process that contributes to chemical bioaccumulation but can only be measured using controlled laboratory conditions.[36] The concept of "bioconcentration" is based on lipid-water partitioning properties of hydrophobic organic chemicals. The applicability of equilibrium partitioning theory has been rejected for ENMs for multiple reasons.[37, 38] For ENMs, organismal uptake routes and biotransformation are either unknown or occur via multiple pathways. As such, the use of the term "bioconcentration" for ENMs would be recommended only in limited occasions where, in well-controlled laboratory conditions, organisms are exposed to ENMs in the test medium without added food and active uptake of ENMs by ingestion does not occur. The term "bioaccumulation" is preferred, as it captures all potential ENM associations with organisms, including sorption to external surfaces and uptake via ingestion. As will be discussed in additional detail below, differentiating between internalized ENMs and those adsorbed to external surfaces is analytically challenging. Sorption to organisms as a specific ENM bioaccumulation mode is included since membrane-adsorbed ENMs have been shown to exert toxicity via released metal ions.[39]

The calculation of a bioaccumulation parameter, such as either the bioaccumulation factor (BAF), bioconcentration factor (BCF) or the biomagnification factor (BMF), is useful for expressing the bioaccumulative potential of ENMs for the purposes of hazard assessment. Considering the possible ENM exposure routes and association modes with cells, tissues, and organisms described above, we recommend using two approaches for deriving bioaccumulation parameters in ENM studies: biodynamic models for representing ENM bioaccumulation in laboratory studies ("kinetic BAF" or BAF_k) and the ratio of tissue or organism-associated ENM concentration to the concentration of ENM in the surrounding media (BAF) in laboratory, mesocosm, or field studies. Note that BAF is ideally measured under steady state conditions when ENM uptake and elimination rates are constant and steady state can be achieved within the lifetime of an organism.[40] However, we are intentionally not constraining the definition to steady state conditions here, as such conditions may be observable under laboratory conditions but may not occur in environmental systems that are open and inherently dynamic. In contrast, in depositional sediment systems, steady-state conditions may occur.

In designing and interpreting bioaccumulation tests, both ENM and test organism characteristics need to be considered (Figure 1). For instance, different test organism sizes and ventilation rates, exposure duration (hours to months), exposure type (flow-through, static, or semi-static), feeding regimes, and elimination periods are several of the many variables that influence the outcome and interpretation of ENM bioaccumulation tests. Additionally, ENM physico-chemical factors and environmental variables affecting ENM fate determine the potential for ENM exposure, uptake and bioaccumulation in biota, as well as biotransformation in the environment and organisms,[41] and thus should be considered when designing and interpreting bioaccumulation tests (Figure 1).

Organism exposure and ENM transformations in different media

The form of a given ENM, which can change in different environmental media and over time, is critical to understanding its potential bioaccumulation by organisms (Figure 1). The transformations that ENMs undergo in different environment media have been thoroughly described.[42-51] As a summary of the field, Lowry et al.[45] discussed four broad types of transformations including chemical, physical, biological and macromolecular interactions. From the perspective of transformations having the greatest impact on bioaccumulation, the three main processes affecting the transformations ENMs experience during exposure are agglomeration, dissolution, and chemical transformation (e.g., oxidation or reduction). While homoagglomeration and heteroagglomeration affect most ENMs in environmental media, dissolution is primarily relevant for ENMs composed of metals (e.g., quantum dots,[52] CuO ENMs,[21, 53, 54] and Ag ENMs[19, 55, 56]). The impact of these processes on bioaccumulation remains unclear but in general larger contaminants or agglomerated ENMs are considered less bioavailable than individual contaminant molecules/ions or individual ENMs.[57] Furthermore, agglomeration generally leads to gravitational settling of particles,[44] increasing their interactions with sedimentary and soil surfaces and associated organisms while reducing their bioavailability to pelagic organisms.[58-61] Disagglomeration may also occur in the environmental matrix or in the gut environment after intake, although these mechanisms are poorly understood.[62] Dissolution also complicates our understanding of ENM bioaccumulation. For example, for metal ENMs, if bioaccumulation is observed by an organism, it is often unclear if the metal accumulated was delivered in the form of ENM or ionic metal.

Like most particles in environmental media, ENMs are likely to agglomerate, especially at higher ENM or background particle concentrations and under saline conditions, leading to sedimentation of ENMs from aqueous solution to the benthos. At higher concentrations, ENMs are more apt to collide and agglomerate, while high saline (i.e., ionic strength) conditions reduce the electrophoretic mobility of ENMs and also promote agglomeration.[46, 63] Other variables influencing agglomeration include the ENMs' surface charge, shape and size along with the pH and temperature of the aqueous media. For metal ENMs, coatings such as citrate and polyvinylpyrrolidone (PVP) are used to stabilize ENMs against agglomeration; for carbon, boron nitride and other hydrophobic ENMs, surfactants, synthetic polymers, and natural organic matter have been used as dispersing agents.[64] However, the environmental stability of these coatings may vary as they can be lost due to environmental degradation (e.g., microbial or photodegradation) or replaced by other natural organic ligands.[65-67] When ENMs undergo agglomeration, the exposed surface area of the particles declines, potentially resulting in decreased ENM-cell contact and thus bioavailability. Agglomeration can also reduce the dissolution rate for ENMs that have dissolvable components.

Many metal ENMs will undergo some degree of dissolution that involves the release of ionic forms of the metal into the aqueous phase.[52-54] The degree of dissolution is driven by the type of ENM including the elemental composition and the ENM size, shape, and surface coating as well as the media characteristics. For example, media pH, temperature, natural organic matter (NOM) concentration, availability of anions such as chloride or sulfide, and salinity will influence dissolution and also the fate of the released metal (e.g., ionic silver will often be sequestered by the chloride ions in seawater to form insoluble AgCl).[19, 55] As suggested above, because of the composition and manner in which they were synthesized, carbonaceous ENMs such as single- and multi-walled carbon nanotubes (SWCNT, MWCNT), GFNs and fullerenes do not undergo dissolution in the same way as metal ENMs although there can be release of ions from metal catalysts if used in the ENM synthesis process.[63, 68]

Chemical transformations of ENMs can occur in the natural environment and during ENM bioaccumulation experiments. For example, graphene oxide can be reduced to form reduced graphene oxide (rGO) by microorganisms,[69, 70] and other GFNs can also be oxidized and degraded under certain environmentally relevant conditions, which can decrease their bioaccumulation and also result in organismal exposure to degradation products.[71] Carbon nanotubes can also be oxidized or degraded by environmental processes,[72-75] although the molecular stability of CNTs often means that degradation requires relatively extreme conditions or is slow.[75, 76] It is also broadly known that metal and metal oxide ENMs can be chemically transformed through oxidation and reduction processes.[28, 77, 78]

Relevant analytical methods

This brief overview of methods for ENM detection and quantification provides context for subsequent discussions of bioaccumulation measurement strategies for different types of organisms. It is essential during bioaccumulation experiments to make accurate quantitative measurements of the ENM concentration in the biota and also the matrix of exposure. This will enable the calculation of bioaccumulation metrics such as BAF values. More extensive reviews of quantification procedures have been recently published for carbon and metal-based ENMs.[63, 79-81] Since many of the methods differ between ENM types (carbonaceous ENMs (CNMs) or metal-based ENMs), the relevant methods will be discussed separately. While some techniques can quantitatively detect various types of ENMs in organisms within certain parameters (e.g., above a certain concentration in organism tissue), they typically do not provide information about the ENM size distribution in the tissue. Also, many techniques do not distinguish between ENMs versus ions in the case of metal ENMs. Other techniques, such as many microscopic methods, can provide definitive identification of ENMs in tissues, but they are typically qualitative or semi-quantitative.

Bioaccumulation of CNMs is often detected using their unique characteristics such as their thermal or spectroscopic properties. In laboratory studies, isotope labeling is a frequently used approach to quantify bioaccumulation of CNTs, GFNs, and fullerenes.[14, 15, 60, 82-86] Unlike CNTs or GFNs which are typically highly polydisperse, fullerenes can be quantified using mass spectroscopic techniques such as high-performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS).[87, 88] In the absence of isotopically labeled samples, it is often necessary to use extraction or separation steps to isolate CNMs from the sample matrix prior to analysis.[59, 89-92] However, few studies have been conducted to develop these methods for CNMs other than for fullerenes and SWCNTs.[79] This remains an important area for future research. There are some methods that can be used for CNT quantification in organisms without extraction, such as a microwave method[93-96] and near-infrared fluorescence for SWCNTs.[97, 98]

Bioaccumulation of metal-based ENMs (e.g., Ag ENMs,[99-103] ZnO ENMs,[104] CuO ENMs[21, 62, 105]) is most often assessed using total elemental analysis after digestion (e.g. acid assisted) with mass spectrometry or spectroscopy techniques. These measured concentrations include the original ENMs and various aged and decomposition products, such as released ions and biogenic/transformed structures. A major challenge with this approach is that these techniques do not distinguish between the background concentration of the main element (except for isotopically enriched ENMs), bioaccumulation of dissolved ions released from the ENMs, and bioaccumulation of the ENMs themselves. Thus, also testing the bioaccumulation of the dissolved metal is usually needed.

For complex matrices such as soils and sediments, it is important to assess the relative availability of the different forms of metal or metal oxide ENMs (e.g., intact ENMs or dissolved ions) in soil or sediment porewater or associated with soil or sediment particles, because ENMs in the porewater may be more bioavailable or easily transported in the environment.[106] For plant exposures, a water-only (hydroponic) design enables the most straightforward ENM characterization, while characterization of ENMs in soils is more challenging as a result of the dynamic nature of ENM behavior in soil,[107] particularly in the rhizosphere due to microbial processes and root exudation (although these processes would still occur to some degree in water-only (i.e., hydroponic) exposures), and the complexity and heterogeneity of the soil matrix.[108] Information on the different forms that contribute to the total metal levels in soils or organisms can be obtained by analyzing the soils using a range of different pore water and weak extraction techniques such as sequential extraction[105, 109] coupled with the use of filtration and/or centrifugation methods to separate particulate and dissolved species. However, the separation approach needs to be evaluated to determine if the procedure would unintentionally remove ENMs located in the pore water, confirm that specific steps can fully remove ENMs if desired, and to assess adsorption of ions or ENMs onto the sidewalls of the containers or to the membrane used for filtration. The resulting fractions can then be analyzed for metal content and possible speciation. Overall, filtering of extracts from more complex matrices (soil, sediment, tissues) may be difficult, because ions, ENMs, and other materials (e.g., NOM) may adsorb to the filter-membrane. This may result in the capturing of smaller materials than expected based on the pore size cut-off of the filter used, and therefore may bias the characterization of the relative concentrations of the different forms of the ENM. Separation of ENMs from soils or sediments using field flow fractionation (FFF) has also been shown to be effective in certain situations.[110, 111] Additional discussion regarding quantification approaches for ENMs in soils, sediments, and organisms and discussion related to spiking ENMs in soils are provided in the Supporting Information.

Stable isotope-enriched metal ENMs have proven useful for assessing the fate and biological uptake of ENMs, especially those based on elements that have high background levels in soil and biota. Studies with isotope-enriched ENMs can be conducted at environmentally relevant concentrations, because elements sourced from such ENMs can be readily separated from the natural background.[112] For example, nominal concentrations up to 6400 mg/kg soil were used in one bioaccumulation study with typical ZnO ENMs,[113] while isotopically enriched Zn allowed for detection of differences compared to the background Zn in soils at a concentrations of only 5 mg/kg to 10 mg/kg soil.[114] However, use of isotope-enriched ENMs does have some limitations. For example, by itself isotope-based discrimination cannot provide information on the ENM form, since, for example, it will not be known whether the isotopes remain present in particles or have formed free ionic species.[114] In some cases, isotopic labelling approaches may be used to distinguish between intact ENMs and dissolved ions released from ENMs through constraining the isotopic compositions of elements taken up in dissolved form where there is a dissolved background of that element with natural isotopic abundance.[115] Dual labelling strategies may provide possible insights into ENM fate and bioavailability when used in different forms.[116] Prior to the use of stable isotope-enriched ENMs, it should be confirmed that uptake kinetics of the different forms of the ENM are similar for the different isotopes.

Another promising approach to characterize metal-based ENMs in organisms is single particle inductively coupled plasma-mass spectrometry (spICP-MS), a technique that can provide size distributions, mass concentration, and number concentration of ENMs in suspensions and distinguish between ENMs and ions.[80, 117-122] However, this technique has only been used in a limited number of ENM bioaccumulation studies and additional research is needed to assess potential biases from ENM extraction processes.[121, 123-127] Additionally, this technique determines particle size based on assumed stoichiometry and crystal structure of particles, and the ENM size detection limit is relatively high for some elements.[29, 128] Recently, the use of spICP-MS has also been optimized to characterize and quantify metal ENMs (concentrations and size distributions) in soil[129] and soil organisms.[20] A key component of this approach is to distinguish ENMs from ionic background concentrations, which requires an optimized dilution of the extracts.[129] Employing spICP-MS for the detection of ENMs in biota may be complicated by the fact that organisms may form biogenic nanostructures of the metals released from ENMs, a finding recently shown using transmission electron microscopy (TEM) and energy dispersive X-ray spectroscopy (EDS) for earthworms exposed to silver ENMs.[20] The assumptions of the assumed stoichiometry and crystal structure for spICP-MS data interpretation are likely not met in such cases. Therefore, particles detected in the organisms may not be the same particles to which the organisms were exposed. In this case, it is essential to also perform spICP-MS analyses on control organisms exposed to ions, which can also contain nano-sized particles of biogenic origin.[20]

Microscopic approaches can provide an alternative or additional methodology to verify the bioaccumulation of ENMs in tissues and cells. However, there are challenges related to providing quantitative information about the mass, particle number, or concentration in the biological sample from microscopic images. Also, microscopy in general can be limited by the ability to locate ENMs within the matrices when the concentrations are low. Nevertheless, EDS can be used for some ENMs to provide elemental information about the particles observed when using scanning electron microscopy (SEM) or TEM. The confidence in microscopic measurements of ENM bioaccumulation can be strengthened by comparing results to those obtained using mature orthogonal measurements such as total elemental analysis when applicable. Additional limitations for analysis using EM are time and labor-consuming sample preparation, and the potential for introduction of artifacts in the samples. In addition to common artifacts like osmium-containing deposit formation in the cells after osmium tetroxide post-fixation, ENM-specific artifacts have been reported in studies with Ag, ZnO, and MgO ENMs.[130] Ag ENMs were shown to react with osmium tetroxide, while staining with uranyl acetate and lead citrate resulted in dissolution of ZnO and MgO ENMs. Thus, it was recommended to test the reactivity between the ENMs and the staining reagents, confirm observed particles by EDS, and use SEM in addition to TEM to confirm the position of ENMs in the sample.[130] Nevertheless, EM methods have been extensively used to uniquely provide visual evidence of bioaccumulation for a wide range of ENMs such as cerium oxide,[131] ZnO,[131] TiO_2,[132] carbon nanotubes,[11, 133-135] graphene family nanomaterials,[14, 24] and Au ENMs[136, 137] in a range of species. EM methods can also provide key information about the distribution of ENMs within cells such as intact CdSe QDs that have been biomagnified,[23] information that can be challenging to obtain using other approaches.

X-ray absorption spectroscopy (XAS) is a technique that can obtain definitive information about the chemical form of metals in biological samples and can differentiate between the dissolved ions, metal or metal oxide ENMs in the initial form used to dose cells or organisms, and transformed ENMs that may have been produced.[138-140] Overall, XAS is perhaps the most frequently used technique to characterize transformations of ENMs in complex matrices such as soils[141-143] and biological matrices[136, 140, 144, 145] and to characterize certain types of transformations in aqueous media such as sulfidation.[146-149] XAS is available at synchrotron user facilities and thus not for routine analysis, yet there are many synchrotron facilities worldwide. XAS measures the local coordination environment of metal centers and the presence of an ENM is inferred from this. The smallest probe size for beamlines capable of performing XAS is ≈ 30 nm, which can enable localization of particles within tissues and provide information about the states of those particles such as if they have been transformed; for example, ENM dissolution can be inferred in cells from the oxidation state of a released component metalloid and its NP form.[150] Assumptions that particles are in nanoparticulate form based on local coordination environment of metal atoms determined by XAS must be justified using deductions based on the XAS spectra or orthogonal measurements[136] such as EM and EDS.[150]

Given that artifacts and biases can impact some measurements, orthogonal approaches are needed wherever possible to provide multiple lines of evidence for quantification and visualization of accumulated ENMs.[29, 151] For example, three orthogonal techniques (scanning TEM (STEM) with EDS, spICP-MS, and ICP-optical emission spectroscopy (OES)) were utilized to assess bioaccumulation of TiO_2 ENMs by hydroponically grown plants.[123] STEM was coupled with EDS analysis to visualize the distribution and confirm the elemental composition of TiO_2 ENMs inside the plants tissues; a similar approach was used for analysis of TiO_2 ENMs in protozoans.[132] ICP-OES analysis was performed to determine the bulk elemental concentration of Ti, while spICP-MS was used to analyze ENM size distribution inside plant tissues.[123] Two plant digestion procedures (i.e. acid vs. enzymatic digestion) were also compared regarding their effects on the spICP-MS analysis. A similar approach was applied to quantify earthworm uptake kinetics of different forms of Ag-nanomaterials (including those biogenically formed from accumulated ions).[20]

Evaluation of detection limits for different analytical methods

The detection limit of a quantification method impacts bioaccumulation methods because lower concentration detection limits will improve quantification of the exposure dose and concentration in the biota, enabling testing at lower and more environmentally relevant ENM concentrations. Decreasing the detection limit will also enable better differentiation between ENMs in biota versus the background from other potentially interfering compounds. This is especially important for ENMs composed of elements which are present at a high concentration in the environment, for example Cu, and for some CNMs.

The lowest achievable mass detection limit when quantifying ENMs in environmental matrices—for many analytical techniques—will be similar to that achieved when using the same technique to quantify the element comprising the ENM. For example, elemental techniques based on measuring carbon to quantify CNMs (e.g., total organic carbon analysis or thermal optical transmittance) will have a lowest achievable detection limit at the concentration for detecting total carbon.[63, 79, 152-154] A similar relationship exists for techniques based on elemental concentration measurements of metal-based ENMs (e.g., ICP-MS). An exception is spICP-MS, which can detect individual ENMs as a result of the substantially shorter dwell times (50 µs to 10 ms) compared to total elemental analysis (approximately 300 ms). Since a spike in the intensity signal is detected in this shorter dwell time windows, spICP-MS has far lower mass detection limits than those for total elemental analysis.[117, 120] In general, the ENM size and concentration detection limits need to be determined on a case-by-case basis for each ENM and matrix combination and depend upon the sensitivity of the instrument to distinguish the ENM from the matrix among other considerations. To further investigate the recovery and detection limit for a particular ENM in a test organism, it is possible to spike a known mass (often applied as a volume of an ENM suspension with a known concentration) or range of masses directly to a mass of organism tissue similar to the mass that will be used in the experiments, and then perform the analytical procedure including any sample digestion steps.[91, 121, 124] However, it is possible that this approach may overestimate the recovery and detection limit if internalization of the ENM within the tissue or cells would lower the recovery of or otherwise bias the analytical method. Furthermore, dissolution of metal ENMs in organisms would increase the ionic background concentration, potentially increasing the smallest ENM size that can be detected.

Theoretically, microscopic techniques such as EM could be used to detect a single ENM particle in an organism. However, detection is not the same as quantification since the latter requires understanding the detection limit if comparative analysis is a goal. In practice, the detection limit (particle concentration of an ENM in a volume of tissue or number of cells) in a specific matrix depends on several factors such as the capacity of a particular microscopic technique to differentiate the ENM of interest from other natural or incidental particles and other materials in the matrix including avoiding false-positive or false-negative results, the number of cells or area of tissue analyzed, and the acquisition of enough visual information in two dimensions such that a three dimensional impression of ENM distribution in tissue can be acquired. The first two challenges are also present for other scenarios where TEM is used quantitatively such as for the standard method for determining asbestos concentrations in air samples[155] or for counting the nanoparticle number concentration in a suspension.[156] In studies assessing whether an ENM can be detected in a biological matrix after exposure, it is not possible to determine the detection limit from the information provided unless the area of tissue analyzed is reported. For the asbestos quantification method, a known area (determined by the number of grids viewed) are analyzed, allowing for calculating the detection limit. Without a similar approach to ENM quantification, it is infeasible to statistically relate the lack of observing an ENM in the tissue to the ENM concentration in that tissue. Thus, a recommendation for EM, if it is to be used quantitatively, is to attend to establishing the NP detection limit. Further, attention to the three-dimensional nature of biological specimens with their bioaccumulated ENMs would be needed, such as by imaging numerous sections representative of the tissue and arriving at a statistically defensible scheme for assembling data across sections into a model of the whole tissue specimen.

Subcellular separation approaches

One approach that can be used to better understand ENM bioaccumulation at the subcellular level (e.g., concentration of an ENM associated with organelles or metallothionein-like proteins) is to perform a subcellular separation technique.[127] This data can improve the potential for toxicokinetic modelling by supporting the selection of appropriate multi-compartment models. Multiple subcellular fractionation techniques have been published for plants and other multicellular organisms.[127, 157] This information may be informative in understanding toxicity mechanisms and the potential for the ENMs to exert toxicity through different adverse outcome pathways. For example, internalization of metals in biota reveals the internal distribution processes that occur during metal accumulation, and may, therefore, provide information on metal toxicity and tolerance after exposure to ions or metal-based ENMs.[157-160] When applying subcellular fractionation for metal-based ENMs, measuring the metal concentration both as the total body burden and in subcellular fractions as a means to assess methodological losses (i.e., comparing the total body burden and the sum of the metal in each of the subcellular fractions) can reveal if an acceptable recovery is obtained. Similar measurements should be performed for CNMs.

There are a number of steps needed for the analysis of tissue compartmentalization. First, the organisms or tissues need to be homogenized, and then the homogenate is subjected to a fractionation procedure such as differential centrifugation. One significant potential complication is if the homogenization process resuspends ENMs, such as those located in the cytosol. These suspended ENMs could then potentially adsorb to other cellular components during the separation steps or be removed from the supernatant by differential centrifugation steps especially if ENM agglomeration occurs. Therefore, appropriate control measurements need to be included such as performing the separation steps with dispersed ENMs added directly to the extraction buffer. In addition, one should conduct the homogenization process on an unexposed organism, spiking in dispersed ENMs, and then perform the extraction process.[158] There is a possibility that the adsorption of a large number of dense ENMs could influence the separation of different organelles if there is a sufficiently large change in density of an organelle to cause it to be removed in a sequential differential centrifugation procedure at a different step. It may be possible to perform calculations using Stokes' Law to theoretically estimate the potential for this to occur using a worst-case scenario such as by estimating the maximum potential loading of the ENMs onto each cellular fraction. However, performing this calculation would require information about the buoyant density and diameter of the organelles and of the ENMs. In addition, ENMs in cells may have their buoyant density decreased as a result of interactions with biomolecules.[161] It is possible to compare results obtained from a subcellular separation process with orthogonal methods such as microscopic analysis using EM[13, 158] or Raman spectroscopy.[162] One approach to avoid some of the issues with sequential differential centrifugation approaches would be to use density gradient centrifugation since only a single centrifugation step is typically performed. Density gradient centrifugation separations rely on the use of centrifugal force to separate particles of different sizes, densities, and masses; larger and denser particles sediment at faster rates than less dense, smaller particles.[163] It is possible to estimate the conditions that should be used for density gradient centrifugation using Stokes' Law as described above if the relevant information is available.[164] To facilitate identification of the ENM-containing subcellular fraction using density gradient centrifugation, using dye-labeled ENMs has been proposed.[165] More information about density gradient centrifugation (e.g., density of ENMs and commonly used media) is provided in the following section when discussing the separation of single-celled organisms and ENMs.

Case studies

Given the different considerations related to making accurate and robust bioaccumulation measurements for various species (Figure 1), multiple case studies will be discussed. Single-celled organisms will be evaluated separately from multi-cellular species given that there are some important considerations for bioaccumulation measurements based on the size and complexity of the organism. In addition, plant species will be discussed separately from other multi-cellular organisms, reflecting differences in their physiology and also specific exposure considerations for studies between multicellular plants and other species. Descriptions of how to prepare and characterize the ENM exposure media (water and soil as examples) are provided in the Supporting Information.

Single-celled organisms

To examine bioaccumulation in single-celled organisms, it is important to consider overarching topics that are relevant for multiple species such as separating them from suspended ENMs and considerations related to bioaccumulation by individual cells or cell populations. To provide more specific examples about how this information can be utilized, case studies are also provided for single-celled organisms without a cell wall and for biofilms.

Separation of single-celled organisms from suspended ENMs

For analytical techniques such as confocal microscopy,[166, 167] coherent anti-Stokes Raman scattering microscopy,[168] hyperspectral imaging,[169-171] X-ray fluorescence,[172, 173] or secondary ion mass spectrometry,[174] separation steps may not be critical or necessary as the detection capabilities of these instruments allow for penetration past the cell surface without destruction of the organism prior to analysis and may allow for distinguishing between particles on the cell surface versus those that are internalized. On the other hand, many techniques that provide quantitative information on bioaccumulation such as the total elemental analysis methods described above require separation of the cells from suspended ENMs prior to analysis. This is critical because insufficient separation of cells and suspended ENMs can lead to biased bioaccumulation measurements since suspended ENMs will be mistakenly interpreted as being associated with the cells.

When separating ENMs from suspended cells using filtration or centrifugation, the primary focus is separation, while a secondary purpose can be to dislodge surface-attached but not internalized ENMs.[121, 169, 172, 175] Repetitive rinsing and differential centrifugation steps have often been applied to algae and bacteria before quantification of the cell-associated ENMs.[39, 150, 176] In studies with protists and algae, repetitive centrifugation, washing with clean medium and filtration though a > 1-µm pore size filter have been applied with similar aims. Some authors have shown that the filtering and rinsing approach is efficient in removing the loosely bound ENMs from cells by confirming that additional washes do not reduce cell-associated ENM concentrations,[177] especially when the ENMs are well dispersed.[178] However, these simple rinsing procedures may not be sufficient to remove suspended particles or their agglomerates from single-celled organisms that could be in the same size range as ENM agglomerates. To further assess ENM removal using these approaches, it may be helpful to perform experiments where the cells and ENMs are mixed, and then the separation step immediately performed to assess the extent to which ENMs are fully removed. This control experiment revealed a lack of full ENM removal with several rinsing steps of multicellular nematode *Caenorhabditis elegans*,[121] although it is unclear if a similar result would be obtained for suspended cells. For larger or agglomerated ENMs, alternative approaches may be required. For example, the mobility of ciliated protozoa can be utilized in separating unicellular organisms from the pellets of CNTs: after pelleting the samples by centrifugation, *Tetrahymena thermophila* were allowed to swim out of the pellet into the supernatant prior to collection.[179] If it is critical to determine if surface-attached ENMs have been removed, it is possible to evaluate the outer surface of a statistically sufficient number of exposed organisms using SEM or TEM to assess the presence of ENMs.

Recently, alternative separation strategies such as the use of density gradient centrifugation, a technique commonly used to achieve size separation and selectivity of ENMs in the post-synthesis and purification steps,[180-184] have been implemented to separate unassociated ENMs from organisms in cases where water or media rinses and differential centrifugation were found to be insufficient.[82, 164, 185] Media of particular densities can be selected to enable separation of the ENMs and organisms based on either their size and mass (rate-zonal centrifugation) or solely on density (isopycnic centrifugation).[164] Rate-zonal centrifugation is similar to differential centrifugation in the sense that the sedimentation speed of the particles depends on their size and mass. The advantage of this approach is that it allows for complete separation of smaller from larger particles[121] unlike in differential centrifugation where cross-contamination of particles of different sedimentation rates may occur.[186] In rate-zonal centrifugation, the cells and ENMs form distinct zones when moving down the density medium as the faster sedimenting larger and heavier particles move ahead of the slower ones.[121] Since the density of the gradient medium is lower than the density of the cells and ENMs, the sample components will pellet if centrifuged for a sufficiently long period. Thus, selecting the centrifugation time and force is crucial for optimal separation.[164] In isopycnic separation, the density of the medium must be in the range of equal to or greater than the density of the sample components so that the cells and ENMs remain in the media layer equal to their buoyant density.[187] Important factors to consider in choosing a suitable density gradient medium include the following: (i) biocompatibility to avoid adverse impacts on cell physiology, behaviors, and viability; (ii) sufficient solubility to produce the range of desired densities; and (iii) easy removability from the purified cells. To optimize this procedure, certain organisms may require gentle centrifugations speeds, while others do not. The density ranges for the most prevalently used gradient media, species that are suitable for use with this separation technique, and the density ranges reported for ENMs are highlighted in Figure 2. If purified organisms are intended to be used in further experiments, such as trophic transfer tests, optimization of the centrifugation time is especially important to ensure complete separation while keeping the centrifugation time short enough not to compromise the viability of the organism. Theoretical approaches based on Stokes' Law have proved useful in optimizing centrifugal times and assessing the likelihood of effective separations in density gradient centrifugations.[164] Calculating the theoretical minimum diameters of the particles that would sediment can guide the optimization of both differential and density gradient centrifugation procedures. However, it must be noted that possible discrepancies between the theoretical and experimental results should be considered in cases where the density gradient medium is expected to interact with cell surfaces or permeate the cell membrane, such as with sucrose,[164] or when coating with biomolecules may change the buoyant density of ENMs.[161] Depending on the size, mass and buoyant density of the particles to be separated, a sequential separation approach that combines differential, size- and buoyant density-based centrifugation may be needed.

Considerations regarding bioaccumulation measurements of individual cells and cell populations

The bioaccumulation assessment of ENMs in microorganisms usually involves planktonic cultures composed of hundreds of thousands to millions of single cells. Unlike tests with larger organisms, such assays enable population-level measurements. Microbial studies offer a unique opportunity of evaluating ENM bioaccumulation across thousands of individuals as well as multiple generations.[188, 189] ENM bioaccumulation measurements using growth assays, sampled at different time points, can provide valuable information on the ENM content associated with the cells at different population growth stages. It has been reported that uptake of ENMs by eukaryotic cells can be influenced by their cell cycle phase.[190] ENMs that are internalized by cells or associated with the cell membrane are split between daughter cells when the parent cell divides. Consequently, in a cell population, the concentration of ENM in each cell varies depending on the cell cycle phase. Similarly, association of ENMs with prokaryotic cells in a growing culture varies depending on the growth phase: in the phase of fast division the bioaccumulation rate of ENMs could be overpowered by the rate of cell division such that the concentration of ENMs in or on individual cells could be diluted in a manner similar to the growth dilution that can occur in plants. Therefore, it is important to consider cell cycle phase (eukaryotic microbes), growth phase (prokaryotic microbes), and thus growth rate, when interpreting the bioaccumulation of ENMs in single-celled organisms.

Often, the addition of ENMs to single-celled organism cultures results in heteroagglomeration. For example, cell agglomeration has been noted when co-incubating CNTs[164] or positively charged ENMs[191] with bacteria, or CNTs[192] or alumina-coated SiO_2 ENMs[193] with algae. Such heteroagglomeration complicates bioaccumulation measurements because (i) determination of cell numbers by direct counting is typically not possible and other approaches, such as ATP concentration of the cells[194] or photosynthetic activity of the algae[193] instead need to be employed, although the potential for artifacts in cell viability assays is well known and appropriate controls should be used;[28, 195, 196] (ii) separation of cells and ENMs not tightly associated with the cells is challenging as described above; and (iii) heteroagglomeration becomes an issue in single-cell analysis methods such as flow cytometry and single cell analysis by ICP-MS. Application of the latter methods for quantification of ENMs associated with cells is discussed in more detail below.

Conventional analytical methods used for quantification of ENMs associated with cells (e.g., ICP-MS, ICP-OES, liquid chromatography/mass spectrometry, fluorimetry, ultraviolet-visible (UV-Vis) spectroscopy) require harvesting at least several hundred micrograms of biological material to provide a sufficient mass for analysis. These analyses yield an average ENM concentration in the cell population. While some of these methods (ICP-MS and ICP-OES) enable detection of trace metal concentrations, they typically do not provide information on ENM distribution among the cells in the population. However, flow cytometry and single cell cytometry by time of flight (TOF) ICP-MS can provide information on the distribution of ENMs in hundreds or thousands of individual cells.[197, 198] Techniques used for ENM quantification at the single-cell level, including flow cytometry, have been recently reviewed from a nanomedicine viewpoint, focusing on ENM bioaccumulation in mammalian cell lines.[199]

In flow cytometry, ENM bioaccumulation is quantified either based on fluorescence (in the case of fluorescent or fluorescently-labeled ENMs) or other optical properties of ENMs. Measurement of non-fluorescent ENMs is achieved based on side scattering (SSC) intensity that correlates with changes in cellular granularity due to the uptake of ENMs. Flow cytometry as a semi-quantitative technique has been successfully used for measuring uptake kinetics of quantum dots (QDs) in protozoa *T. thermophila*[200] and algae *Ochromonas danica*[167] and of TiO_2 ENMs in *Paramecium caudatum*.[201] One of the challenges in using flow cytometry for measurements of single-celled species exposed to ENMs is avoiding misinterpreting signals from ENM agglomerates as those from ENM-coated cells. The latter is especially important with bacteria or small protists. It may be possible to minimize this impact if separations are performed first as described above. Aggregated cells, heteroagglomerates of cells and ENMs, and ENM association with cell debris can also complicate analysis and signal interpretation. It is also important to note that some ENMs have been shown to cause false-positive or false-negative results in a viability assay to test for apoptosis or necrosis using flow cytometry and thus careful control experiments also need to be included for bioaccumulation measurements to avoid artifacts.[202]

More recently, ICP-MS has been developed and commercialized for the analysis of single cells.[203-205] Similar to spICP-MS, in single-cell ICP-MS (SC-ICP-MS) the cell suspension is nebulized through an ICP-MS sample introduction system, each cell is ionized, and the metal ions originating from a single cell are detected. Considering that SC-ICP-MS is a new technique, it is not surprising that the applications for ENM quantification are still in the development phase and relevant literature is limited. SC-ICP-MS has been successfully applied for the detection of QDs in mouse cells[206] and Au ENMs in algae,[204] and laser ablation ICP-MS (LA-ICP-MS) has been used for measurement of Au and Ag ENM bioaccumulation by and within mouse cell lines.[207, 208] Considering that concentrations of trace elements in various other environmental single-celled species have been studied using SC-ICP-MS,[209-211] there is substantial promise for the use of this technique to assess cellular ENM bioaccumulation. Important considerations when using this method include a careful separation of non-associated ENMs from the cells prior to analysis so as to ensure that the measured signal originates from within the cells, and adjusting the cell concentration in the sample and instrument dwell time so that only one cell is detected at a time. Similar to flow cytometry, one of the limitations of SC-ICP-MS is that no distinction can be made between internalized and cell surface-attached ENMs. Coupling ICP-MS with laser ablation provides information about the spatial distribution of ENMs in cells, although resolution at the nanometer scale remains a limiting factor.[205]

Microscopic methods that can resolve ENMs associated with the cells are often used for confirming ENM localization within cells.[23, 167, 200, 212] Intracellular ENM quantification methods that are particularly suitable for protist model organisms that are relatively large (e.g., *Tetrahymena* sp., *Euglena* sp., and *Ochromonas* sp.) include optical microscopy (i.e., bright field, phase contrast, and darkfield microscopy with hyperspectral analysis)[82, 200] and EM.[132] Such techniques can also be used semi-quantitatively or quantitatively for ENM bioaccumulation measurements. Semi-quantitative approaches include measurements of ENM area or fluorescence per cell. In quantitative microscopy, ENMs are counted in cells or the measured ENM area per cell is converted to mass or number concentration based on the size, shape and density of the ENM. In

ENM research, high-resolution techniques are desired for the visualization of single ENMs in cells. In addition to being a valuable tool for characterizing ENM-cell interactions, EM can be used quantitatively. For instance, TiO_2 ENM accumulation in the food vacuoles of *T. thermophila* was quantified from the scanning transmission electron microscopy (STEM) images of *T. thermophila* thin-sections.[132] Based on the geometries of *T. thermophila* food vacuoles with accumulated TiO_2, the ENM concentration per cell volume was calculated using the volume and number of food vacuoles per cell and the density of TiO_2. Similar to making quantitative microscopic measurements of cells for other purposes, there are a number of sources of uncertainty in microscopic imaging relevant to understanding the precision of these measurements for ENM bioaccumulation: (i) the impact of microscopic imaging parameters (e.g., focus),[213] (ii) image quality such as the signal to noise ratio for the ENM area compared to the background, (iii) determining the adequate number of cells to analyze to sufficiently reflect the behavior in the larger population; and (iv) the precision and reproducibility of image processing algorithms to calculate the ENM area;[214-217] assessing the image processing algorithms could be performed by comparing manual measurements of the ENM area for a certain number of images to those calculated by the computer program to assess the accuracy of the algorithm.

Although light microscopy cannot resolve single ENMs, it is suitable for visualizing ENM agglomerates when these are larger than the resolution limit of light microscopes with a conventional lens, i.e., approximately 200 nm. This may occur if ENMs are packed into agglomerates in the food vacuoles of particle feeding (phagocytosing) single-celled species.[82] This phenomenon provides a good opportunity for using quantitative optical microscopy for ENM uptake and elimination kinetics measurements. Dark field microscopy coupled with hyperspectral analysis also enables identification of ENMs in cells, confirming that only the intracellular agglomerates composed of ENMs are measured.[171] Since single-celled species vary in physiology and ENM uptake mechanisms, it is advisable to validate microscopic image-based quantification with another analytical method. For example, uptake of carbonaceous nanomaterials in the protozoan *T. thermophila* was quantified in parallel by image analysis and measuring ^{14}C labelled MWCNTs, and the two methods were found to correlate well.[82]

Single-Celled Species Case Study #1: Species without a cell wall (protozoa)

The lack of a cell wall makes the membrane of single-celled species such as protists and some mixotrophic algae directly accessible to ENMs. ENMs can adsorb onto and associate with the cell membrane and subsequently be internalized by endocytosis.[167, 177] In addition to endocytosis, some protists and mixotrophic algae acquire nutrients by phagocytosis, a mechanism by which particulate materials (organic particles, bacterial, yeast and small algal cells) are internalized by the formation of food vacuoles. Thus, in contrast to microorganisms with cell walls that cannot internalize particulate matter, protists and some algae are expected to take up ENMs and their agglomerates at sizes larger than 50 nm[218] by natural feeding mechanisms, as reported for various species and different types of ENMs.[82, 132, 167, 171, 200, 219] Food vacuoles containing ENMs are trafficked through the cell similarly to those containing nutrients. For inert ENMs or non-toxic ENM exposure concentrations, the contents may be subsequently expelled through the cell membrane. Therefore, from the perspective of bioaccumulation assessment, food vacuoles in protists function similarly to the digestive system of multicellular organisms and thus, the experimental design warrants the inclusion of an elimination phase before quantification of bioaccumulated ENMs (Figure 1). So far, only a few studies have measured elimination of ENMs in single-celled species, including those without a cell wall.[167, 171, 200]

Single-Celled Species Case Study #2: Biofilms

Biofilms (Figure 3) comprise surface associations of microbial cells embedded in hydrated extracellular polymeric substances (EPS).[220] Biofilms are prevalent forms of microbial growth in all compartments of natural and built environments.[221] Yet they are less studied in the realm of microbial-ENM interactions, including assessments of ENM bioaccumulation, than free living microorganisms.[222] EPS appears to trap ENMs, as demonstrated for ZnO ENMs in activated sludge flocs,[223] and Ag ENMs in bacterial monocultures under laboratory conditions.[224] Because EPS is a physical structure surrounding the cells, the association of ENMs with EPS influences exposure of biofilm cells to ENMs, and may affect direct ENM bioaccumulation. For example, Au ENMs in estuarine mesocosms[16] and TiO_2 in paddy microcosms[225] were shown to accumulate in biofilms with subsequent transfer to higher, predating organisms such as grazing snails. The quantification of such ENM bioaccumulation within biofilms is currently largely unresolved; this may be significant if ENMs are compartmentalized in biofilms with preferential association either on cells or in the EPS. As shown in Figure 3, ENMs associated with EPS or cells would be quantified in a total biofilm mass-based accounting of prey in a grazing experiment. However, trophic transfer and biomagnification may hinge on ENMs being firmly associated with cells, especially in cases where a predator's digestion of EPS and prey differ. In environmental microbiology, it is an established convention to separate biofilm cells from EPS and to quantify toxicant association with each of these two broad biofilm components separately, such that increased EPS production—a common stress response in biofilm bacteria—can be assessed along with toxicant accumulation.[226] A future recommendation in the assessment of ENM bioaccumulation for biofilms would be to adopt a similar approach. This would allow the normalization of ENM accumulation in the biofilm to total cell count and also to EPS dry mass, rather than wet-mass which can be system- and condition-dependent. This approach, coupled with ENM quantification for each biofilm component (EPS and cells), would allow determining overall biofilm bioaccumulation assessments in terms of ENM distribution. Furthermore, it would allow trophic transfer or biomagnification factors to be better expressed according to either the whole biofilm (in the event that ENMs are evenly distributed across EPS and cell components), EPS (if ENMs are mainly concentrated there), or cells (if ENMs are preferentially adsorbed to their external surfaces).

Multicellular organisms (excluding plants)

For multicellular organisms, it may be important to distinguish between the total body burden in the absence of voiding the gut (as the ENM concentration in the gut tract can readily be voided), the ENM concentration adhering to an epithelial surface (e.g., gut microvilli), and the ENM concentration that has been truly adsorbed through an epithelial surface, for example in daphnids (Figure 4). Which of these fractions is relevant for an individual assessment may be context dependent (Figure 1). For example, trophic transfer studies may consider all fractions, while toxicokinetic and mechanistic toxicology studies may focus only on the absorbed fraction.

However, even in the latter case it is important to bear in mind that it is entirely possible that the ENMs may cause adverse effects during simple passage through the gut tract (or while in contact with gills), and thus concentrations in the gut tract and in other tissues may be important to measure, depending upon the other endpoints that are measured and the ultimate purpose of the experiment. The importance of such considerations is illustrated through a set of relevant case studies provided for fish, soil invertebrates, *Daphnia*, and marine bivalves.

Another key approach that can be used to elucidate the bioaccumulation of ENMs is to evaluate the toxicokinetics of uptake and elimination behaviors of whole organisms or specific organs or tissues. With regards to the elimination rates, one key difference between ENMs and dissolved organic chemicals or metals for multicellular organisms with a digestive tract is that the majority of the ENMs can be loosely associated with the digestive tract and, therefore, potentially subject to rapid egestion within the early part of an elimination phase. Therefore, taking additional time points close to the conclusion of the elimination period may be valuable for discerning if all of the ENMs associated with the organism after the uptake period can be eliminated by voiding the gut tract. Depending upon the organism's physiology, feeding during the elimination period may be needed for voiding the gut tract. For some species, the time period needed to void the gut tract has been measured (e.g., *Lumbriculus variegatus*[227] and earthworms or enchytreaids[228]) or visually inspected in semi-transparent organisms (e.g., *Capitella teleta*[229]) and is, hence, relatively well understood. However, such information is not always readily available for other species. If the gut voiding kinetics are unknown for a species, it is possible to assess this for soil and sediment organisms by measuring the rate of soil/sediment elimination by the organism. This can be measured during a depuration experiment by determining the ash content after combustion of organisms to determine the quantity of soil or sediment remaining,[227] or by measuring the amount of a non-bioaccumulating rare earth metals such as lanthanides in the test species and comparing that concentration to the amount in the soil or sediment to determine the soil or sediment mass remaining in the organism.[230] For smaller species, such measurements may require population cohorts rather than individuals to meet detection limit thresholds. One important consideration is the need to balance gut voidance time with the potential for elimination of ENMs from the tissues being investigated. Hence, longer elimination periods are not necessarily better, because there can be rapid elimination in the time period shortly after the cessation of exposure. The initial kinetics of elimination may be overlooked if longer elimination periods to void gut contents are used.[231] Thus, it is recommended to make measurements during the elimination time series to initially include smaller steps (hours to days) to assess gut voiding and then longer steps (days or weeks) toward the end of the elimination period.

For ENMs that dissolve (e.g., Ag ENMs) or for ENMs composed of an element that is present in the exposure matrix (e.g., Zn in a sediment experiment), measuring the elimination rate at additional time points may be important to assess if there is a biphasic elimination process such as rapid elimination of the ENMs followed by a slower release of the accumulated dissolved ions or indeed the reverse case of fast eliminating labile and slower released particulate pools in cells. As described above, these measurements can potentially be refined by evaluating the ENMs associated with the organism such as by conducting spICP-MS analysis after digestion, or by measuring isotopically labeled ENMs for metal or metal oxide ENMs using isotope specific mass spectrometry. For ENMs that dissolve, it can be informative to compare the toxicokinetic rates obtained to those for a metal ion exposure using similar conditions. This can allow differences in toxicokinetic rates to be identified based on model fits and parameters values for different single compartment and multiple compartment kinetic models. These quantitative methods could be coupled with imaging techniques to obtain a better estimation of actual particles versus dissolved fractions in the organism tissues.

Multicellular Species Case Study #1: Fish

Measurement of the bioaccumulation potential for ENMs in fish requires special attention because the principle regulatory bioaccumulation test is a fish bioaccumulation assay (OECD TG 305[30]). Fish are a group of organisms that are large enough to facilitate dissection of the internal organs to identify the 'target organs' and the ENM biodistribution.[49] However, there remains a substantial problem: the relationship between the exposure concentration and the internal dose leading to adverse effects remains unclear. The absence of routine measurement methods for ENMs in tissues has prevented unequivocal demonstration of cause and effect.

The initial step in the case of waterborne exposure after the exposure period is the removal of any excess water containing the ENM from the body surface. Experience so far suggests that there are no special or additional steps needed to do this for ENMs compared to traditional chemicals. For trout, netting the fish into a closed bucket of clean water with dilute anaesthetic to calm the animal and facilitate handling is needed. Typically, the fish is rinsed for about a minute in one bucket, and then transferred to another bucket of water containing a more sufficient level of anaesthetic to enable terminal anaesthesia (i.e. euthanasia in preparation for later dissection). Once the fish is euthanized, larger fish can be further triple rinsed in ultrapure water or completely immersed in a series of beakers of ultrapure water for smaller fish. This procedure will remove loosely bound material and dilute away any residual water from the tank. However, this procedure may not fully remove ENMs trapped in the mucus layers on the gill, skin or gut.

Fortunately, there are methods available to quantify the surface-associated ENMs in the mucus of the gill microenvironment and for the gut mucosa. These 'Surface Binding Experiments' have been well established for metals and other solutes[232] and are the experimental basis for the biotic ligand models (BLM[233, 234]). The technique involves a separate short experiment with previously unexposed fish tissue. The tissue (e.g., gill filaments or piece of intestine) is allowed to instantaneously adsorb the ENM onto the surface of the epithelium over a few seconds (i.e., before true uptake can occur). Then the total metal concentration in the tissue is determined. This method has been used successfully to measure the surface-bound TiO_2 ENMs, for example, on the mucosa of the mid and hind intestine of rainbow trout.[235] This study revealed that surface adsorption can be significant and, when exposure concentrations of 1 mg/L or less are used, it is likely that approximately 20 % of the apparent total tissue Ti is adhered to the surface of, not within, the tissue. Instantaneous adsorption measurements therefore become a vital consideration when interpreting data on ENM uptake by the gill, skin, gut or other external barriers of organisms (Figure 1).

Multicellular Species Case Study #2: Marine Bivalves

Marine bivalves (e.g., clams, mussels and oysters) are ideal candidates for the study of ENM fate and effects and have been exposed to a wide range of ENMs.[236-241] Their physiology is well studied, and they are generally tolerant to varying environmental conditions and therefore relatively easy to culture and test. These species are commonly used as monitoring organisms because of their sessile and widespread nature. In addition, they serve as a food source for many higher trophic level aquatic and non-aquatic organisms including humans. Bivalves are unique in that their internal organs are often bathed in external or environmental media. In addition to direct exposure of external media, their capacity to filter large volumes of water ensures their exposure to large quantities of contaminants present in the water column, and for burrowing bivalves exposure at the sediment-water interface and in sediment interstitial water.

Assessing the biodistribution in these organisms via dissection enables a better understanding of what organism tissues are exposed to ENMs and if absorption of ENMs across epithelial surfaces has occurred. The gills are often the first organ to be exposed due to their filtering role, and studies have shown that bivalve gills have the capacity to differentiate among particles as a result of particle sizes and surface characteristics,[242-244] although ENMs are subsequently translocated into the digestive system. For example, *Mytilus edulis* had a progressive uptake and transport of SiO_2 particles from the gills to the digestive gland and then to hemocytes.[245] Similarly, Au ENMs accumulated primarily in the digestive gland (93 %) of *M. edulis* with smaller amounts in the gills (3.9 %) and mantle (1.5 %).[246] Similar findings have been observed for TiO_2 ENMs[247] and Ag ENMs (although Ag ions were not distinguished from Ag ENMs[241]), while a study on ZnO ENMs showed higher Zn concentrations in the gill compared to the digestive gland.[248] Once ENMs enter the organism, they have been shown to transfer across cell membranes and interact with key internal cell organelles causing cellular damage.[49, 249, 250] In addition, while pristine ENMs may be smaller than the preferred size for uptake by bivalves, either homo- or heteroagglomeration may change the bioavailability of the ENM based upon the filtering capacity of the gills or particle capturing apparati. Therefore, a number of researchers point out the importance, particularly in high ionic strength marine waters, of characterizing the ENM agglomerates to which organisms are exposed.[244, 251, 252]

There are some important considerations for both laboratory procedures and data interpretation when working with bivalves. Bivalve organs typically dissected include the gills, digestive gland as well as the gonad tissue in mature animals. The hemolymph can be collected via a syringe from the adductor muscle.[234] There is a concern that these invertebrate animals have an open circulation system and any ENM will bathe all the internal organs in an undirected manner (i.e., not via a blood vessel[253]). Direct contact with the organs in an open circulatory system may change the interpretation of both the internalized dose and the notion of a true target organ. Practically, at the bench, it becomes even more important to ensure that all of the internal organs are suitably washed, as without this step the hemolymph may contaminate all tissues and lead to erroneous estimate of actual tissue burdens. In bivalves, because of this, there is also a concern that excretory products may incidentally contaminate the tissue sample. Special attention needs to be given to the pseudofeces or biodeposits produced by bivalves. In the animal's normal biology, biodeposits are an efficient way of preventing the accumulation of unwanted naturally occurring particulates and insoluble metal deposits. These biodeposits alter the ENM form when it reenters the environment, as the ENMs will be packaged in a carbon rich, dense, mucous bundle that most likely enters the sediments and will be reprocessed by deposit feeders or organisms that filter larger particles. During bivalve bioaccumulation experiments, only a minute contamination of bivalve tissue with such biodeposits can lead to overestimation of the tissue metal concentration. There is also concern about particles settling onto the external surfaces of the body organs in the elevated ionic strength conditions of the hemolymph or in seawater.[254] However, surface-binding experiments such as those conducted on trout tissue have not been performed with shellfish.

Careful dissection and detailed washing procedures are needed to avoid this contamination, and such methodological details should be reported for ENM studies with bivalves.

Multicellular Species Case Study #3: Daphnia

Daphnia species have been widely used in bioaccumulation studies, as they represent a key level in trophic chains while feeding on unicellular organisms and serving as prey for second consumers. Uptake, elimination and bioaccumulation studies with *Daphnia magna* have been described in the literature for a broad range of metal-based ENMs and CNMs.[11, 12, 15, 71, 255-258] Bioaccumulation experiments with *D. magna* have been conducted using experimental designs that include an uptake followed by an elimination phase in clean media, or by independent experiments evaluating both processes. Exposure through media only or via contaminated food (e.g. algae) are also experimental setups available in the literature.[257] Uptake phase durations range between 1 h to 48 h, while elimination phases last similar periods or can be extended up to 10 d.[259]

The organism age varies substantially among studies of ENM bioaccumulation (<1 d[256] to 14 d[260]) which impact ENM bioaccumulation results as a result of different body morphometrics; similar findings were observed for bivalves as described in the Supporting Information. It has been suggested that differences in body burden that result after MWCNT exposure may stem from differences in the sizes of the organisms: smaller organisms, for which the gut tract is a larger fraction of the total organism, may have higher body burdens than larger organisms if the gut tract is not voided.[255] Within this variability regarding age, the organism's growth and reproductive status should be considered in ENM bioaccumulation experiments, avoiding as much as possible different life-cycle stages within sampling times. Before the uptake phase, some studies also report the need to void daphnids' guts,[96, 258] while other studies report a short feeding period prior to ENM exposure.[261] These practical details can complicate comparing data, as differences in age, exposure time and gut status (voided or not) can cause substantial differences in bioaccumulation patterns among studies. There is also a relationship between ENM uptake, size of the organism, and volume of the ENM test media as described in more depth in the Supporting Information.

Daphnids sampled for analysis are expected to adsorb ENMs to their carapace. Several studies have already identified the presence of attached ENMs in moult samples.[96, 262] Therefore, several procedures have been described for sampling daphnids for chemical analysis. These methodologies range from a gentle wash[96] to a vigorous agitation by pipetting daphnids in and out of the water,[261] to collecting daphnids with a small sieve and rinsing them with Milli-Q water [257] or with the exposure media.[12, 258] Although different procedures are described, little evidence is provided on method effectiveness. While adsorption onto the carapace can be seen as an external accumulation that will typically not directly harm the organisms (unless by impacting molting), external accumulation can be important to trophic transfer.

Multicellular Species Case Study #4: Soil invertebrates

Soil is considered a major sink for chemicals and also for ENMs, which may reach this compartment through direct ENM application as an agrochemical (e.g, fertilizer pesticide, or biocide), or from solid waste including sewage sludge.[106] Soil is an extremely complex matrix, and the transformation and fate of ENMs in soils are similarly complex.[106, 263, 264]

Soil invertebrates can accumulate ENMs or dissolved, or otherwise transformed, materials from the soil or soil porewater both through direct dermal contact or orally via ingestion with food.[114, 265] Key soil properties such as pH, organic matter content, clay mineralogy and cation exchange capacity, as well as the specific physiology of the species, can all potentially influence ENM bioaccumulation potential. For assessment of bioaccumulation of ENMs in these species, ENM characterization and quantification both in soil and organisms can help to understand routes of uptake and modes of action and also to gauge the potential for trophic transfer. Similar to fish and bivalves, key tissues that are recognized as key sites of ENM accumulation can be readily dissected including tissue associated with the posterior gut and surrounding chlorogogenous tissue of earthworms and mid-gut gland of snails.[265] Many soil-dwelling organisms, similar to bivalves, may produce inorganic biominerals in response to ENM exposure either directly for accumulated intact particles or, more often secondarily after initial dissolution. The production of the metal rich granules has been investigated for species including earthworms, soil arthropods and molluscs.[266-269] Results have shown that the specific routes of metal ion trafficking may vary between metals, with some forming inorganic mineral deposits (e.g. phosphates ligands) and others associating into metal ion clusters with sulfur rich ligands. The biogenic production of nano-structures has also been shown for Ag ENMs and Ag ions in earthworms.[20] The potential toxicological availability and potential for trophic transfer can vary between these different forms.

Soil invertebrates can be hard bodied or soft bodied, depending also on their life stage. These differences are important with respect to bioaccumulation, as the presence of a hard integument can greatly affect the balance between the two major routes of uptake across the dermal and oral pathways.[270] Soft bodied organisms may accumulate chemicals through skin (dermal uptake),[271] which is less likely for hard bodied organisms. Furthermore, hard bodied organisms that shed their integument during growth have this additional and potentially efficient route of excretion that may not be available to soft bodies species.

It has been shown that in (soft bodied) earthworms uptake of Ag ENMs is both dermal as well as through the gut, and that the distribution of the Ag within the organisms differed for Ag ENMs and Ag ions.[265] In contrast, earthworm uptake of stable isotope labelled ZnO ENMs was dominated by uptake from the gut, as earthworms precluded from feeding only accumulated approximately 5 % of the Zn assimilated by feeding individuals.[114] The two metals used differ with respect to their physiological requirement, with Zn being an important essential nutrient, and thus potentially subject to efficient gut assimilation, while Ag has no known physiological function. Hence, earthworms may be particularly efficient at assimilating Zn from their diet to meet physiological requirements, which may also contribute to the apparent differences between the two studies of these ENMs with different compositions. Another study of the uptake of different forms of Ag (ionic, pristine and sulfidized nanomaterials) has shown that uptake was primarily related to ionic Ag.[20] Uptake of non-dissolving Ag_2S-ENMs was minimal, while uptake kinetics of Ag-ions and pristine, rapidly dissolving, ENMs were more or less similar.

For hard bodied organisms, studies with isopods have indicated that uptake can occur both via food, by direct contact of the body integument with the soil, and by soil ingestion.[100] Establishing the dominance of these two exposure routes under environmentally relevant scenarios is difficult as it can be influenced by the release form and environmental fate of the tested ENMs. Some studies have shown that metals derived from ENMs can be accumulated in the hepatopancreas of isopods in the S-cells, along with S and Cu granules.[100, 272] Hence physiological mechanisms may play an important role in determining ENM partitioning and intracellular fate that ultimately govern bioaccumulation potential.

Multicellular plants

The potential bioaccumulation of ENMs in plants is of obvious concern for trophic transfer in the food chain and risks to food safety. One important consideration for plant bioaccumulation studies is the accumulation metrics (Figure 1). In the literature, BAF values for plants have been estimated by calculating the ratios of ENM concentrations in plants to ENM concentrations in the exposure media (e.g., hydroponic solution or soil).[41] For plants, it is important to provide accumulation metrics using both the ENM concentration and the total EMN mass in the tissue of concern. By plotting the data using both metrics, one can address the potential for growth dilution, as well as physiological changes as the plant moves from vegetative to reproductive growth stages. In addition, one should measure the dry mass of the plants given that some ENMs such as MWCNTs can alter water accumulation.[273] To assess ENM bioaccumulation, either root (through hydroponic or soil exposure) or foliar exposures have been studied. The following case studies address the major considerations for measuring ENM bioaccumulation in plants under each exposure scenario.

Plant case study #1: Hydroponic exposure

Hydroponic (growing plants in liquid culture media[274]) exposure is often used in nanotoxicology research, since its less complex but defined exposure medium composition facilitates ENM characterization. Hydroponic exposures ensure a relatively greater bioavailability of ENMs to plants, in comparison to exposures via the soil matrix which can sorb or otherwise change ENM bioavailability.

To conduct a hydroponic exposure, the test medium can either be reagent water[123] or a defined nutrient medium for plant growth such as Hoagland's solution of different strengths.[275] Water has been commonly used in short-term exposure (e.g. < 7 d), although nutrient media is more often used for longer experiments.[151] The medium selected should be fully characterized, as its properties (e.g. pH and ionic strength) can affect ENMs behavior and bioavailability. For example, TiO_2 ENMs may undergo significant agglomeration (measured as hydrodynamic diameter increase with time) in plant growth media.[276] This may result in ENM settling and heterogeneous ENM exposure concentration within the test medium. Although TiO_2 agglomeration has been found to decrease linearly with the dilution of the plant growth medium,[276] solutions with low ionic strength may physiologically stress the test plant species.[277] Therefore, the choice of the specific test medium may depend on the purpose of study and the requirements of the plant species. In some cases, assessing ENM bioaccumulation using a series of test media with different composition and characteristics may allow investigating the effects of environmental conditions on ENM behavior, bioavailability, and bioaccumulation.[108]

The quantification and characterization of ENMs during exposure may raise another issue: how to maintain a constant ENM exposure for plant bioaccumulation measurements. The U.S. EPA guideline OCSPP 850.4800 for testing plant uptake and translocation specifies that during exposure, the chemical concentration in the test medium should not change by over 20 % as compared to the initial (or nominal) dose.[278] This is in accordance with the OECD guidelines for aquatic toxicity testing.[279] However, this may be challenging to implement and perhaps not even environmentally relevant for ENM testing, given the dynamic transformations that may occur for many ENMs (e.g. dissolution and agglomeration) in aqueous exposure media.[279] In addition, plants continue to take up water from the medium and evapotranspire during exposure,[277] which may gradually concentrate the ENMs within the test medium. In some hydroponic studies, water or nutrient solution was added to the system to compensate for water loss due to evapotranspiration.[280] In other studies, the test medium was periodically renewed during a relatively long period of exposure (e.g., 15 d[275] and 4 weeks[281]). In any case, the specific procedure used during exposure should be appropriate for the questions being asked and should be clearly described. It is worth noting that ENM behavior and bioavailability may be significantly modified in the presence of plants, due to the influence of root exudates (including amino acids, organic acids, and sugars) and a microbial community that develops in the solution.[282, 283] Therefore, one should quantify and characterize ENMs in the medium during and after plant exposure,[123, 277] which may enable a better understanding of the actual exposure conditions and may assist in the possible interpretation of bioaccumulation results relative to ENM concentrations and speciation.

During hydroponic exposure, ENMs are in immediate contact with plant roots, and may attach extensively to the root surfaces prior to accumulation.[151] Therefore, one major consideration in assessing ENM bioaccumulation in plants is to distinguish absorbed ENMs from that adsorbed on the surfaces of root tissue. If the purpose of the study is to visualize the interactions between ENMs and root surfaces, then no washing may be needed.[284] If, however, the ENM concentration within the roots is of interest, then proper washing to remove surface associated ENMs before analysis is necessary to avoid overestimating bioaccumulation. Washing has been conducted using distilled or deionized water,[123, 275, 281, 285] phosphate buffer,[286] dilute acid (e.g. 0.01 M HNO_3),[287] and complexing agents,[288]; notably, few studies actually investigated the removal efficiency of the washing steps. For example, nearly 80 % and 10 % of ceria initially measured in unwashed cucumber roots was removed in the first and second round of washing by deionized water, respectively, with negligible removal in the subsequent three rinses.[285] Metal complexing agents (NaOAc and Na_4EDTA) have been found to be more effective than water, as they compete for metal ions. Similarly, a surfactant desorbed CuO ENMs from wheat root surfaces, with the mode of action being acceleration of CuO ENM dissolution and subsequent efficient complexation with dissolved Cu ions.[288] Even after washing, it is possible that there may be some ENMs fraction that is strongly adsorbed on the external root surface.[123, 151, 288] When measuring ENM bioaccumulation in aboveground tissues, washing may not be necessary, given that these tissues were not in direct contact with ENMs during exposure.[151]

Plant case study #2: Soil exposure

Although hydroponic studies have advantages such as simple and defined exposure media which allow for increased bioavailability, this design does lack a certain degree of environmental relevance.[151] Soil matrices can affect ENM fate and bioavailability[57] due to the interactions with complex soil components including microorganisms.[107] In addition, some plant species may develop different root morphologies (e.g. a lack of root hairs) when grown under hydroponic conditions,[289] and may have different ENM accumulation patterns in soil than for experiments using hydroponic exposures. Therefore, it is necessary to assess ENM accumulation in plants grown to maturity in soil to full characterize potential risk to food safety. Some of the considerations in hydroponic exposure are also applicable to soil; therefore, those specific to soil will be emphasized here. The choice of a particular soil type needs to be fit for the purpose of the experiment. Both the OECD Test No. 208[290] and the U.S. EPA guideline OCSPP 850.4100[291] describe that either natural or artificial soil (with a high sand content and up to 1.5% organic carbon) may be used in the terrestrial plant seedling emergence and growth tests. Additionally, the

OECD standard artificial soil (10% sphagnum peat, 20% kaolin clay, 69.5% sand, 0.5% $CaCO_3$) specified for earthworm acute toxicity testing[292] has also been used in assessing ENM uptake in soil-grown plants.[293] Since standard artificial soil is of known and less complex composition than natural soils, its use may better allow interpretation and reproducibility of the bioaccumulation tests, as well as benchmarking across different studies.[108] However, artificial soil not only lacks the physicochemical composition and complex structure of natural soil, but it is also biologically limited with regard to natural soil microbial communities that are known to interact with plants and to affect ENM behavior.[57, 107] Thus, natural soil would be a more environmentally relevant exposure matrix for assessing ENM bioaccumulation. In either case, the soil used should be sufficiently characterized for parameters including texture, pH, organic matter, major nutrients, cation exchange capacity, moisture content, and redox potential.[108, 294] This is necessary because soil characteristics affect both plant growth and ENM behavior,[295] including uptake by plants.[296] Standard natural soils such as the LUFA soils (http://www.lufa-speyer.de/) are available and have been used in ecotoxicity tests.[101, 297, 298]

In natural soils, there are a large number of plant-root symbioses, such as mycorrhizae. Rhizosphere microbial communities, including populations that form symbioses with plants, can affect local geochemical characteristics relevant to ENM dissolution or similar physicochemical processes that in turn affect exposure at the plant root and therefore plant uptake of ENMs. Notably, this applies to the leaf surface as well, where a phyllosphere community exists. Plants may respond to rhizosphere plant-microbe interactions by changing their exudate chemistry, which can in turn further alter ENM bioavailability and uptake.[299] Conditions of the rhizosphere or phyllosphere microbial communities—including changes from sampling and storing (e.g. refrigeration) of field soil, or including growing plants under variable conditions that would change phyllosphere physiochemistry—could alter ENM fate and distribution to plants, which in turn affects bioaccumulation. Given these complex interactions, investigations should ideally acknowledge such complexities in study designs by carefully designing exposures and sampling practices. It is also important to archive samples (e.g. of soil) that can be analyzed to reflect the realistic conditions of the plant and matrix (and therefore associated microbial communities) *in situ* so that changes leading up to the actual exposure can be considered when interpreting results. For example, Chen et al.[300] showed that a significant reduction of microbial biomass and a shift in microbial community composition occurred during storage of soil plus biosolids mixtures for six months at 4 ºC.

During long term soil exposure, irrigation using either water[57] or nutrient solution (e.g. Hoagland’s solution)[295] will be necessary. When quantifying uptake of metal or metal oxide ENMs, it is important to quantify the background concentration of elements of the same composition as the ENMs in both the irrigation water or other irrigating solution and soil;[301] it should be noted that there is a potential for loss of sensitive tissues during washing which may decrease the biomass. It is also useful to place a tray under the pot to collect any leachate from irrigation, so that any potential leaching of ENMs can be monitored quantitatively.[302]

The overall sample preparation procedures and analytical techniques for ENM quantification and visualization in soil-grown plants are similar to those used in hydroponic studies. One specific consideration for soil exposure is that additional care is needed to fully recover the root system from the soil with minimal root system disturbance; this can be particularly difficult with species that have fibrous root systems.[57, 281, 301] If a significant amount of belowground biomass is lost, ENM bioaccumulation (based on total mass) might be underestimated. Washing belowground harvested biomass using tap or deionized water is commonly used to remove the surface associated soil particles and ENMs.[57, 281, 301, 302] After exposure, it is important to dissect the plants to obtain the different tissue types so as to fully characterize *in planta* translocation processes (e.g., stem, leaves, pods, roots, seeds, and nodules).

Plant case study #3: Foliar exposure

While most work conducted thus far on plant-ENM interactions has focused on root exposure through soil or hydroponic media, foliar exposure is another significant pathway by which terrestrial plant species may interact with ENMs. This pathway encompasses a wide range of exposure routes, including aerial deposition of industrially derived materials such as nanoceria from vehicle combustion, airborne particles from tire or paint weathering, resuspension of contaminated soils, and direct application of nano-enabled agrichemicals such as nanopesticides to suppress pathogens and pests and nanofertilizers to enhance growth yield. In the foliar exposure literature, a limited number of studies have a toxicity focus but a larger body of work has addressed issues of intentional application, largely through nano-enabled agrichemicals. Importantly, within a given experimental design, the precise nature of the exposure (dose, concentration, application regime, etc.) will vary with the questions being investigated and the overall goal of the study.

In studies seeking to evaluate toxic response, isolating the exposure route is recommended. For example, one study compared the *in planta* accumulation and distribution of TiO_2 ENMs in rapeseed and wheat after both separate foliar and root exposures.[303] The authors noted that particles accumulated in the plants through both pathways, although toxicity was negligible by both routes. Studying both routes of uptake simultaneously is possible but would require ENM exposure in one pathway using an isotopically enriched or labeled material. Care may also be needed to prevent, or at least be aware of, stem exposure; many species have stomata on stem tissue and contamination there could confound attempts to mechanistically describe *in planta* movement of particles from exposed leaves to other tissues. Although some work has been done on ENM transformation in soils and within plants (see above), reactions on the plant leaf surface remain almost completely unexplored. In certain studies, it may be important to differentiate between surface adsorbed materials (on or within the cuticle, attached to the outer epidermis) and that fraction which has been truly absorbed into the tissue by diffusion through the cuticle and epidermis or through the stomata. In such cases, a number of techniques for the removal of the surface adsorbed particles could be used, including mild acid rinsing or washing with specific organic solvents (given the hydrophobic nature of the cuticle). Importantly, the use of any such removal technique would first require validation of the method through the appropriate quality assurance and quality control checks. This could include injecting materials into the tissue to ensure that the rinsing procedures do not impact the absorbed particles or using labeled particles on the surface only to ensure complete or near complete recovery. Separately, in an experiment involving foliar exposure of TiO_2 ENMs to lettuce in pristine form or from a weathered paint product, both particles were found in exposed plants.[304] Alternatively, lettuce exposed to foliar treatment of Ag ENMs exhibited ENM entrapment within the cuticle, followed by entry through the stomata.[305] Importantly, either *ex planta* or *in planta* oxidation resulted in significant complexation of Ag ENMs to thiol-containing biomolecules by a potentially significant series of biotransformation reactions. Additional important considerations for this type of work include possible physical or oxidative damage to leaf structures or morphology, as well as the role of the phyllosphere in potential ENM transformations and the impact of ENM exposure on the associated microbial community. It should also be noted that species-specific properties such as cuticle thickness and stomatal distribution on shoot tissues will significantly impact the uptake and accumulation of ENMs. In studies where determining the mechanism of uptake is of interest, being able to determine the distribution of ENM across the leaf surface could be important. EM with EDS can be used for this purpose, although labelled or fluorescently-tagged ENMs facilitate use of other analytical and visualization methods. Laser ablation ICP-MS may also be a useful technique in these studies.

For foliar exposure studies designed to exploit nanoscale size properties, environmental conditions such as moisture status, water potential, or UV light impacts may be important as they will influence leaf physiology. Importantly, these factors are dynamic during growth and exposure. For example, in an early study, leaf stomata were shown to readily permit entry of materials as large as 50 nm, although not all stomata were functionally equivalent, with only some structures allowing particle entry.[306] The authors speculated that the wettability of the guard cell cuticle was the key factor controlling activity. Alternatively, ENM exposure may alter stomatal function. Foliar Fe_2O_3 ENM application increased stomatal opening, with subsequent increases in soybean photosynthesis and growth.[307] Both particle size and particle number were key factors impacting uptake and translocation of ENMs upon delivery to watermelon leaves with an optimized aerosol platform.[308] Again, understanding species-specific properties of the plant such as stomatal distribution on the leaves, stems, and other tissues plus cuticle thickness, will be important.

One other area of interest is the use of foliar applications of nano-enabled agrichemicals in response to infection or disease. It is also important to note that the majority of commercial agrichemicals intended for foliar application have additional materials in the formulation, including surfactants or "stickers" to promote retention on the leaf surface.[309] The activity of these potentially complex formulation materials will also influence the nature of the exposure under realistic conditions, and their activity must be taken into consideration. A final consideration is the role of pathogens in affecting uptake as these may affect leaf or stem tissue leading to necrotic damage. These changes can result in the loss of the cuticle barrier, and ENM entry through those tissues may change the amount of ENM bioaccumulation in comparison to plants not impacted by pathogens.

Trophic transfer

Laboratory trophic transfer studies

Many of the considerations in trophic transfer studies are similar to those which have been described in feeding studies, yet there are also a number of specific considerations. Trophic transfer studies involve exposing one population of organisms to an ENM and then feeding the prey with bioaccumulated ENMs to a predator type of organism, for example in a simulated laboratory food chain. Because synchronization of the exposures of the populations of two or more species is challenging, researchers may be tempted to simply "spike" the organisms from the lower tropic level with ENMs. An example of this could be spraying an ENM onto a leaf and then feeding it to an insect, or growing algae and then simply spiking a suspension of the algae with an ENM. Two studies have demonstrated that this approach can underestimate the bioavailable fraction of ENMs for the predator species. For example, the assimilation of Au ENMs by tobacco horn worms from tobacco plants which had taken up the ENMs hydroponically was significantly higher than assimilation from leaves onto which Au ENMs had been sprayed.[18] Similarly, bullfrogs accumulate Au ENMs more efficiently from consuming earthworms raised in Au ENM contaminated soil than when they were exposed to pristine Au ENMs via oral gavage.[40] There are many possible explanations for this behavior including biological modifications of the particles, such as acquisition of a protein corona, that favor their cellular uptake. In a third study with SWCNTs, ambiguous results were reported when algae were amended with a SWCNT suspension and then fed to bivalves which were then fed to polychaetes.[310] No evidence of trophic transfer was detected. As noted in the previous studies with Au ENMs, there are several possible explanations for these results such as analytical interferences and poor uptake of SWCNTs by the algae.[310]

Numerous challenges exist in preparing ENMs for inclusion in trophic transfer studies via food consumption. Researchers must balance loading prey items with ENM concentrations high enough to observe an effect at the next level and keeping ENM concentrations low enough to avoid unwanted toxicity to the prey organisms and to stay environmentally relevant. Exposure time of prey to the ENMs must also be balanced to maximize the uptake concentration before elimination occurs and decreases the concentration. It should be noted that, in the case of food web accumulation, ENMs that are attached to organisms or in their gut but not fully assimilated in the tissues are still of importance. Hence, decision about the preparation of plant and animal food items for the consumers species should be sensitive to such considerations depending on the aims of the study.

Algae or bacteria are often starting points in trophic transfer studies as they are relatively easily cultured and are common food items for many invertebrates. Sorption to or uptake by unicellular organisms is affected by surface charge of both the ENM and the organism, as well as by the presence or absence of cell walls and membranes which may serve as a barrier to ENMs.[311] Coatings on ENMs such as citrate or other organic compounds increase the stability of the ENMs in aquatic environments and play a critical role in the interaction of ENMs with an algal or bacterial cell.[191] Sorption to the outside of single-celled organisms is another mechanism to move ENMs through the food chain; however, care should be taken through multiple washing steps and analysis of the prey media to ensure that the ENM is thoroughly bound to the prey organism and not easily dislodged to prevent exposure to the next trophic level through direct contact with ENMs rather than by food uptake. Collection of ENM-exposed prey can be performed using procedures that include various methods of filtration, centrifugation and rinsing steps. Density gradient separation is described in detail in the single cell species section and is a robust method for separating single-celled organisms from suspended ENMs.

For uptake at the next trophic level(s), the same concerns exist with respect to determining the length of exposure to reach maximal uptake with a minimum of elimination and toxicity to the prey organism. Using an elimination period for prey organisms is not generally recommended, because many consumers will usually eat prey whole and as such exposure will be both to prey tissue and also via the gut load. However, consumption of the gut content does not occur for some organisms such as the European mole (*Talpa europaea*), which will often squeeze the gut contents from earthworm prey before consuming them.[312] The timing of introducing ENMs to prey and subsequent transfer of the ENM through a food web must also be considered. Researchers have generally exposed protozoans and crustaceans used as secondary trophic level prey for periods of 1 d to 7 d. While most researchers rinsed the prey, the decision could be based upon the objective of the exposure. It can be argued that rinsing the organisms may represent the ENM that is truly incorporated within the prey while, conversely, not rinsing the organisms may be more representative of the body burden that the organisms may experience in the field. Generally, some rinsing is necessary to ensure that ENMs are transferred via the food and not via exposure media. Additionally, when composite ENMs, such as QDs, are being transferred, it is important to assess if the composite ENM has decomposed inside the prey organism or between transfers.

Mesocosm and Field Studies

Inherently, quantifying bioaccumulation is a step towards understanding the potential for ENM trophic transfer and biomagnification, both of which are important concerns in ecotoxicology. Although many controlled, multiple-population based, trophic transfer studies regarding ENM biomagnification have been performed for food chains of microbial[23, 82, 132] and higher[17, 40, 313] organisms, the assessment of ENM distribution in complex food webs consisting of many biotic trophic levels with multidirectional nutrient flows is more rare. In some studies, ENMs are isotopically labeled to allow for specific quantification of low ENM bioaccumulation abundances, as would occur with initially low exposure concentrations,[82, 314] although the use of stable isotopes does not necessarily indicate that the bioaccumulated material is still nano-sized. However, use of isotopically-labeled ENMs in large scale mesocosm studies is unrealistic as the synthesis of labeled ENMs is specialized and typically expensive, and radioactive isotope use is more safely conducted at small scales under highly controlled conditions.

Determination of trophic status in mesocosm or field studies can be challenging, a challenge not restricted to studies on ENMs.[270] Furthermore, many organisms feed from multiple food chains and trophic levels during their lifespans or even simultaneously in the case of omnivory. Stable isotope (e.g. ^{13}C and ^{15}N) and ENM bioaccumulation measurements of organisms at various trophic levels in a food web may be used to infer predator-prey interactions that may influence final ENM distributions, such as has been utilized in a study of TiO_2 in a paddy mesocosm.[315] However, stable isotope methods need to be used with caution as they can only be used to determine trophic structure of relatively simple food webs. For example, only two sources of coupled nitrogen and carbon administered into a food chain can be traced with conventional ^{15}N and ^{13}C studies.[316] If more sources exist at the base of food chain or if nitrogen and carbon cycling are decoupled, then erroneous determinations of trophic status result.[317] In such cases, traditional methods, such as the examination of stomach contents, may provide more reliable information.

Study designs would ideally be well-informed by an existing understanding of the system ecology. For example, CeO_2 ENMs were traced through an aquatic food web by using temporally and spatially dense sampling, since ENMs quickly compartmentalized by settling into sediments, then redistributed within food webs starting from the benthos.[318] In this case, understanding the dynamics of physicochemical processes affecting ENM compartmentalization, relative to feeding and organismal reproductive rates, allowed for judiciously designing a biotic sampling program that revealed ENM distribution across multiple trophic levels.[318]

Future work and next steps

The recommendations discussed here are intended to inform the design (Figure 1) and interpretation of studies examining ENM bioaccumulation. While the best practices for conducting nanomaterial bioaccumulation assays have been described for a broad range of ecological receptors, additional research described throughout this manuscript can further refine these methods. One key factor is the further development of analytical methods to quantify ENMs in the test species. Different methods can be refined to quantify ENMs in individual single-celled organisms, populations of these organisms, or multicellular species. These include a range of different analytical and microscopy methods that can be used for assessment ranging from determination of overall concentrations to assessments of localization and chemical form.[81] This is especially important for ENMs that may be transformed in which case it is valuable to quantify the different forms. One promising approach that is increasingly being utilized for the detection and quantification of ENMs in biological samples is spICP-MS. The value of this method is that it can distinguish between dissolved ions and ENMs and for directly measuring particle number concentrations. In addition to continued refinement of this technique to improve its robustness, research is needed to develop effective extraction techniques, which minimally change the ENMs for different types of organisms. One challenge with these measurements though is that there typically are not readily available orthogonal techniques to evaluate the size distribution of ENMs in the organisms for comparison.

Separation of ENMs from suspended particles is another critical consideration for research on ENM bioaccumulation by single-celled organisms, small multicellular organisms, and in subcellular fractionation studies using cells or tissue samples from larger species. The need for more effective and complex separation procedures such as density gradient centrifugation is among the main differences in the analytical methods for bioaccumulation of ENMs by these species as compared to studies with dissolved chemicals. Additional research is needed to evaluate the conditions under which sequential differential centrifugation is sufficient for separating ENMs from the test species or different cellular fractions and when density gradient centrifugation is needed. In addition, the application of density gradient centrifugation to separate freely dispersed ENMs from ENMs associated with different cellular fractions as compared to sequential differential centrifugation procedures need thorough evaluation. This will require the development and testing of density gradient centrifugation procedures to separate organelles for different types of tissues or cells and determining how interactions with ENMs affect the buoyant density of organelles and cells. This can result in a set of clear recommendations on the application of this approach in ENM bioaccumulation studies.

One of the challenges with providing guidance on bioaccumulation studies with ENMs is that the recommended protocol depends to a large degree on the purpose of the measurements. In some instances, a fit for purpose method would include voiding of the gut tract while for other situations, it would be helpful to measure the body burden without voiding the gut tract. Even when the aim is to assess the exposure of consumer in trophic transfer studies it may be necessary to treat samples in a different way depending on, for example, whether the predator consumes or avoids eating the prey gut content. Quantifying the kinetics of the uptake and elimination processes can provide key insights into the bioaccumulation processes and is recommended as opposed to measuring a bioaccumulation-related factor (e.g., BAF) at a single time point. For comparison to results with dissolved species, voiding the gut tract of multicellular organisms is an appropriate step. Results from plant ENM bioaccumulation studies should be reported both in terms of ENM concentration and the total mass of ENM in the plant tissue. When testing ENM bioaccumulation in soils and sediments, it is important to assess how bioaccumulation factors and bioaccumulation kinetics relate to the soil or sediment porewater concentrations as compared to the total soil or sediment concentration, because the porewater concentrations may be more bioavailable.

The robustness of ENM bioaccumulation methods in general can be improved. Given that the methods among studies vary regarding how to conduct these experiments, it would be helpful to know the sensitivity of bioaccumulation methods to changes in the protocol. For example, it has been shown that organism size can impact ENM bioaccumulation studies with bivalves, and it has been proposed that the daphnid size can impact bioaccumulation measurements in the absence of gut voiding. However, to date there have not been systematic studies to specifically evaluate how the age of the daphnid used in bioaccumulation studies impacts on the results. Hence, it remains unclear whether the use of standard age and size organisms is needed and the extent to which studies conducted with different age cohorts can be directly compared. In plant bioaccumulation studies, a step of the assay protocol that often varies is the washing procedure used to separate weakly-attached ENMs from the roots. However, the impact of these different washes procedures on ENM bioaccumulation results and their comparability across studies is unclear. It is likely that no one method can be the requirement to fully remove all loosely attached ENMs, while fully retaining root fine tissue structure integrity. The reproducibility of results (e.g., to what degree would a similar result be obtained if the experiment was repeated) is unclear and often not reported. If a bioaccumulation experiment is repeated within a single laboratory, it would be helpful if these results were reported, such as in the Supporting Information which typically do not have length limits. Another important topic within each study is to ensure that there is an adequate number of replicates to make robust statistical comparisons among conditions tested. It is also important that sufficient detail is provided about if each replicate within a measurement is from a single organism or the average of multiple organisms.

The practices and discussion described here will enable researchers to make more accurate ENM bioaccumulation measurements using a broad range of species. This will help advance the field of environmental nanotoxicology through supporting regulatory decision making and elucidating interactions of ENMs with organisms. Careful attention to the key topics discussed throughout this paper will facilitate researchers making results that are comparable across studies and reproducible, a key issue in science in general[319, 320] and also especially in nanotoxicology.[321-323] Overall, these measurements will support the sustainable commercialization of nanotechnology.

Author contributions

All coauthors contributed to discussions, writing and revisions of this manuscript.

Conflict of interest

There are no conflicts to declare.

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NIST disclaimer

Certain commercial products or equipment are described in this paper in order to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that it is necessarily the best available for the purpose.

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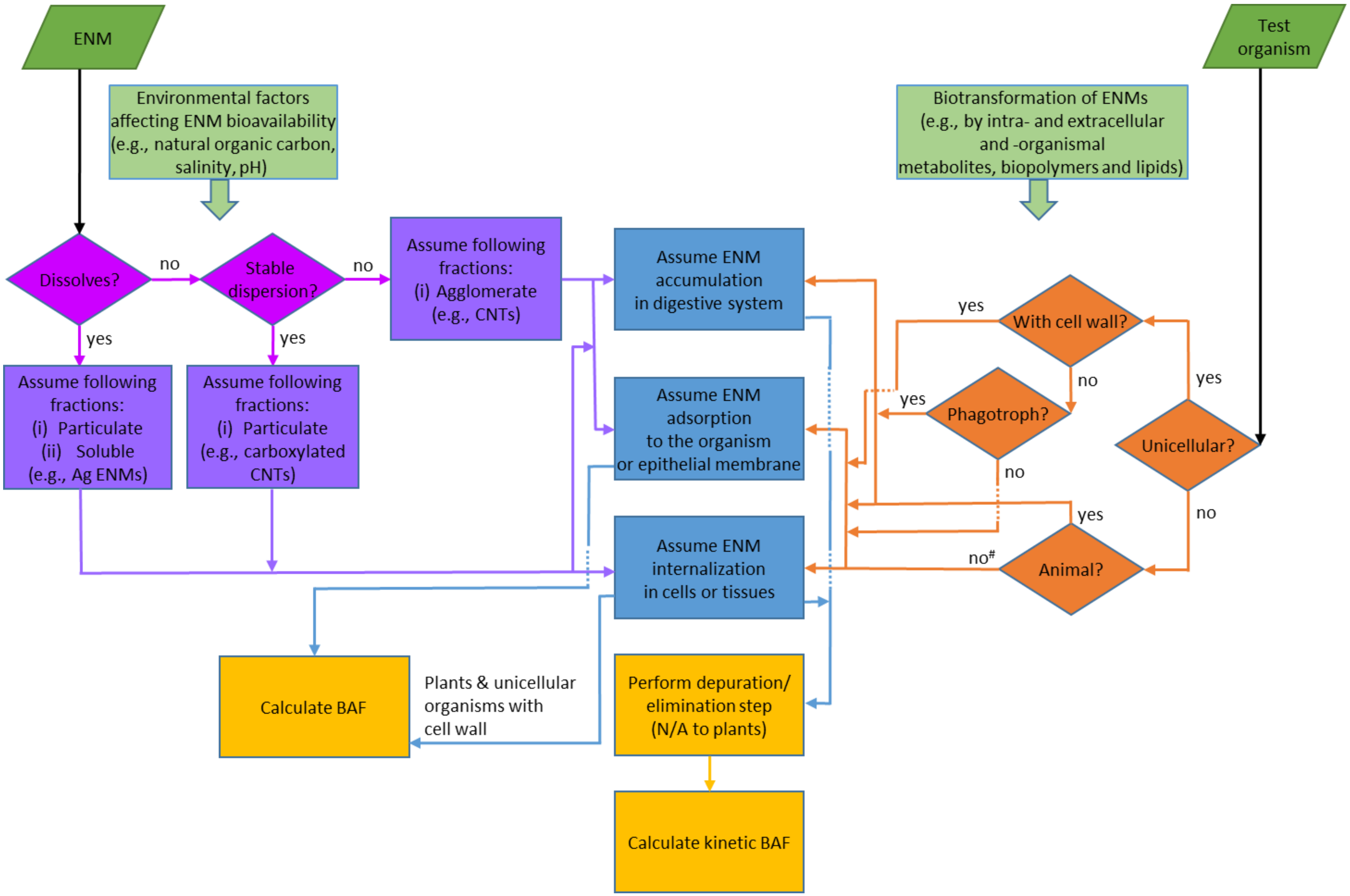

Figure 1. Scheme of decision steps, processes and factors important to consider in designing engineered nanomaterial (ENM) bioaccumulation tests and calculating bioaccumulation factors. The scheme depicts how the physicochemical properties of ENMs (purple boxes and violet

diamonds) and the physiology of the test organism (orange diamonds) influence ENM internalization or adsorption to organisms or cell membranes (blue boxes) and the consequent steps for calculation of single metrics of ENM bioaccumulation (yellow boxes).

ENM interactions with cells and organisms (blue boxes) have been grouped based on the potential of ENMs to adsorb or become internalized into cells or tissues. Accumulation into the digestive system has been presented as a special case because ingestion is a significant uptake pathway of ENMs for certain types of organisms (e.g., filter feeders, phagotrophs, and fish). Whether or not ENMs are assimilated into the tissues or cells, or merely adsorbed on the epithelial membrane of the digestive system depends on the ENM physico-chemical properties and biotransformations in the digestive system. Regardless of their fate in the digestive system, ingested ENMs contribute to the total body burden of ENMs that can be transferred to subsequent trophic levels, and should be taken into account in bioaccumulation measurements. Based on the potential of ENMs to either dissolve or form stable aqueous dispersions (purple diamonds), ENMs can be divided into (1) water-soluble ENMs, such as ZnO, Cu, CuO, and Ag, with particulate and dissolved fractions interacting with organisms, (2) insoluble ENMs, such as carbon nanotubes (CNTs), graphene, boron nitride nanotubes or flakes, and TiO_2, which are not water-dispersible and tend to agglomerate in environmental matrices and thus are less likely to be internalized into cells and tissues but may be adsorbed to organisms or cell membranes, and (3) insoluble ENMs that form stable aqueous dispersions, such as functionalized carbon or boron nitride nanotubes, graphene oxide, and TiO_2 with hydrophilic coatings, and may interact in nanoparticulate forms (violet boxes) with organisms. In addition to intrinsic ENM properties, environmental factors affecting ENM bioavailability and ENM biotransformations need to be considered in the test design (light green boxes). Conversely, the ENM interaction with organisms depends on the structure and physiology of the latter (orange diamonds). For example, ENMs can accumulate in multicellular animals by entering the digestive system, adsorption to the organism, and internalization in the tissues (blue boxes). The pathway of ENM accumulation in the digestive system is excluded for multicellular plants (non-unicellular organisms which are not animals), unicellular organisms with cell walls (bacteria, fungi and green algae) and non-phagotrophic unicellular organisms without cell walls (some protists and mixotrophic algae). If no internalization of ENMs in organisms is assumed (e.g., in the case of insoluble poorly dispersed ENMs interacting with bacteria) or in case of plants and unicellular organisms with cell wall, an elimination step may not be necessary before quantifying bioaccumulated ENMs (yellow boxes). In this case, a bioaccumulation factor (BAF) can be calculated. If accumulation in the digestive system or internalization of ENMs is assumed, it is advisable to perform an elimination step for calculating a kinetic BAF.

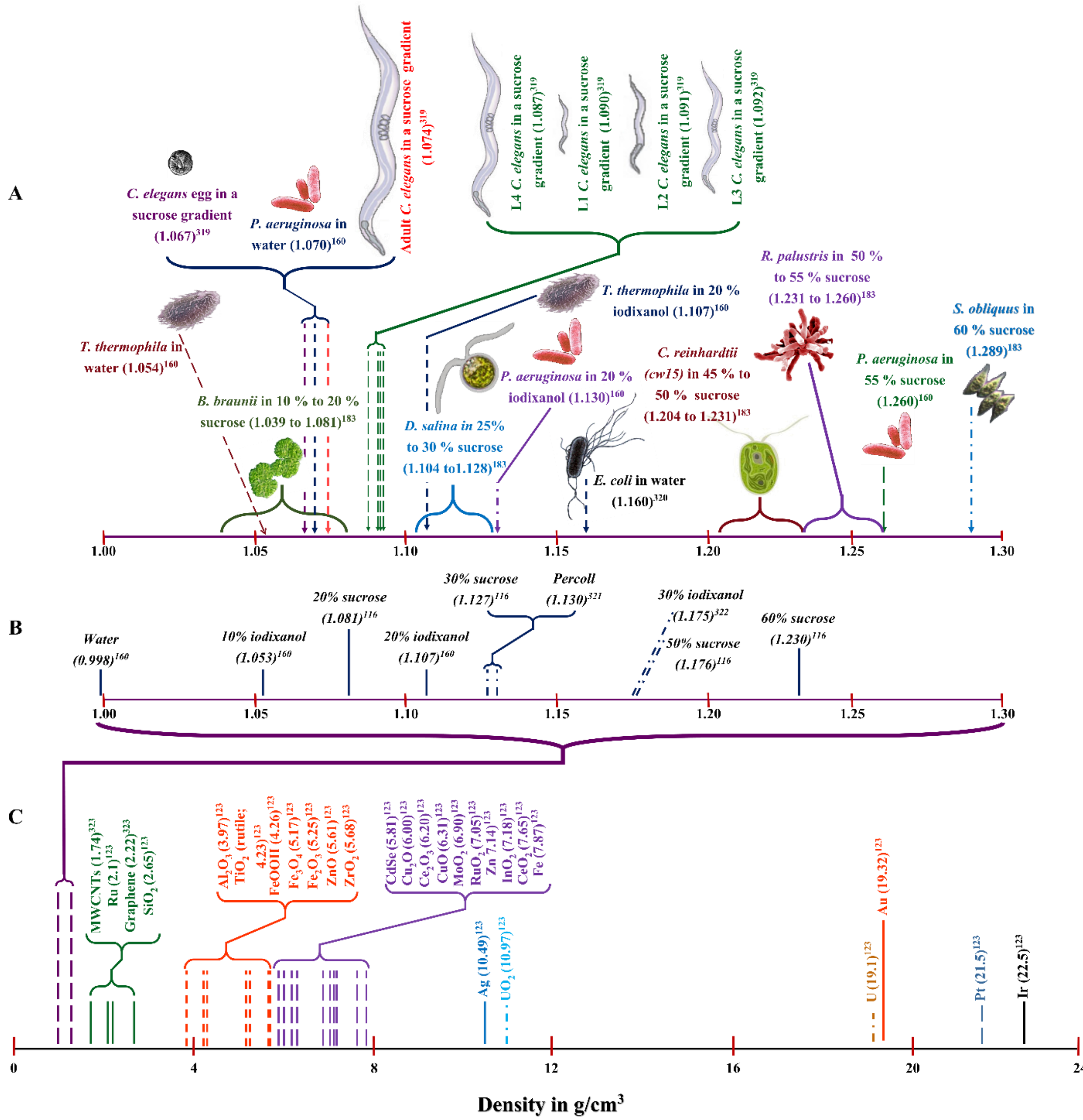

Figure 2: Comparison of densities among (A) biological organisms in density media, (B) media used for density gradient centrifugation separations, and (C) ENMs (bulk). Densities for gradient density media are represented in percentages of weight by volume (w/v; 10 % iodixanol, 20 % iodixanol, 30 % iodixanol, Percoll (23 % coated silica spheres in water), 20 % sucrose, 30 % sucrose, 50 % sucrose, and 60 %sucrose). *T. thermophila: Tetrahymena thermophila; B. braunii: Botryococcus braunii var. Showa; C. elegans: Caenorhabditis elegans; P. aeruginosa: Pseudomonas aeruginosa; D. salina: Dunaliella salina; E. coli: Escherichia coli; C. reinhardtii (cw15): Chlamydomonas reinhardtii (cw15); R. palustris: Rhodobacter palustris (CGA009); S. obliquus: Scenedemus obliquus* [128, 324-328]

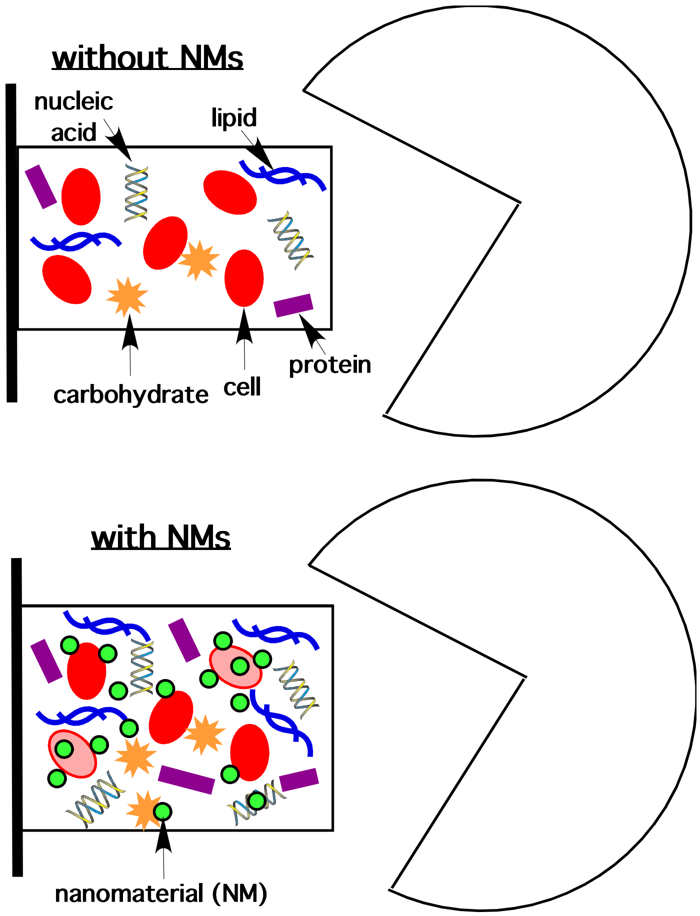

Figure 3: Conceptual representation of microbial biofilms (left) subject to predation by grazing (right) without (top) or with (bottom) ENMs accumulated in the biofilms. Note that the extracellular polymeric substances (EPSs) are depicted as macromolecules (lipids, nucleic acids, carbohydrates, and proteins) that are hydrated, surrounding biofilm cells. In the presence of ENMs that impose cellular stress, EPS accumulations may increase (bottom) which could increase the overall abundance of retained ENMs in the vicinity of prey (biofilm cells) and predator (grazer or similar).

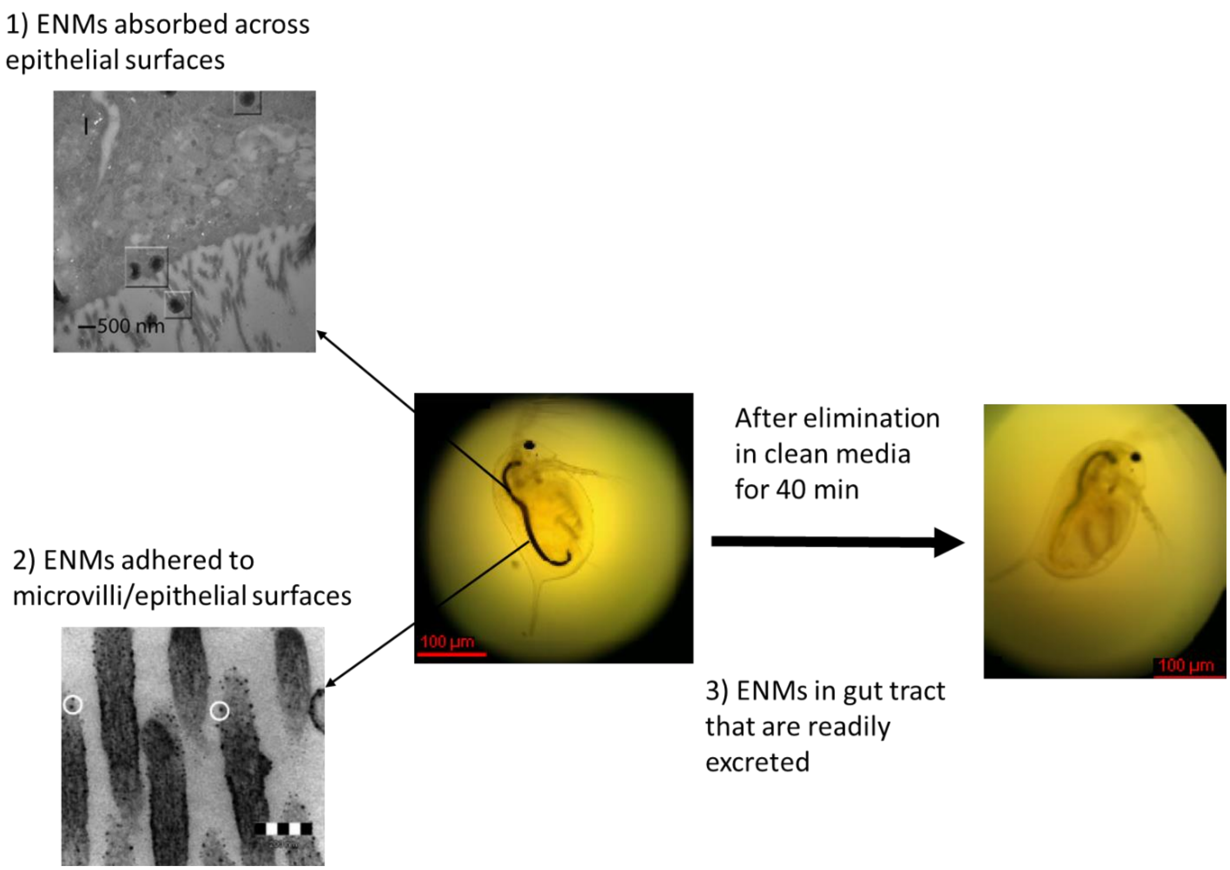

Figure 4: Fractions of engineered nanomaterials (ENMs) that can be detected in organisms with a digestive tract: 1) ENMs absorbed across epithelial surfaces; this figure (upper left) shows carbon nanotubes (CNTs) that had been absorbed by microvilli (see squares) although additional analysis using high resolution transmission electron microscopy (HRTEM) revealed that these particles were amorphous carbon and not CNTs.[11] 2) ENMs adhered to microvilli; this figure (bottom left) shows apparent fullerene particles adhered to the microvilli.[12] 3) ENMs in gut tract that are readily excreted; this figure (far right) shows that the gut tract of the *Daphnia magna* turned from black (as a result of uptake of few layer graphene for 24 h) to transparent or green after an elimination period of 40 min with algae feeding;[256] adapted with permission from [256] 2013 American Chemical Society.

Box 1. Definitions of key terms used in the current review. [30, 35, 329] (The term “ENM” includes ENMs and its transformation products.)

Assimilation efficiency – a measure of the proportion of ingested ENMs assimilated into (initially) the alimentary epithelium of the feeding animal; the amount absorbed per amount ingested from the diet.

Bioaccumulation – the process and phenomenon of ENM accumulation in or on an organism, regardless of exposure regime (i.e. whether ingesting or otherwise taking up ENMs via water, food, sediment, soil, or air).

Bioaccumulation factor (BAF) – (1) the ratio of the ENM concentration associated with the organism exposed through all possible routes (C_B, g ENM/kg dry mass) and the concentration in the exposure medium (air, water, soil or sediment) or food (C_S, g ENM/kg wet mass or volume), or (2) the ratio between the uptake rate coefficient (k_1) and elimination rate coefficient (k_2), termed “kinetic BAF” or BAF_k. Note that steady state is not assumed here, unlike in conventional BAF definitions, because steady state is likely not reached in ENM exposures, particularly in field studies.

Bioavailability – the ability of ENMs to interact with organism biosystems.

Bioconcentration – the process and phenomenon of ENM accumulation in an organism from the ambient environment via uptake through all routes excluding diet.[330]

Bioconcentration factor (BCF) – for aqueous ENM exposures in the absence of food, (1) the ratio of the ENM concentration associated with the exposed organism (C_B, g ENM/kg dry mass) and the concentration in water or (2) the ratio between the uptake rate coefficient (k_1) and elimination rate coefficient (k_2), termed “kinetic BCF” or BCF_k.

Biomagnification – the increase in whole-body ENM concentration from one trophic level to the next resulting from ENM accumulation in food.

Biomagnification factor (BMF) – the ratio of ENM concentration in an organism (trophic level n, C_B, g ENM/kg dry mass) to that of the diet (trophic level n-1, C_D, g ENM/kg dry mass), using organisms of known or assumed trophic status.

Biodistribution – ENM distribution within an organism.[331, 332]

Body burden – the ENM concentration in, or on, an organism at a given time.

Elimination rate coefficient (k_2) – the numerical value defining the rate of decrease in the ENM concentration in the test organism, or specified tissues thereof, following the test organism transfer from a medium containing the ENM to an ENM-free medium.

Elimination – the combined process of metabolism, excretion, and degradation which results in ENM removal from an organism.

Growth dilution – the decrease in ENM concentration in a growing organism because the amount of tissue in which the ENM is distributed is increasing at a faster rate than the increase in ENM amount in the organism.

Gut voidance – ENM loss from the gut lumen when an organism is removed from ENM-contaminated media and placed into clean media free of ENMs or is fed an ENM-free diet.

Toxicokinetics – the study of organismal rates of ENM uptake, transfer between biological compartments, biotransformation and elimination.

Trophic level – a conceptual level in a food web such as primary producer, primary consumer or secondary consumer, recognizing that omnivorous organisms do not have discrete trophic levels.

Uptake – that part of the bioaccumulation or bioconcentration process(es) involving ENM movement from the external environment into an organism, either through direct exposure to an ENM-contaminated medium or by consumption of food (including prey) containing the ENM. This can be defined as an uptake rate (e.g., mass of ENM per day), an uptake rate coefficient or, particularly for plants, as the total uptake over the course of an exposure.

Uptake rate coefficient (k_1) – the numerical value defining the rate of increase in ENM concentration in or on the organisms, or specified tissues thereof, when the organisms are exposed to ENMs.

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