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Nanomaterials in the environment

Nanomaterials in the Environment: Behavior, Fate, Bioavailability, and Effects—An Updated Review

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Abstract: This review covers developments in studies of nanomaterials (NMs) in the environment, since the much-cited review of Klaine et al. (2008). It discusses novel insights on fate and behavior, metrology, transformations, bioavailability, toxicity mechanisms and environmental impacts, with a focus on terrestrial and aquatic systems. Overall the findings were that: i) despite the substantial developments, there remain critical gaps, in large part due to the lack of analytical, modelling and field capabilities and in part due to the breadth and complexity of the area; ii) a key knowledge gap is the lack of data on environmental concentrations and dosimetry generally; iii) there is substantial evidence that there are nano-specific effects (different from both ions and larger particles) in the environment in terms of fate, bioavailability and toxicity, but this is not consistent for all NMs, species and all relevant processes; iv) a paradigm is emerging that NMs are less toxic than equivalent dissolved materials but more toxic than the corresponding bulk materials; v) translation of incompletely understood science into regulation and policy continues to be challenging. There is a developing consensus that NMs may pose a relatively low environmental risk, however, with the uncertainty and lack of data in many areas, definitive conclusions cannot be drawn. In addition, this emerging consensus will likely change rapidly with qualitative changes in the technology and increased future discharges.

Keywords: Nanomaterials, Nanoecotoxicity, Hazard/risk assessment, Nanometrology, Aquatic and soil organisms

INTRODUCTION

In 2008, we produced one of the first comprehensive reviews of nanomaterials (NMs) in the environment (Klaine et al. 2008), that has since been very highly cited. The pace of NM research, development and application that was evident at that time has continued and increased. Rapid increases in citations and patents granted are evident. The widely cited Nanotechnology Consumer Products Inventory showed that nano-enabled products increased from 50 to nearly 2000 (Vance et al. 2015) and continue to grow. Despite the limitations of the database, it provides evidence of substantial increases in nanotechnology use. In addition, data suggest that there is a large and growing body of nano-environmental and nano-ecotoxicological research (Selck et al. 2016). This research is driven by questions of environmental risk because of the predicted rapid increases in environmental concentrations (Gottschalk et al. 2013), the known bioavailability (Luoma et al. 2014) and deleterious biological effects (Fabrega et al. 2013), the suspected novel behavior of some NMs in environmental and biological systems (Luoma et al. 2014; Taylor et al. 2016) and the consequent complexities of risk assessment (Laux et al. 2018). Additionally, modelling projections using a ‘business as usual’ scenario, suggest that production will more than triple by 2020, with much of the output eventually being discharged to the environment (Sun et al. 2017). It is therefore timely to review progress over the previous decade. In particular, we will consider the advances that have been achieved, where the most critical gaps and opportunities are, and where improvements in our knowledge base are needed.

With the increasing use of NMs from early 2000, the question of whether they pose a risk to the environment has loomed large. In 2008, there was a lack of basic data on fate and behavior, ecotoxicity and related issues (Klaine et al. 2008). The field has provided a great deal of high quality advances in all of these areas, but there remain substantial uncertainties and data gaps. Regulatory agencies needed (and still need) to decide whether NMs as a class must be managed differently to larger micron-sized particles or the dissolved phase (Pettit and Lead 2013; Hunde-Rinke et al. 2016). This relevant question can be posed as follows: does the nanoscale imply a greater risk to aquatic and terrestrial ecosystem health through novel behavior related to extra reactivity, increased transport and interaction with sub-cellular components, leading to more serious adverse interactions with organisms? The novel properties necessary for technology and innovation, such as the spatial constraint of electronic properties and high specific surface area might, but do not necessarily, imply novel environmental and biological behavior. For example, we now know that the critical effector of the antibacterial activity of Ag NMs is the released silver ions rather than the nanoparticles themselves (Xiu at al. 2011), at least for single species cultures. For biofilms and eukaryotes, the situation is not as clear cut and there is likely to be a role for the NMs. Accordingly, any effect of Ag NM size, coating or shape on single species bacteria is indirect, since these factors mainly influence transport, bioavailability and the rate and extent of Ag^+ release (Xiu et al. 2011) (for details, please refer to the discussion in the Supplemental Information). However, many studies show significant size-dependent effects, which are not readily explained by metal ion dissolution, the speciation chemistry of the solutes released, or their chemical reactivity. Nonetheless, the NMs show a bioavailable fraction and biological effects (van Hoecke et al. 2009; Croteau et al. 2011a, b; Yin et al. 2011; Pan et al. 2012; Al-Jubory et al. 2013; Pang et al. 2013; Cozzari et al. 2015; Taylor et al. 2016). For instance, Taylor et al (2016), showed that tightly constrained (in size and oxidation state) and well-characterized ceria NMs gave different algal transcriptomic and metabolomic responses compared to larger scale particles and dissolved phase Ce, although in all cases toxicity was low.

Confounding factors to such studies lie in: i) ensuring that NMs and larger particles are only different in one variable, which is difficult when physical and chemical properties are size dependent as is the case for ceria and oxidation state; ii) rapid transformations of NMs in complex media such as environmental and exposure media; and iii) precipitation of the dissolved controls after exposure. In addition, the limited knowledge of hazard and exposure (Klaine et al. 2012) create large uncertainties in understanding risk. Details of potential nano effects are given in later sections.

Issues such as nanometrology, dosimetry and transformations, especially in complex media, which were just coming to the attention of the scientific community, have been brought more sharply into focus (Baalousha et al. 2012; Lowry et al., 2012; Selck et al. 2016). Transformations may reduce toxicity (Levard et al., 2012) although this is not always the case (Wang, Ebbs et al. 2013). Nevertheless, questions remain about timescales and reversibility of transformations, and effects on persistence and bioaccumulation. In addition, the many new variants of NMs entering the market (with their system-specific transformation products often unknown) hinder the accurate modelling and prediction of NM fate and impacts.

This review aims to examine the new knowledge gained since Klaine et al. (2008) on NMs in aquatic and terrestrial systems especially, and to discuss new questions arising from research conducted since 2008, in terms of the environmental fate and effects of NMs.

SELECTED RECENT ADVANCES IN NANOMATERIAL COMPOSITION AND METROLOGY

Surface modification of nanomaterials

The study of coatings to modify surface properties and alter aggregation behavior in the environment has advanced significantly since the last review in 2008 (e.g., Tolymat et al. 2010). There have been a number of papers investigating the effects of citrate, polyvinylpyrrolidine (PVP), polyethylene glycol (PEG) and other coatings for stabilizing NMs (Angel et al. 2013). Some, but not all, of these coatings are known to have associated biological activity. Natural organic matter (NOM; particularly humic substances, HS) has been shown to perform a similar function with a range of NMs affecting both fate and biological effects (Yin et al. 2015), although often with concomitant effects on the core material (Merrifield et al. 2017a). The use of coatings on carbon-based materials is rarer, with changing properties commonly achieved via surface functionalization (Balasubramanian and Burghard 2005). Questions related to the changing nature of surface chemistry e.g., the formation of eco-coronas and protein coronas are being addressed (Manciulea et al. 2009; Mudunkotuwa and Grassian 2015), but require further work. There are limited or no data on the kinetics and extent of eco-corona and protein corona exchange with synthetic coatings, for instance.

New nanomaterials

While silver, zinc oxide, copper oxide, cerium dioxide, titanium dioxide, iron oxide, fullerenes, carbon nanotubes and a small number of others remain the most widely used and researched NMs, a number of newer NMs have been produced in recent years. The greatest interest and development has been in broad classes of materials including nanocomposites and nanohybrids, sometimes referred to as either multiple component nanohybrids (Wang et al. 2015), core-shell nanocrystals (Chaudhuri and Paria 2012) or oligomer NMs (Miao et al. 2016). The terminology is confusing but one distinction is that nanocomposites are NMs enclosed or encapsulated with other materials, which may not have nanoscale features, while nanohybrids are the linking of two or more discrete NMs to give different functionality (Saleh et al. 2015). Such NMs can be based on semiconductor substrates such as GaAs, CdSe, CdS, SiGe, etc., modified with shells and coatings, along with mixtures of carbon-carbon and carbon-metal or metal oxides (Wu et al. 2015). Quantum dots are one such hybrid and have been in use for several decades. Their applications are widespread including medical diagnostics, solar cells, nanoelectronics, detectors and photocatalysts. For many composites and hybrids, however, their current use is relatively small in many sectors and their current likely impact on the environment is low, except in the hypothetical case of a major spill. Nevertheless, use is likely to rapidly increase and they are therefore likely to present a significant concern in the near future. Future research will need to focus on release rates, persistence, bioavailability and toxicity of these nanohybrids.

Klaine et al (2008) called for the development of new standards of various sorts. At around the same time, there was much discussion of test materials, reference materials and certified reference materials (CRMs) between producers of such materials and ‘consumers’ (toxicologists, ecotoxicologists and others). Relatively simple test and reference materials of good quality are now available from bodies such as NIST, JRC and others, including commercial sources, in North America, Europe and elsewhere. Clearly, study aims drive the choice of such materials while researchers must choose the correct parameters by which the materials are referenced (size, concentration etc.).

Nanometrology

Characterization and metrology has been recently reviewed (Baalousha and Lead, 2015) in detail and detailed discussions about technical advances can be found there. Improved metrology is required to refine understanding of environmental concentrations and the forms of NMs, as well as to enable more accurate dosimetry in ecotoxicology testing, therefore defining the dose-repsonse relationship more exactly. Current methods are limited for various reasons, including inadequate sensitivity and selectivity in relation to the complexity of both NMs and environmental conditions, lack of resolution (for imaging techniques), an inability to provide full quantification and a lack of broad applicability. For instance, uv-visible spectroscopy is highly selective for metals such as Ag, Au and Cu in relatively simple systems (Zook et al. 2011), but produces lower quality information in more complex systems and is not very sensitive. Sensitivity can be paritally alleviated by using longer path lengths for transmission through the sample. Modern transmission electron microscopy (TEM) systems have sub-nm resolution and can perform single particle analysis for a wide variety of properties (size, composition, shape, chemical speciation). Although not widely available, in-liquid imaging can be performed (Zeng et al. 2017), while sample handling methods can overcome drying and beam damage issues (Prasad et al. 2015) and atomic force microscopy can be used to analyse liquid samples (Lead et al. 2005). Dynamic light scattering (DLS) is frequently used for size measurement and again it works very well for simple samples which are monodispersed, but size data are often inaccurate in aggregating/aggregated systems for a variety of reasons (Liu et al. 2012).

The development of new methods in the intervening years such as nanoparticle tracking analysis (NTA), hyphenated methods such as field flow fractionation with inductively coupled plasma mass spectrometry (FFF-ICP-MS) (Meisterjahn et al. 2016) and especially single-particle

ICP-MS (SP-ICP-MS) (Reed et al. 2012; Bi et al. 2014; Yang et al. 2016; Tharaud et al. 2017) have improved the situation. NTA may give more accurate data compared to DLS (Domingos et al. 2009), while SP-ICP-MS allows the single and ensemble analysis of particle number and concentration on an ion-specific basis (Merrifield et al. 2017a). More recently, single cell ICP-MS (Merrifield et al. 2018) has allowed the quantification of cellularly internalized concentrations of NMs and these ICP-based developments are proving to be extremely powerful. Hyphenated methods such as FFF-ICP-MS and SEC-ICP-MS, which have been widely used (Meermann 2015), could also be combined to provide extra information. A limitation of the SP-ICP-MS method is the lowest size NM that can be quantified (Lee et al. 2014), which is routinely higher than 20-40 nm and is element dependent. Various current developments such as very sensitive detectors, removal of ions during sample introduction, desolvation of the sample prior to the plasma and use of isotopically pure materials should reduce the detectable NM size to approaching 1 nm in the near future. SP-ICP-MS has been adopted for a range of environmental studies (Pace et al. 2011, 2012; Hadioui et al. 2015), while its importance in validating exposure modelling has been discussed (Nowack et al. 2015). It can be clearly seen that all methods have advantages and limitations and there is no metrological method which gives all required data. A multi-method approach (Domingos et al. 2009) has been developed and discussed widely, which brings to bear a wide range of techniques for sample analysis, allowing more detailed, complete, accurate and unbiased data production on NM physico-chemistry. This multi-method approach, employed on pristine and potentially transformed NMs, is recommended.

NM reactivity and toxicity is largely dependent on their physical and chemical properties such as size, shape, specific surface area, elemental composition, surface functionalization and modification, crystalline structure and other factors (Derfus et al. 2004; Lesniak et al. 2005).

Depending on exposure conditions, such properties can affect the propensity of NMs to aggregate, attach to surfaces, release toxic metal ions, become passivated or interact with various environmental or biological constituents such as humic substances, (muco)polysaccharides and cellular debris, in a manner that affects their bioavailability, uptake, and toxicity (Liu et al. 2013; 2016; Sayes et al. 2014), and so the need for metrology is clear.

As an example of why metrology is needed, the relationship between NM surface properties, such as surface energy and reactivity, and toxicity has received little attention. Some research shows that, despite faster dissolution of toxic metals such as Cd^{2+} and higher affinity for proteins, CdS NMs with higher surface energy are less toxic than lower-energy NMs. This unexpected difference is possibly due to the NMs greater propensity to adsorb to or react with biological protective barriers and/or background constituents that alter behavior and effects (Liu et al. 2016). Such reactions passivate their reactivity and decrease their bioavailability and toxicity. The need for appropriate metrology and linking to behavior and effects is clear.

Further discussion on nanometrology and its advances are provided in the Supplementary Information and in cited references.

ENVIRONMENTAL FATE AND BEHAVIOR OF NANOMATERIALS

The environmental fate and behavior of NMs is fundamental to understand their effects on environmental health and is a key aspect of environmental risk assessment (Stone et al. 2010; Hartmann et al. 2014). Knowledge of the sources, pathways, transformations and sinks for NMs will provide information on the compartments (water, sediment and biota) that are exposed to the NMs. Furthermore, understanding the behavior of the NMs in the environment will enable specific hazards to be identified, such as the bioaccumulation potential in aquatic food webs (Luoma et al. 2014; Selck et al. 2016).

Physico-chemical properties

The role of NM characteristics in environmental risk is widely appreciated (Hasselov and Kaegi 2009), although the methods to quantify these properties are not always appropriate or deployed appropriately. In our earlier paper (Klaine et al. 2008), the role of transformations was known and was briefly mentioned. However, physico-chemical properties such as size, composition, surface energy and specific surface area of the original, pristine (as-made or as-used) NMs are still important for two reasons. First, for risk assessment and management purposes, along with an assessment of NP risks and benefits, an understanding of the original (and transformed) NMs is required. Secondly, transformation behaviors and rates are critically dependent on these original properties. However, it is now known that the properties of the transformation products are far more significant than originally thought (Lowry et al. 2012) and these are discussed in detail.

Processes and transformations affecting nanomaterial fate and behavior in surface waters

Transformations of NMs are analogous to the problem of changes in speciation in metal fate, behavior, bioavailability and effects. They have been reviewed (Lowry et al. 2012; Hartmann et al. 2014), although, in 2008, we were only beginning to think about these issues. Dramatic improvements in our knowledge have taken place and the importance of transformations in complex media such as the environment is now better understood. Transformations can be subdivided into physical, chemical and biological processes. Physical processes include aggregation, agglomeration, sedimentation and deposition (in porous media). Chemical processes include dissolution and subsequent speciation changes, redox reactions (oxidation and sulfidation), photochemical reactions and corona formation. Biologically-mediated processes include biodegradation and bio-modification, most likely microbially mediated. These are encapsulated in a conceptual process model (Figure 1). Although it is clear that transformations will be dependent on the nature of the NMs and of the environmental conditions, the complexity and variability of both make understanding and prediction extremely challenging.

Dissolution and solubility

The solubility of NMs has a major influence on their fate and toxicity, for some NMs such as zinc oxide, where toxicity has been largely attributed to the ions (Franklin et al. 2007). For other NMs such as silver, copper oxide and some quantum dots, intermediate dissolution and solubility suggests a role for both ions and particles (Hartmann et al. 2014; Leclerc and Wilkinson 2014), possibly where the ions have the direct biological impact, but NMs increase transport to the cell and locally increase ionic concentrations. For carbon-based NMs and many inorganic NMs, such as ceria and titania, where solubility is low, dissolution and solubility become less important. Nevertheless, dissolution in micro-environments such as cellular vacuoles, where pH is reduced, may be important even for these NMs.

For the purpose of this review and for nanoscience in the environment, the importance of the ion relative to the particle should be judged against their relative behavior and effects over relevant timescales. In toxicology, for instance, the importance of dissolution can judged over the exposure time period and this may be different from the equilibrium solubility. The solubility of nano-CeO_2 is extremely low, making it a useful material for studying the NM effects directly without the complication of accounting for ionic behavior. For instance, they can be used to investigate the role of nano- compared to micron-sized materials on behavior and toxicity (Rogers et al. 2010).

In addition to composition, several studies have shown intrinsic NM properties to affect dissolution and solubility including size (Tsiola et al. 2017), coating (Toncelli et al. 2017) and doping (Adeleye et al. 2018). A major issue in this area, as in other areas of correlating properties and behavior has been the difficulty of varying single properties of NMs, while leaving other confounding factors unchanged, although this is being doen more successfully now than in 2008 (e.g., Adeleye et al. 2018). In addition, studies have investigated the impacts of external factors such as NOM on NM behavior, concluding that dissolution may be enhanced (Xiao et al. 2017), along with particle ripening and precipitation of new NMs (Merrifield et al. 2017b). Organic materials can also significantly impact NM dissolution (Luoma et al. 2016), but their influence is complex and differing study data have not been fully rationalized (Aiken et al. 2011). Natural waters from a variety of systems have been studied (Odzak et al. 2015; 2017) and these complex systems substantially affect dissolution (Wasmuth et al. 2016).

Aggregation, sedimentation and deposition

The interaction between charged colloidal particles in solution has been described by the DLVO (Deryaguin, Landau, Verwey and Overbeek) theory as the combination of repulsive and attractive forces. The application of this theory to NMs has been somewhat confounded by both their polydispersity, complexity and non-sphericity (Hotze et al. 2010), although in general the theory works well (Aich et al. 2016) in understanding charge (de)stabilization. It is now clear that the key factors in water chemistry that apply to natural colloids, can also largely be applied to manufactured NMs. The key factors in particle aggregation of charge-stabilized NMs in environmental systems are pH, ionic strength, the presence of divalent ions, and the type/concentration of organic matter (Handy et al. 2008a; Bian et al. 2011), along with the concentration of manufactured NMs (Baalousha et al. 2016). For charge stabilized systems, the effect of these variables is mediated by charge, so pH may lead to different behaviors, while increases in ionic strength will lead to aggregation. The role of NOM is discussed later. Homoaggregation/agglomeration (where agglomeration is usually defined operationally as a weaker interaction than in aggregation) is commonly studied with NMs and may be essentially instantaneous on addition to test solutions, yielding aggregate/agglomerate sizes in excess of 100 nm. Many studies have demonstrated the role of NOM such as humic and fulvic acids in stabilising NMs against aggregation (e.g., Domingos et al. 2009; Angel et al. 2013; Yang et al. 2017), through charge repulsion and steric repulsion.

The importance of heteroaggregation (aggregation between non-similar particles) at high NM concentrations was demonstrated by Quik et al. (2012) using 1 mg/L of nano-CeO_2 added to filtered and unfiltered river waters. In unfiltered waters, heteroaggregation with natural colloids led to 80-85% removal of the ceria by sedimentation over 4 days, following first-order kinetics. By contrast, NOM in the filtered waters stabilized the NMs for up to 12 days. At higher ceria concentrations, 10 and 100 mg/L in unfiltered waters, more than 99% settled out, largely by homoaggregation. In a filtered algal medium, increasing additions of NOM increasingly stabilized nano-CeO_2 by adsorption, reducing homoaggregation by increased electrosteric (i.e., combined electrostatic and steric) repulsion, as evidenced by an increasingly negative zeta potential (Quik et al. 2010), and steric stabilization alone is also likely to be important (Diegoli et al. 2008). Recall that ceria concentrations are likely to be in the µg/L range for freshwaters (Figure 2), several orders of magnitude lower than the experimental concentrations in most studies. The demonstration of first-order kinetics for both sedimentation and dissolution was a useful input for future modelling exercises, however, rate constants could not readily be estimated (Quik et al. 2011).

In a later paper (Quik et al. 2014), heteroaggregation rates with natural colloids and sedimentation rates were estimated for C60, nano-CeO_2, PVP-coated nano-Ag and SiO_2-coated nano-Ag for a range of river waters from the Netherlands. System-specific parameters such as these will be important for site-specific modelling. Such studies led to other investigations of heteroaggregation with clay particles using high concentrations (0.1, 0.8 mg/L) of nano-TiO_2 with natural clays (Labille et al. 2015) and humic acid colloids (Praetorius et al. 2014). While they provide useful starting data, these may substantially overestimate the importance of homoaggregation, given that aggregation kinetics are strongly related to the initial concentration (in particular the number concentration) of the dispersed NMs (Merrifield et al. 2017b).

The nature of the NMs in different sinks and receptors may also be fundamentally different because of aggregation and dispersion. The water column can be expected to contain smaller, dispersed NMs, while the benthos are exposed to aggregated NMs, at higher mass but likely lower toxicity per unit mass (Romer et al. 2013). Although aggregation often reduces bioavailability from waterborne NM exposures (Khan et al. 2012), there is no evidence that it affects bioavailability from ingested NMs (Croteau et al. 2011a, b). In some cases, aggregation can enhance bioaccumulation by making particles accessible (Ward and Kach 2009) or by increasing ingestion rates (Croteau et al. 2014).

Recently the concentration-dependence of aggregation (and dissolution) has been shown (Figure 3) by measurement of number concentration and NM mass (size) of core-shell NM using SP-ICP-MS (Merrifield et al. 2017c). Homoaggregation was shown to be quantitatively unimportant at realistic environmental concentrations (<1 µg/L; see below) and relevant timescales, suggesting that heteroaggregation may be a more important mechanism, given the higher concentrations of natural colloids.

NMs stabilized by other mechanisms, primarily steric interactions, are largely unaffected by solution conditions such as ionic strength and are far more stable, especially in hard and marine waters (Badawy et al. 2010;). NOM has been shown to provide additional colloidal stability through replacement of original coatings and subsequent electro-steric repulsion (Diegoli et al. 2008), as well as additional charge repulsion. However, sterically stabilized NMs are prone to aggregation at higher concentrations (Al Abresm et al. 2017) usually significantly higher than likely environmental concentrations, possibly due to polymer entanglement and bridging mechanisms.

Both charge and steric stabilization of NMs can influence aggregation as illustrated by El Badawi et al. (2012) for Ag NMs. Both uncoated and citrate-coated nano-Ag were stabilised by the negative surface charge on the particles caused by adsorbed hydroxyl ions and citrate molecules, showing slow aggregation over short time periods in low ionic strength (10 mM) NaCl or $NaNO_3$ solutions. Polyvinylpyrrolidone (PVP) coatings sterically stabilized nano-Ag, with very little change in size even in 1M NaCl. Such effects were not predicted by DLVO theory, of course, although there are models that can predict steric behavior. Coating with NOM also afforded stabilization due to steric repulsion (Domingos et al. 2009), although for some NMs, depending on solution pH, effects on zeta potential were evident. Similar results have been found by others (badawt et al 2010) and the role of both stabilization mechanisms should be considered. The literature reveals an over-reliance on concepts of charge stabilization over steric stabilization; zeta potential is often used as surrogate for colloidal stability, for instance. Additionally, although increased colloidal stability is usually found and most often discussed, it does not always occur and NOM under certain conditions can cause aggregation (Baalousha et al. 2007 Omar et al. 2014). Understanding the conditions under which NOM increases stabilization or destabilization, and the mechanism by which this is effected is a key point in colloidal stability in environmental systems.

Sulfidation and redox behavior

Sulfidation is a major chemical transformation for many metal NMs particularly in the presence of enhanced sulfide concentrations as found in parts of wastewater treatment plants (WWTPs) or in anoxic or sub-oxic sediments (Kim et al. 2010; Kaegi et al. 2011). The reactions can result in changes in particle size, surface charge and solubility and often is thought to be caused by core-shell ($Ag@Ag_2S$) formation, where the Ag_2S layer gradually increases. Ultimately these changes will influence the fate, bioavailability and effects of the NMs.

The identification of silver sulfide (Ag_2S) NMs in sewage sludge (Kim et al. 2010), provided field evidence of sulfidation of the widely used silver NMs, while microcosm studies have shown sulfidation to occur (Auvinen et al. 2017). The reaction mechanism requires both oxygen and sulfide and may be either a fast direct surface reaction or a slower indirect reaction involving release of ionic silver and more rapid precipitation of Ag_2S (Liu et al. 2011; Levard et al. 2013). Oxysulfidation is the preferred route where sulfide concentrations are high (mg/L) (Liu et al. 2011) and NOM concentration plays a protective role in reducing sulfidation rates (Baalousha et al. 2015). The extreme insolubility of Ag_2S ($Ksp = 6 X 10^{-51}$) means that Ag^+ will likely exchange with other less soluble sulfides (ZnS, FeS) and toxicity is generally reduced (Devi et al. 2015), although this process may not lead to coherent core-shell NMs (Baalousha and Lead 2015). In addition, preservation of the original NMs has been observed (Baalousha and Lead 2015; Pettibone and Liu 2016; Romer et al. 2016), while reversibility (e.g., via transport from sediments to overlying waters through bioperturbation) is poorly understood; examination of the literature on trace metals will be a useful starting point to understand such processes for inorganic NMs.

Nano-ZnO can undergo slow sulfidation by a surface dissolution and reprecipitation mechanism (Ma et al. 2013). As with partially sulfidized Ag NMs, the solubility of zinc oxide was not quenched by a partial coating, although coating will likely play a key role in controlling dissolution rates. With increased sulfidation, solubility was reduced, although the transformation process does generate Zn^{2+}. The newly formed, sulfidized nano-ZnO particles were found to be smaller, but, with a reduced surface charge, they are often more susceptible to aggregation. Nano-CuO sulfidation also occurs over several days with the formation initially of copper(I) sulfide (Cu_2S) with possible transformation to CuS (Ma et al. 2014). Unlike nano-Ag and nano-ZnO, the sulfidized form of the original nano-CuO has greater solubility and releases more Cu^{2+} that the parent NM (Ma et al. 2014) and, as a consequence, has been shown t be more toxic to aquatic biota (Li et al. 2015).

More generally, oxidation is not a major transformation pathway for most NMs, although it is an essential step in the dissolution of metals such as silver, while redox transformations of metal oxides such as iron oxide and ceria are important in determining behavior. The effectiveness of Ag NPs as bactericides likely relies in part on the surface oxidation of elemental silver, and recent studies suggest that photochemical oxidation can enhance the formation of a surface layer of Ag^+ (Grillet et al. 2013), while again NOM has a protective role on Ag NMs (Romer et al. 2016).

Modeling the exposure and fate of nanomaterials into the environment

Fate and behavior considerations necessarily start with discharges to the environment, which are in principle known, although very poorly known in practice. There are limited, hard to find and poor quality data on who is manufacturing, using and discharging NMs and in what amounts. Regulations such as the European UNION'S REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) and USEPA's Toxic Substances Control Act (TSCA) should improve this situation, at least potentially, by making these data more publicly available. The main discharge routes include point or diffuse sources to freshwaters, including treated waste water, sludge application to soils and landfill leachate. Other primary sources of NMs to the environment comprise emissions associated with production, spillage during transportation, handling and storage, and discharges associated with waste handling.

There have been increasing numbers of publications directed towards modelling the concentrations and fate of NMs entering the environment. The current status of modelling and the differences in their underpinning assumptions have been reviewed in recent publications by Gottschalk et al. (2013) and Baalousha et al. (2016), with distinctions were drawn between mass flow analysis (MFA) and fate and behavior (FB) models. The former tend to concentrate on providing input data, while the latter tend to focus on within-environment processes such as aggregation. Although computationally challenging, the nesting of these models such that the MFA models provide input parameters to the FB models, would be ideal and has already partly begun (Baalousha et al. 2016).

A major limitation for the MFA models remains a lack of adequate input data. Models clearly require data on NM production and usage in industrial and consumer products. Subsequently, there needs to be an evaluation of the extent to which NMs in these products are released to the environment (Wohlleben and Neubauer 2016); additionally, calculation of release rates and later transformation rates and types are needed. The lack of analytical capabilities for detection and quantification of NPs in real environmental systems currently also makes validation against actual environmental concentrations impossible (Nowack et al. 2015). Input data for the MFA models must be laboriously collected (Mahapatra et al. 2015) or must be estimated within quite wide bounds. The outputs from these models are therefore not definitive, as they are often portrayed, and should be used as a guide and with circumspection.

The earliest modelling by Boxall et al. (2007) relied on global production data, with a focus on products having free, engineered NMs in such product categories as cosmetics, paints and coatings, catalysts and lubricants, water treatment, food and food packaging, human and veterinary medicines and plant protection products. Mueller and Nowack (2008), in considering environmental impacts in Switzerland, used global production estimates converted on the basis of the Swiss population compared to that of the industrialized world, but there were clear limitations in approaches in large put due to the difficulty of gathering reliable data. As newer data on production volumes became available and as models became more sophisticated, more reliable estimates have been achieved (Keller et al. 2013; Sun et al. 2014). For instance, global production data, regional projections, and information on local production were used (Gottschalk et al. 2009, 2011; Keller and Lazareva 2014; Sun et al. 2014). In addition, models developed probabilistic approaches that considered the distribution of concentrations at various stages of the material flow analysis (Gottschalk et al. 2009, 2010; Sun et al. 2014), to account for the large uncertainties and variability in model input parameters.

A generalised structure of an MFA model is shown in Figure 1 (Sun et al. 2014), where the releases from primary sources go principally to elements of the engineered environment, namely WWTPs, waste incineration plants, landfill or recycling, and also directly to the natural environment (air, soil, water and sediment). There is transfer from these two broad compartments as well as transport within each of these compartments. For instance, aggregation and settling will remove NMs from the aqueous phase into the sediments, with likely resuspension in many cases. WWTPs are often generally assumed to be the major recipient of many NMs, although significant misconnection of drains (Mahapatra et al. 2015) and other sources mean that there are a number of other environmental receptors.

Fate processes were largely ignored in the earlier studies (Boxall et al. 2007). Bottom-up approaches (Mueller and Nowack 2008) are more fully life-cycle based, while recent FB models include more detailed processes such as dissolution, sedimentation and aggregation, often linked to stream flow and other physical processes (Praetorius et al. 2012; Liu and Cohen 2014; Sun et al. 2014; Dale et al. 2015; Sani-Kast et al. 2015; Ellis et al. 2016, 2018). Despite these advances, there remain many uncertainties and deficiencies. Most models assume steady-state concentrations in various compartments, however, the variability in the dynamics of transformation are very important and not well understood.

In addition to the lack of methods for the analysis of environmental concentrations, laboratory studies of NM fate are usually undertaken under simplified conditions, using concentrations much higher than those expected in the environment. The concentration effect on dissolution, aggregation and more complex transformations has recently been quantified for Ag NMs (Hadioui et al. 2015; Baalosuha et al. 2016), where a dissolution-dominated regime occurs below 1 µg/L and an aggregation-dominated regime above 10 µg/L for Ag NMs (Merrifield et al. 2017a). This change has consequences for bioavailability which is also concentration dependent (Croteau et al. 2014). The effects are likely mediated through changes in aggregation behavior. Similarly, unusual behavior of the diffusion coefficients of TiO_2 NMs with concentration may be related to aggregation (Holmberg et al. 2011).

Predicted environmental concentrations

As mentioned, analytical measurements on real-world systems are almost non-existent. Because of analytical challenges, detecting and quantifying NMs in real environmental systems remain essentially impossible. As a result, there are few data on actual concentrations against which to validate FB and MFA models (Nowack et al. 2015). In assessing NM risk to the environment, probable (or predicted) environmental concentrations (PECs) are required that can be compared to predicted no effect concentrations (PNECs). In the absence of measured data, modelling has been our sole method of providing estimates of exposure concentrations. Examples of PECs for nanomaterials in fresh waters determined from a range of modelling approaches, together with measured data, are shown in Figure 2. Note that while the examples provided are for a freshwater compartment, similar estimates have been made for soils, sediments and wastewater treatment effluents and sludge, as reviewed in many of the papers cited here. Even for these freshwater systems, the limited number of data sets shows a wide range of values, possibly reflecting real differences in the environment, differences in the methodological approaches or both. Ranges of up to four orders of magnitude for nano-TiO_2 and nano-ZnO, to two orders for nano-Ag and carbon nanotubes (CNTs) are shown in Figure 2.

It has been suggested (Gottschalk and Nowak 2012) that models based on top-down approaches can easily be in error because they rely on imprecise estimates of market penetration. Nevertheless, estimates based on bottom-up modelling at a local scale can be equally variable with ranges such as 11-1600 and 4-320 ng/L being estimated for TiO_2 NMs and Ag NMs respectively (Gottschalk and Nowack 2012), the upper values of which exceed measured concentrations (Gottschalk et al. 2013). Recent modelling of releases to European rivers of nano-ZnO and nano-Ag (Dumont et al. 2015) found that half of the river stretches had predicted long-term (months to years) average concentrations exceeding 0.002 ng/L for nano-Ag and 1.5 ng/L for nano-ZnO, noting that these were based only on household products and so are likely underestimates of environmental concentrations. In addition, these values are based on recent estimates of discharges of NMs, not on likely increased future discharges, again underestimating likely concentrations in the long term.

This large variability has disturbing implications for the reliability of risk estimates. Importantly, it reinforces the need, already noted by several authors (Gottschalk and Nowack 2012; Gottschalk et al. 2013) for more localized monitoring of NMs in waters, sediments and soils, provided that appropriately sensitive analytical methods are available.

Fate and behavior of NMs in marine ecosystems

The review above specifically refers to freshwater systems, although processes operative are similar for marine systems and here we discuss differences between the two systems. Theoretical concerns centre on the higher ionic strength (and to some extent on lower NOM concentrations), which would lead to charge screening, aggregation and particle settling, for charge stabilized NMs (Klaine et al. 2008). Thus, coastal sediments and those in the deep ocean are considered as likely sinks (Klaine et al. 2008). However, microbial and physico-chemical activity in marine sediments will likely transform NMs and/or remobilize them into the water column. There is little direct field evidence, but mesocosm and laboratory studies and read-across from other contaminant behaviour suggests this is likely (Galloway et al. 2010). In addition, near-shore environments might be expected to have higher concentrations than the open ocean given the likely terrestrial discharge sites, although again there is little or no direct data. The viscous properties of ultrafine particles in the sea-surface microlayer are also a concern and may be an important sink for certain types of NM. Little progress has been made in this area either.

Progress has been made on understanding the settling rates of NMs in saline conditions. Some of this knowledge is derived from studies on physiological salines that also have relatively high salinities. For example, Al-Jubory et al. (2013) showed that artificial gut saline for trout rapidly precipitated TiO_2 particles in a size-dependent manner, leaving only the smallest size fraction (mainly primary particles) in suspension; sterically stabilized NMs are often far less susceptible to aggregation and are more likely to remain in the aqueous phase (Hitchman et al. 2012). Particle settling rates have also been measured in seawater (nano-TiO_2: Brunelli et al. 2013; C60, nano-CeO_2 and various nano-Ag-containing particles: Quik et al. 2013), almost always at high concentrations. The settling behavior is dependent on the particle type, with aggregation kinetics increasing at higher mass concentrations of each material, in general agreement with DLVO theory. However, for nano-TiO_2, at least in one study, the settling rates in seawater appear to be similar or only a little faster than those in artificial freshwater (Brunelli et al. 2013).

The accumulation of chemicals in marine and estuarine (and freshwater) sediments is generally well-known (Honeyman and Santschi 1992). However, details of the sedimentation behavior of NMs are poorly quantified, particularly at environmentally-relevant concentrations, and in mixing zones in freshwater, seawater and in estuaries. In addition, how NMs would be altered by these interactions and the fate of the NMs within these processes is largely unknown. However, it is hypothesized that NMs in the pore water of sediments will behave similarly to in the overlying water, and the binding of NMs to natural minerals and organic matter in the structure of the sediment will be broadly similar to that of natural colloids and other chemicals.

For example, dissolution and complexation processes should still occur in the sediment pore water, and the mobility of NMs in the sediment will depend on the sediment grain size and its chemical composition, temperature, salinity, and the effects of bioturbation.

There are only a few studies on the behavior of NMs in marine sediments. Bradford et al. (2009) showed that serially dosing intact sediment samples from Plymouth Sound in the UK with Ag NPs, to simulate a daily effluent discharge with the tide, resulted in rapid loss of silver metal from the water column and accumulation in the top 1 cm of the sediment. The total silver remained trapped in the surface, without transfer to deeper parts of the sediment. There were also no discernible effects on the microbial biodiversity in the sediments based on molecular methods such as density-gradient gel electrophoresis of the nucleotides. A follow-up study on Plymouth Sound sediment showed that the antibiotic resistance of the microbes was also unaffected (Mühling et al. 2009). At high concentrations, nitrification processes have been reduced over short-term studies, but not at lower concentrations over similar timescales (Beddow et al. 2017). In freshwater sediments, the cumulative respiration rate and microbial biomass of the sediment was unaffected by exposure via the overlying water containing up to 250 mg/L of Ag as Ag NPs (Colman et al. 2012). In the same study, the freshwater sediments showed normal microbial services with no loss of sulfatase, phosphatase, or lucine aminopeptidase activity. Such detailed studies of ecosystem services from microbes remain lacking for Ag NPs in marine sediments. In addition, the limited data sets available show somewhat contradictory conclusions which need to be rationalized by appropriate choice of NMs, conditions and endpoints in further studies.

Mesocosm data have shown that Au NMs are present mainly in sediment biofilms in estuarine systems (Ferry et al. 2009). Marine sediments are also subject to bioturbation by polychaete worms and shellfish living on/in the sea floor. However, a quantitative analysis of how bioturbation alters the fate and behavior of NMs in marine systems is yet to be performed, but it is clear that the behavior of marine organisms in sediment can sometimes be affected upon NM exposure. For example, polychaete worms can show a decrease in casting rates (nano-TiO_2: Galloway et al. 2010), or alternatively burrowing behaviors may not change very much (nano-CuO: Buffet et al. 2013). Similar observations are also being made for sediments in freshwater lakes. Pakarinen et al. (2014) showed that up to 9% of fullerenes deposited in surface lake sediments, were remobilized over several days as a consequence of surface currents, as well as by bioturbation in the natural environment. However, the rates of bioturbation along with tidal flows and wave action suggest that such mixing and remobilization effects will be much higher in coastal marine ecosystems.

Fate and behavior in terrestrial systems

There are a number of processes that govern the fate of NMs in soils that are common with those that govern fate in aquatic systems, namely dissolution, transformation and aggregation/disaggregation. However, some of the processes governing NM fate and behaviour in soils are quite different, e.g., straining, deposition/mobilisation and diffusive transport (Figure 5). These vary in importance depending on the characteristics of both the NM and the soils (Cornelis et al. 2011, 2014).

For some NMs, dissolution may be very important as it degrades the NMs so that fate and bioavailability become more aligned with the soluble components. For example, it has been demonstrated that bulk ZnO dissolves rapidly in soils (Smolders and Degryse 2002; McBeath and McLaughlin 2014), so that ZnO NMs are also likely to be ephemeral in soils unless coated with agents to restrict dissolution. Heggelund et al. (2014) showed an absence of NP-specific effects in soils, with bulk and nano-ZnO behaving similarly in terms of fate, toxicity and dissolution, with pH being the most important control on dissolution. ZnO NMs can dissolve and/or be transformed into a mixture of species such as ZnS, $Zn_3(PO_4)_2$, Zn-cysteine, Zn-substituted ferrihydrate and Zn^{2+} adsorbed to mineral surfaces. The reported proportions of these components will be different depending on the wastewater treatment configuration (Lombi 2012; Ma 2013; Wang, Menzies et al. 2013; Brunetti 2015; Judy 2015b).

It is worth noting that dissolution of NMs in soils is not easily predicted by batch water solubility tests, as soil surfaces provide a sink for anions or cations released from the NMs (promoting dissolution), as well as providing strong pH buffering of the soil solution. Thus dissolution of metal oxides is much faster in soils than in water. Carbon-based NMs are generally insoluble (in water) but may degrade though photolysis or microbial decomposition (Chouhan et al. 2016; Navarro et al. 2016).

In a recent review of the fate of NMs in soils, Cornelis et al. (2014) performed a useful comparison of typical pH and ionic strengths of soil saturation extracts (using a global database) in relation to typical critical coagulation concentrations (CCCs) for NMs. They concluded that homoaggregation of NMs would be slow in most soils as the pH and ionic strength of most soil solutions would lie below the CCC of most NMs. Heteroaggregation is likely to be very important in soils, as with aquatic environments, as soil pore waters often contain higher concentrations of natural colloids in suspension. Numerous studies have observed strong heteroaggregation of NMs with soil colloids (Cornelis et al. 2010, 2011, 2012; Hotze et al. 2010; Huynh et al. 2012; Hoppe et al. 2015; Klitzke et al. 2015; Labille et al. 2015; Smith et al. 2015) which has significant implications for limiting NM transport through soils as straining will be enhanced (Figure 5). On the other hand, the presence of NOM in soil pore waters has often been found to stabilize NMs and inhibit both homo- and heteroaggregation (Praetorius et al. 2014).

This means that for the less soluble NMs, accumulation will likely occur in topsoils with little movement to depth in most soils. Few studies have examined transport under field conditions (Kasel et al. 2013), and this is probably the key gap for more accurate assessment of the real risk of NM transport through soils.

Over the last decade, studies of NM transport through soils have progressed from using inert stationary phases (e.g., quartz beads) in columns (Lecoanet et al. 2004) to the use of natural soils, so that we now have a much better appreciation of the potential transport of NMs in terrestrial systems (Jaisi et al. 2008; Darlington et al. 2009; Fang et al. 2009; Jaisi and Elimelech 2009; Wang, Jaisi et al. 2015; Wang, Li et al. 2010; Cornelis et al. 2012, 2013; Coutris et al. 2012; Sagee et al. 2012; Zhao et al. 2012). CNTs appear to be retained in soils due to their high aspect ratio leading to significant straining (Jaisi and Elimelech 2009; Kasel et al. 2013; Zhao et al. 2016). Fullerenes are also strongly retained in soils, likely through strong interactions with soil organic matter (Wang, Li et al. 2010; Navarro et al. 2013). Where some mobility was observed, this was usually in pure sand media or very sandy soils with very low OM contents (Zhang, Hou et al. 2012).

NANOMATERIAL BIOAVAILABILITY AND BIOACCUMULATION IN AQUATIC ORGANISMS

Studies on bioavailability and uptake are critically important to link the environmental chemistry of NMs to biological effects. The assumption is that the presence of a NM on or in an organism will lead to a biological response, and this can be informed by how the NM in the environmental media initially interacts with the external surfaces of the organism. In 2008, it was quickly identified that the broad concept of substance behavior in water, adsorption of a bioavailable fraction to the epithelial surface of the organism, and then membrane uptake to internalise the substance, could be applied to NMs (Handy et al. 2008a,b; Klaine et al. 2008). The steps for net uptake or absorption to the internal body fluids are summarised in Figure 6. The key steps involve how the NM behaves in the external media (e.g., water or gut lumen) and is presented to the external surface of the organism. The latter is a dynamic microenvironment where secretions from the organism can interact with the external media, and/or act as a concentrating layer for the substance. Transformation processes like those described above for water and soil might also occur. Figure 6 shows the uptake across an idealised epithelium such as a fish gill, but conceptually many organisms have uptake pathways for solutes (metal transporters are highly conserved across species) and also for particulates via endocytosis pathways. The experimental evidence for the bioavailability and the uptake mechanisms of different NMs in aquatic organisms is far from complete, and there are inherent differences in how, for example, invertebrates compared to fishes process metal particles.

Bioavailability and uptake studies in invertebrates

Effect studies largely dominate the scientific literature on aquatic invertebrates and engineered NMs (Selck et al. 2016). Although important, these studies provide a limited understanding of the processes linking the sources and transformations to bioaccumulation, and ultimately toxicity. Fewer studies address NM bioaccumulation, for which bioavailability is a driver. The ability of aquatic invertebrates to accumulate NMs has been unequivocally demonstrated (Garcia-Alonso et al. 2011; Khan et al. 2015a). Clearly, NMs provide a unique type of exposure that is not fully considered in the risk assessment process for metals alone (Luoma et al. 2014). For instance, the internalization of NMs is poorly understood, although much progress has been made since 2008, and there is significant evidence of nano-specific effects.

The properties and behaviors of NMs (as described earlier) are important drivers of bioaccumulation in invertebrates. For example, particle size has been shown to influence bioaccumulation, although NM size may not be indicative of exposure if particles aggregate. There are many studies that show that bulk or micron-size particles are less bioavailable to invertebrates than their dissolved or nano-sized counterparts (Pang et al. 2013; Cozzari et al. 2015). Numerous studies have shown that metal uptake rates are faster for ionic forms than for nano-sized forms (Zhao and Wang 2010; Croteau et al. 2011a; 2014a; Ramskov et al. 2015). For example, silver uptake rates were 2-10 times faster for dissolved silver than for silver in nanoparticulate forms for the estuarine snail *Peringia ulvae* (Khan et al. 2012), the freshwater snail *Lymnaea stagnalis* (Croteau et al. 2011b), the water flea *Daphnia magna* and the freshwater oligochaete *L. variegatus* (Khan et al. 2015b).

Particle composition also has an important influence on NM bioaccumulation in invertebrates. In general, Au, TiO_2 and SiO_2 NPs were less bioavailable and toxic than CuO NPs, ZnO NPs or Ag NPs (S.N. Luoma, UC Davis, Davis, CA, USA, unpublished manuscript). For example, *D. magna* can efficiently ingest Au NPs, but its gastrointestinal tract can be largely purged after 1 h of depuration in clean water in the presence of food (Khan et al. 2014). In contrast, citrate-capped Ag NPs accumulated after ingestion of diatoms mixed with NPs were retained in the tissues of the snail *L. stagnalis* with no detectable loss after transfer to clean media for up to 14 days (Croteau et al. 2011b). Likewise, the cadmium accumulated after ingestion of quantum dots (CdS and CdSe) was retained in the tissues with negligible elimination (Khan et al. 2013b). Comparison of data from studies conducted with different approaches and particle properties should be performed with due care; however, these results show a lack of (or slow) elimination of metal-based NMs have important implications for bioaccumulation; slow metal efflux rates of NMs will typically cause high accumulation of metals within cells or organisms.

In addition to particle size and composition, the shape of the NMs (e.g., rods, spheres and platelets of CuO NMs; Dai et al. 2015; Ramskov et al. 2015), the synthesis method and the nature of the polymer used to stabilize the NM can affect bioaccumulation. These influences are much less studied partly because of the difficulty to constraining one NM feature (size, shape, etc.) while maintaining constant all other NM properties which might affect uptake and toxicity. Collaboration between materials scientists and toxicologists is still needed to fill this knowledge gap.

Bioaccumulation of NMs is furthermore influenced by the behavior of the NM in the environment. Dissolution, for instance, may contribute to the total uptake of metal from NM exposure. This was shown, for example, in aquatic snails exposed to nano-Ag (Croteau et al. 2014b; Khan et al. 2015; Stoiber et al. 2015); nano-CuO (Croteau et al. 2014a) and nano-ZnO (Khan et al. 2013a). However, in most cases, bioavailability (and/or toxicity) cannot only be explained by the metal concentrations in the dissolved phase. Aggregation can also influence bioavailability. For example, aggregation can change the dominant exposure route from water to sediment (or food) by removing NMs from the water column. As a result, potential impacts are shifted from pelagic to benthic organisms (Selck et al. 2016). However, aggregation does not eliminate bioavailability. Aggregated NMs appear bioavailable when accidentally ingested by deposit-feeders and grazers. For example, using assimilation efficiency (AE) as a measure of bioavailability from diet, bioavailability of aggregates (or agglomerates) of NMs associated with the food ingested by the snail *L. stagnalis* ranged from 49-58% for Ag NPs (Croteau et al. 2011b); 41-83% for CuO NPs (Croteau et al. 2014a) and 80% for ZnO NPs (Croteau et al.

2011a). Water hardness did not influence the dietary bioavailability of NPs (Lopez-Serrano et al. 2014). In some cases, aggregation of NPs can even enhance bioavailability by forming larger particles that are preferentially retained by filter-feeding invertebrates such as mussels (Ward and Kach 2009). To the extent that bioaccumulation and toxicity are linked, exposure to highly bioaccumulative NMs is likely to elicit adverse effects more readily than exposure to other NMs.

Bioavailability and uptake studies with fishes

Similar to the studies on invertebrates, aspects of the water chemistry are known or expected to influence NM bioavailability to the gills of fishes. There are several explanations as to why a NM in the water column may become bioavailable to a fish gill (reviews by Handy et al. 2008b). These include particle mobility – a stable dispersion of primary particles may collide frequently with the epithelium resulting in attachment of particles to the membrane surface. The attachment and transport mechanism might be related to steric (shape of NM), charge or diffusive hindrance in the mucus layer of fish and other organisms (Smith et al. 2007), or electrostatic attraction of positively charged particles to the polyanionic epithelium (although in practice most NMs are negatively charged and there will be charge repulsion). Alternatively, an unstable dispersion forming larger aggregates may simply precipitate onto the epithelium (e.g., TiO_2 particles, Johnston et al. 2010). Finally, particles may also dissolve (e.g., Cu NPs in acidic fresh water, Al-Bairuty et al. 2016) and be taken up according to traditional free ion activity models and the affinity of the dissolved form for solute transporters (Figure 6). Dependent on coating and solution conditions, Cu NPs can form reasonably dispersed phases in freshwater (Shaw et al. 2016) with greater uptake from the more highly dispersed. However, particle settling and the greater mass concentration in larger particles or aggregates best explain TiO_2

accumulation in/on the gut epithelium of trout (Al-Jubory et al. 2012), while titania can also be found in the water column embedded with mucus, produced by fish (Johnston et al. 2010).

The evidence for uptake in the particulate form versus the ionic or dissolved form of a NM is often circumstantial in fish, unlike in invertebrates, based on the dissolution behavior of the material in water or the differences in total metal accumulation in the gill between animals exposed to metal salts or the equivalent NMs. For example, with CuO NPs the dissolution is a small fraction of the total metal in the particle dispersion in freshwater (Al-Bairuty et al. 2016) and thus it might be argued that the metal is initially taken up mainly in the particulate form.

The uptake mechanism can also be investigated pharmacologically in these larger vertebrate animals. In trout intestines, the apparent epithelial uptake is blocked by both ion transport inhibitors and agents that interfere with endocytosis pathways, demonstrating that both solute and particle transport are involved in the absorption mechanism (Al-Jubory et al. 2013. Proof can be found, at least qualitatively, in the identification of intact particles inside the epithelial cells, although the formation of NMs *in vivo* cannot be discounted. For example, many aquatic species, especially shellfish, use the biomineralization processes as part of their normal biology for the formation of shell and the sequestering of metals in the tissues (Brown 1982). Metal granules can also be observed in the livers of fishes (Lanno et al. 1987). There is therefore a need to differentiate manufactured NMs from the particles already present in the tissue. This is difficult but the availability of isotopically labelled NMs and MS-based approaches are currently yielding important results (Thit et al. 2016).

In addition, techniques available to identify and partially quantify such processes include electron microscopy coupled with energy dispersive x-ray spectroscopy (EDX), electron energy loss spectroscopy (EELS), or other measurements for percentage elemental composition (see review in Handy et al. 2012b) as well as speciation (Merrifield et al. 2017c). Such methods allow direct determination of the particles in the tissue. However, there is a need to consider the prospect of finding small numbers of NMs in a grid made from only a handful of cells from the original tissue sample. For example, the gill surface area of a 10 g teleost fish is approximately 10,000 mm^2 (Hughes and Al-Kadhomiy 1988) and, with a profile area of a typical cell in the epithelium of around 300 µm^2 (Laurent and Hebibi 1988), one might estimate some 3.3 x 10^6 cells facing the external media in a single fish. For TiO_2 NPs in freshwater, a typical 1 mg/L dispersion contains ~10^6 particles/mL (Ramsden et al. 2013) of which, at best, less than one third would be bioavailable (settling, losses to mucus secretion, etc.). This equates to 0.2 x 10^6 particles in a freshly dosed 20-L fish tank, and with typically 15 fish there would be only 5 x 10^7 gill cells available, or one particle in every 250 cells. Thus, even at relatively high concentrations the probability of visualizing even a single NM in an electron micrograph of a gill epithelial cell is remote. Consequently, microscopy is not likely a useful tool for determining the presence of particles in the tissue as the risk of false negatives is high.

As discussed earlier, SP-ICP-MS is being developed to enable particle detection in tissues after digestion of the tissue in strong alkali (Gray et al. 2013). More recently direct measurements have been performed by single cell ICP-MS (SC-ICP-MS) (Merrifield et al. 2018) at sub µg/L aqueous concentrations with 0-3 NMs per cell (in algae), in agreement with indirect measurements by Piccapietra et al. (2012). Application to fish has not been performed but is feasible. In addition, targeted Raman spectroscopy has been able to identify intact particles in or on the surface of fish gills from waterborne exposures (Johnston et al. 2010). Furthermore, studies with cultured mammalian epithelial cells show, in principle, that intact NMs can be taken up by endocytosis mechanisms (e.g., Caco-2 cells, Busch et al. 2011; Gitrowski et al. 2014).

Evidence is also mounting for fish epithelial cells from gills (Felix et al. 2017) and intestines (Geppert et al. 2016), where particle internalization was reduced at low temperature, indicating an energy-dependent uptake process. However pharmacological proof of the uptake mechanisms in the gills of aquatic organisms such as fish and bivalves is largely absent.

Dietary exposures

Hou et al. (2013) reviewed the existing literature on NP bioaccumulation by invertebrates and fish. Out of 65 reviewed papers, half dealt with aquatic invertebrates. The pelagic crustacean *Daphnia* sp. was by far the most studied taxon. However, sediments are a likely sink for NPs because of the settling behaviors of particles. Benthic organisms such as worms, insect larvae and mollusks are thus expected to be more exposed to NMs than pelagic species (Selck et al. 2016). However, sediment exposure is under-studied compared to waterborne exposure studies conducted with pelagic taxa such as water fleas (Hou et al. 2013). The difficulty of separating the NM contribution to bioaccumulation from that of background largely explains the paucity of dietary studies. For example, distinguishing newly accumulated copper or zinc from background levels in organisms is difficult unless extremely high, and thus environmentally irrelevant exposure concentrations, are used (Dybowska et al. 2011; Misra et al. 2012). The application of tracer techniques can overcome this complication (Zhao and Wang 2010; Croteau et al. 2011a, 2014a; Ramskov et al. 2015; Thit et al. 2016). For example, Croteau et al. (2014b) used isotopically labelled Ag NPs to characterize silver uptake rates in the freshwater snail *L. stagnalis* across a wide range of aqueous and dietary exposure concentrations. Results showed a concentration-dependent shift in the relative importance of dissolved versus nano Ag uptake to the snails that would not have been detectable working with unlabeled silver. Likewise, Khan et al. (2013a) exposed estuarine snails to isotopically labelled zinc in the forms of ZnO NPs, ZnO bulk particles and aqueous zinc and concluded that bioaccumulation of zinc from ZnO NPs was primarily dependent upon solubility. Weak copper accumulation by the freshwater oligochaete *Lumbriculus variegatus* exposed to isotopically labelled copper (ionic copper and CuO NPs) in water and sediment was also reported by Ramskov et al. (2015) who concluded that this species was an inefficient bioaccumulator of copper, explaining in part the tolerance of the species. The use of tracers to detect metals that originate from metal-based NMs is a growing field of study.

Techniques have also been developed to examine trophic transfer of nanoparticles in the absence of solubility in the water column. Using methods developed by Cresswell et al. (2017), the uptake, assimilation and trophic transfer of dietary nano CeO_2 particles along a freshwater food chain represented by an alga (*Pseudokirchneriella subcapitata*), a grazing snail (*Potamopyrgus antipodarum*) and a prawn (*Macrobrachium australiense*) were studied using particles containing the gamma-emitting radioisotope Ce-141 (L.A. Golding, CSIRO Land and Water, Kirrawee, NSW, Australia, unpublished manuscript)). Using gamma spectrometry, pulse-chase experiments showed a rapid elimination of CeO_2 in both nano- and micron-sized forms from the digestive systems of both the snail and the prawn. More sensitive autoradiography confirmed that no Ce-141 could be detected in the prawn tissue outside the confines of the digestive tract, evidence that the particles were not transported across the gut wall and were therefore not assimilated by the biota. Nonetheless, from the perspective of predator-prey interactions, a prey item with a gut lumen full of NMs will represent a neat package of NM-contaminated food for the predator.

Dietary studies have been conducted on invertebrates and fish, although the data remain sparse (Croteau et al. 2011a,b; 2014b; Khan et al. 2013b). Feeding studies do show that fish will eat food contaminated with NMs and continue to grow (e.g., nano-TiO_2, Ramsden et al. 2009;

quantum dots, Blickley et al. 2014), but the details of changes in individual nutrients or the long-term health of the gut (or animal) is not known. Processes critical to feeding and digestive physiology are disrupted by ingestion of NMs by snails, sometimes at very low concentrations (e.g., Ag concentrations in diet ranging from 1-5 µg/g: Croteau et al. 2011b, 2014). This is important because disruption of gastro-intestinal function and a reduction in feeding might cause harmful changes that can affect processes such as growth and reproduction. Ultimately these changes can cause population and community changes. Uptake across the gut epithelium of metal from oral exposure to NMs has been confirmed (e.g., trout, Al-Jubory et al. 2013). However, some unexpected observations have been made, e.g., in the case of nano-TiO_2, the uptake rate across the gut of rainbow trout is dominated by a partial pressure of carbon dioxide effect (Al-Jubory et al. 2013). The effects of barometric pressure, gas pressures, or water depth are simply not considered in environmental risk assessment for any chemicals, but this observation raises a concern that NMs may be more bioavailable at >10 m depth than at the surface. Another interesting observation has been with regard to the gut microbiome of fishes. Silver NMs especially, are noted for their antimicrobial properties but how this alters the natural microbiome of animals is not clear. Merrifield et al. (2013) demonstrated that the microbiome in the zebrafish gut does vary between NMs (Ag NPs and CuO NPs), but also showed a nano effect in that dietary $CuSO_4$ resulted in a different microbial biodiversity to that of CuO NPs in the fish gut. However, the toxicological or nutrition consequences of such changing microbiology of the fish gut remain to be investigated. Chronic studies at environmentally realistic concentrations also remain a significant data gap for most aquatic organisms. A combination of isotopically labelled NMs, new methodologies models, using mesocosms, could start to address these areas in an environmentally realistic manner.

Target organs for nanomaterials

The internal target organs for NMs in aquatic species still remain poorly understood for most organisms. This is especially true of small invertebrates where dissection for quantifying organs is impractical. In addition, the form of the materials in the internal organs (i.e., dissolved or particulate) has also not been established in the studies so far due to the technical challenges of measuring particles inside tissues. However, the microscopic imaging of invertebrates has proven useful. For example, Zhu et al. (2009) showed images of multi-walled carbon nanotubes in the gut tract of *D. magna* after a 24-h exposure. Using transmission electron microscopy, Heinlaan et al. (2011) demonstrated the presence of CuO NPs in the midgut of *D. magna*. Gomes et al. (2011) proposed a so-called 'Trojan horse' delivery and release of ionic copper following cellular internalization of CuO NPs by the marine mussel *Mytilius galloprovincialis*; similar to the suggestions for the uptake of nano Ag and nano CuO by human cell lines (Cronholm et al. 2013). While microscopy approaches can demonstrate this, the reliable identification of internalised NMs in epithelial cells from invertebrates has proven difficult. Hull et al. (2011) successfully used micro x-ray fluorescence spectrometry to show Au NMs in the gut epithelium of the freshwater Asian clam, *Corbicula fluminea.* Garcia-Alonso et al. (2011) observed electron-dense particles resembling Ag NPs in the gut epithelia of estuarine polychaetes exposed to sediment spiked with citrate-capped Ag NPs and the presence of silver in these particles was confirmed by energy dispersive X-ray analysis. SC-ICP-MS may prove useful here in future.

The main effort on internal target organs has been with fishes which are large enough to dissect. The target organs and effects on individual body systems of fishes have been reviewed (Handy et al. 2011). In general, the target organs for NMs are similar to those for their nearest equivalent traditional chemical, with possible exception of the spleen which appears to have an important role in processing particulate materials to prevent toxicity to the internal organs (TiO_2 NPs:Ramsden et al. 2009; Boyle et al. 2013; CuO NPs: Al-Bairuty et al. 2014). Concerns that NMs may be acutely immunogenic or neurotoxic in fishes have been proved largely unfounded from a functional perspective, although, changes in the immune cells and/or white pulp of the spleen may be observed with NMs (e.g., Al-Bairuty et al. 2013). These latter effects are mostly well within the physiological scope of the animal. So far, the potential hazards to the immune systems of fishes have been theoretically described (Handy et al. 2011; Jovanović and Palić 2012), but data (for example) on *in vivo* microbial challenges to the immune system during, or following NM exposure, are lacking. Currently there is no strong or consistent evidence of acute hypersensitivity reactions or immunosuppression in fishes exposed to NMs. Alternations in the brain biochemistry (Ramsden et al. 2009) and brain pathology (Al-Bairuty et al. 2013) have been observed in trout, but it is hard to link this to changes in animal behavior (Boyle et al. 2014). However, there have been some surprises. Nanomaterials may block the olfactory canals of fish such that they cannot chemically taste alarm substances in the water (Sovová et al. 2014). Manufactured NMs also interfere with the sensory lateral line functions of fish (McNeil et al. 2014). Such effects on their own are of limited direct toxicological consequence for the individual animal, but the ecological significance is far reaching. For example, loss of sensory perception might alter foraging behaviors, susceptibility to predation, the avoidance of contaminated water, or even fish migrations.

NANOMATERIAL HAZARD AND ASSESSMENT IN AQUATIC ECOSYSTEMS

In the last decade, there have been numerous reviews on the ecotoxicity of NMs (Handy et al. 2008a, 2012a,b; Klaine et al. 2008; Kahru and Dubourguier 2010; Fabrega et al. 2011; Shaw and Handy 2011; Levard et al. 2012; Schultz et al. 2014; Adam et al. 2015; Bour et al.

2016a,b; Garner et al. 2015; Semenzin et al. 2015; Hjorth et al. 2017a). A number of systematic reviews have discussed ecotoxicity databases and the quality and regulatory adequacy of data therein (Jugenson et al. 2015; Hartmann et al. 2017; Hjorth et al. 2017b). Unfortunately, most of the data obtained over the past decade have been for acute effects, with algal bioassays providing the majority of chronic endpoints. The acute assays have been reported largely for freshwater organisms used in OECD tests, often with limited dosimetry. Notably, even the OECD sponsorship program to systematically test a variety of NMs (http://www.oecd.org/chemicalsafety/nanosafety/testing-programme-manufactured-nanomaterials.htm) has been criticised for a lack of endpoints, variable quality assurance on individual test methods, and substantial data gaps in toxicity such that firm conclusions could not be obtained (Hansen et al. 2017). This section does not intend to reiterate the findings of the acute toxicity data for NMs that have been summarized in the reviews above, but instead to evaluate progress on some of the data gaps that are especially important to hazard assessment.

Progress on chronic studies and identifying no effect concentrations

Robust predicted no-effect concentrations (PNEC) or water quality guideline values for NMs in aquatic ecosystems are still not available to any great extent. PNECs ideally require quality-assured *chronic* toxicity data with ecologically relevant endpoints based on lethality, immobilization, growth, development and reproduction, expressed, for instance, as no observed effect concentrations (NOECs). The lack of chronic toxicity data is problematic and a key research gap.

Chronic effects of NMs on reproduction have been studied on invertebrates, but the data so far are mostly the OECD-style chronic tests in freshwater using model organisms such as algae or cladocerans. These limited studies do show effects on growth and reproductive success

(TiO_2 and ZnO NPs: Wiench et al. 2009; Ag NPs: Zhao and Wang 2011), with chronic reproductive endpoints more sensitive than acute mortality. Developmental defects are reported for planktonic stages of sea squirts, marine mussels and sea urchins with iron particles (Kadar et al. 2013). Fullerenes are also known to interfere with the development of oysters, *Crassostrea virginica* (Ringwood et al. 2013).

Chronic effects on reproduction and offspring development have been studied to some extent in fish or amphibian models. The literature on amphibians has mainly focussed on developmental toxicity to the growing tadpoles and their subsequent morphogenesis into the body form of adult frogs. Similar to fish embryos (Shaw et al. 2016), exposure of frog embryos to NMs prior to hatching has limited effects in terms of acute mortality, but at very high concentrations there can be some subsequent developments defects (e.g., exposures to 1000 mg/L for TiO_2, Fe_2O_3, CuO, and ZnO NPs: Nations et al. 2011). Clearly, in unhatched embryos of both fishes and amphibians, the chorion and the mucilage of the perivitelline matrix is a formidable barrier to exposure. Animals therefore become more vulnerable when they have hatched. Early studies on tadpoles of *Xenopus laevis* found that double-walled CNTs compacted the gills and gut to impair growth (Mouchet et al. 2008). There are also concerns that co-exposure of frogs to UV light and TiO_2 NPs may enhance ecotoxicity (Zhang, Wages et al. 2012). However, the science is still at the observational and hypothesis-formulation stage for amphibians. There are only a few mechanistic studies, and to our knowledge only one of these is near environmentally relevant concentrations. Carew et al. (2015) used low µg/L concentrations of Ag NMs to investigate the chronic effects on amphibian development. Delayed and abnormal development occurred. Critically, they identified interference with the thyroid hormone signalling that controls development as a key mechanism. More studies of this kind are needed on vertebrate and invertebrate animals. The usual assumption in ecotoxicology is that hazardous chemicals delay or adversely alter developmental anatomy. However, this does not have to be the case. One recent report showed that chronic exposure to Au NPs in the wood frog (*Lithobates sylvaticus*) can accelerate amphibian development rather than slow it (Fong et al. 2016).

Mesocosm studies

As with traditional chemicals, acute laboratory tests offer little environmental realism and mesocosms provide potentially useful platforms for generating more realistic data. This realism is likely more important for NMs, given the system complexity and can be more easily generated by non-traditional testing methods (Hjorth et al. 2017), although replication and isolating individual variables is challenging. In 2008, there were almost no data on mesocosms, but now some progress has been made. Mesocosm studies have been performed in freshwater and terrestrial systems (Lowry et al. 2012; Colman et al. 2013). Interestingly, Bone et al. (2015) compared a freshwater mesocosm exposed to a range of Ag NPs with exposure in equivalent laboratory conditions in the same study. They found that the laboratory studies did not replicate the findings in the mesocosms, and highlighted the complexity of organic carbon processing and UV light penetration as a potential explanation for the differences. In addition, mesocosm results (Colman et al. 2014) suggest that all forms of silver (dissolved and different NM types) ultimately have similar effects, while experimental laboratory data suggest that there is a nano effect (Leclerc and Wilkinson, 2014), and that Ag NP data are scattered (S.N. Luoma, UC Davis, Davis, CA, USA, unpublished manuscript), dependent on nature of the NM, organism and media. However, mesocosm dosing also affected outcomes with lower concentrations over time resulting in more suspended NMs compared with single high doses (Baker et al. 2015), in agreement with concentration-dependent laboratory studies (Merrifield et al. 2017a). The agreement between mesocosm and laboratory data is encouraging where it happens, but further work is needed to rationalize data where they do not.

Freshwater mesocosm studies have shown that additions of Au NMs will cause total gold concentrations to increase in the sediments and/or associated surface biofilms (Ferry et al. 2009). These studies showed that elevated total metal concentrations may also be found in the primary producers and aquatic invertebrates (Lowry et al. 2012), but whether or not this represents a hazard by food-chain transfer or simply exposure due to particle settling/direct exposure is not clear. In other work, Bour et al. (2016a,b) serially dosed a freshwater mesocosm with CeO_2 NPs over four weeks and detected total cerium in the biofilms of primary producers (bacteria, fungi, etc.), the grazing chironomid larvae, and *Pleurodeles* larvae (amphibian tadpole) as the top predator in the experiment. However, the bioaccumulation pattern for cerium did not explain the observed toxicity in the mesocosm, and parallel experiments excluded trophic transfer/oral toxicity. Bour et al. (2016a,b) concluded that a more complex set of processes was occurring in the mesososm, perhaps with indirect toxicity due to alterations in the microbial community in the test system.

Steps toward assessment of environmental risk

Toxicity data, used in a species sensitivity distribution (SSD), allow the derivation of a PNEC as the concentration that is hazardous to a given percentage of species, typically 95% (HC5). Ideally, chronic EC/IC10 data or NOECs are required from at least 8 species representative of at least 4 taxonomic groups, but where many of the data are only acute LC/EC50 values, these are typically converted to chronic NOECs using a default acute-to-chronic ratio of 10 (Warne et al. 2015), although 100-1000 may be used for NMs, given the uncertainty. A review of published toxicity data for NMs found that few datasets met these criteria (Batley et al. 2013). Deficiencies included reporting only acute toxicity data (LC50 values), reporting EC50 rather than EC10 values, and reporting nominal rather than measured concentrations, along with a lack of information on dosimetry, aspects of which have been discussed. Often, the tested NMs differed in their physico-chemical properties and transformations are not monitored, making comparisons between studies difficult. There is a pressing need for multi-species toxicity data for each of the common forms of each NM, especially for the most toxic form of each so that we can at least derive a conservative HC5 value for use in risk assessments.

Of the published PNEC value derivations (Adam et al. 2015; Garner et al. 2015; Semenzin et al. 2015), those of Garner et al. appear the most reliable and the mean values calculated from the the plots in that paper are summarized in Table 1, but even these rely heavily on converted acute data. For nano TiO_2, Semenzin et al. (2015) derived an HC5 of 0.02 mg/L much lower than the value in Table 1, but their value is very conservative as conversion of sensitive chronic algal IC50 data to NOECs used a factor of 100 (not 10), while acute LC/EC50 data to chronic NOECs used the same factor. For nano-CuO, Adam et al. (2015) derived only an acute HC5 of 0.15 mg Cu/L (0.19 mg CuO/L) above the 0.04 mg/L chronic HC5 value. Dissolution is a confounding factor with CuO and ZnO NPs and to a lesser extent Ag NPs, but not for CeO_2, TiO_2 and the carbon-based NMs. For uncoated Ag NMs, Batley (unpublished results) derived a value of 0.1 µg/L, which is lower than the Table 1 value. Much of this variability might be explained by different NM types or other parameters. Because of the large variability in observed toxicity as a function of particular coatings, and as a function of NM specific surface area, particle-specific guideline values (GVs) or PNECs are necessary. Van Hoecke et al. (2009), for example, found that the chronic toxicity of nano-CeO_2 to reproduction of the cladoceran, *D. magna* decreased by a factor of 2 as the nominal particle size increased from 12-29 nm (hydrodynamic diameter from 479-552 nm) as shown in Figure 7.

The simplest screening-level hazard assessment, requires that the PEC/PNEC ratio should not exceed 1. In the above cases, the modelled and measured environmental concentrations (PECs) in waters (Figure 2) were generally well below the derived PNEC values in Table 1, indicating a low risk to aquatic ecosystem health. It is anticipated that the same would be true for sediments. Nevertheless, given the uncertainties around issues such as dosimetry and chronic effects, and on-going developments in NM sophistication and increased production levels, there is concern that this risk is severely underestimated and/or may increase in the near future.

A higher level hazard assessment of chemicals would normally consider the persistence of the substance in the environment and its potential for bioaccumulation and toxicity (i.e., PBT criteria). These issues also apply to NMs, but there are some additional considerations. First, the myriad of shapes, sizes and chemical composition of NMs precludes the evaluation of every single NM as a new substance in the testing strategy (Handy et al. 2012b). A more pragmatic approach is to group materials for hazard assessment and/or to estimate the hazard relative to existing substances of similar chemical composition (S.N. Luoma, UC Davis, Davis, CA, USA, unpublished manuscript). Thus, here we discuss whether or not patterns are emerging in the biological data that might allow this grouping and read-across to traditional chemicals. In terms of understanding the role of NM properties on a more fundamental level, clearly the production of tightly constrained NMs, whose properties can be varied is essential (Baalousha and Lead 2013). This has not currently been performed to any great extent, but is feasible. For example, the synthesis of NPs using enriched stable metal isotopes allows for traceability in bioaccumulation experiments at low concentrations (Dybowska et al. 2011; Larner et al. 2012;

Croteau et al. 2014b), whereas core-shell NPs provide a new tracing tool to characterize the mechanisms controlling bioavailability (Merrifield and Lead 2016). Secondly, a decade ago, there were many data gaps on hazard such that uncertainty was high, and thus systematic reconsideration of the data gaps is now warranted.

One difficulty with grouping hazard by chemical substance, is how to classify composite materials, including NMs that have a coating that is different to the core or core-shell NMs. Individual studies on aquatic species have shown coating-dependent toxicity and clearly these may be considered as different materials, with the coating affecting many of the physico-chemical and biological properties, as already discussed. Coatings can also influence the dissolution of toxic metal ions from NMs (Zhao et al. 2012) and the exposure route (Croteau et al. 2011b). However, differentiating the indirect effects of NM behavior on toxicity from the inherent toxicity of the coating itself is challenging, and there are as yet no clear trends with respect to toxicity of surface coatings across a range of different organisms or NMs. In deciding how to treat these new materials, a key question relates to their persistence: are nanohybrids conserved over reasonable environmental timescales or do they dissociate into their component parts rapidly?

A final point is that, in the effort to establish the facts with laboratory testing, the overarching ecological principles of protecting most of the organisms most of the time, as well as preserving biodiversity and ecosystem function may have been overlooked. The essence of environmental protection is to ensure the survival of the maximum number of species at the population level such that biodiversity and ecosystem functions remain intact. In freshwater systems, there are insufficient data to predict the prospects of long-term survival of most organisms. The situation is more uncertain for other compartments. The effects of NMs on processes in river sediments and the biota at the base of aquatic food webs remain a barrier to understanding both the fate of NMs in ecosystems, and the functionality of biogeochemical processes such as the nitrogen cycle.

Regardless of the type of ecosystem or model organism, the ecotoxicity of organic NMs (other than CNTs) also remains poorly explored, as does the next generation NMs. Progress on using quantitative structure-activity relationships (QSARs) for estimating uptake potential, or ranking materials by hazard has been slow, mainly due to the lack of measurement methods to validate the QSAR models (Puzyn et al. 2011; Mu et al. 2016) with experimental data.

Hazard of nano- versus micron-sized materials in the aquatic environment

There were concerns in 2008 about novel materials generating previously unknown mechanisms of toxicity. It is now clear that there are nano-specific aspects to the bioavailability and toxicity of NMs. *In vivo*, significantly different bio-uptake rates (Croteau et al. 2011b, 2014a; Khan et al. 2012, 2013a,b; Thit et al. 2015) and molecular behaviors (Taylor et al. 2016) have been observed when the same element is in the nanoscale phase, compared to the dissolved or larger particle phase. The composition of an inorganic NM appears to be an important property determining bioavailability and toxicity (S.N. Luoma, UC Davis, Davis, CA, USA, unpublished manuscript), possibly suggesting the importance of NM-related impacts on speciation/transformations and delivery to an organism. For example, SiO_2 and TiO_2 NMs are, in general, less toxic (Katsumiti et al. 2015; Lacave et al. 2016) than CuO, CdS and CdSe NMs (Khan et al. 2013b; Katsumiti et al. 2014). Other properties, including of the transformation products, are likely to be important in determining nano-specific effects (Romer et al. 2013).

This potential for nano-specific effects that would necessitate them being regulated differently with respect to their potential hazards to environmental health, is a major concern for regulatory agencies; as discussed there is clear evidence that there can be differences in the bioavailability and toxicity of NMs imparted by their smaller size. Concerns have been raised, for instance, about enhanced toxicity of traditional chemicals in the presence of NMs, due to the so-called Trojan horse effect (e.g., Luoma 2008; Ferreira et al. 2014).

In general, core composition drives relative hazard (S.N. Luoma, UC Davis, Davis, CA, USA, unpublished manuscript). The *acute* toxicity to aquatic species has been reported in the µg/L to mg/L range for a wide variety of NMs. At the upper end of this range, NMs might be considered as a low acute hazard compared to chemicals in the non-nano size range. For example, certain metals such as cadmium or mercury, and organics such as endosulfan have higher acute toxicities in general. However, there are substantial limits on understanding transformations and dosimetry, as discussed earlier, which make the dose-response relationship much more complex than for these chemicals (Merrifield et al. 2017a).

Nonetheless, some common features are emerging. In many cases, for metal-containing NMs, the metal salt is more toxic than its equivalent nano form (e.g., CuO NPs ; Shaw et al. 2012) unless the particle dissolves and therefore shows similar toxicity to the free metal ion (Zn NPs: Brun et al. 2014). However, there are a number of exceptions to this general rule. In some cases, toxicity can be attributed entirely to dissolution of the free metal ion (Zn NPs: Franklin et al. 2007). Silver toxicity is often thought to be due the silver ion, although the data here are more equivocal and a wide range of toxicities exist dependent on test and NM properties. The most definitive demonstrations of the effect of nano-size (compared to micron-sized particles) are where the confounding effects of solubility are minimized, as is the case with CeO_2 NPs (Batley et al. 2013). Here, distinctly greater toxicity of the nano form was evident. This is best illustrated in Figure 8 by the chronic toxicity of ZnO, CeO_2 and Ag NMs to the sensitive microalga *Pseudokirchnierella subcapitata* in a synthetic soft water. Toxicity data for nano-TiO_2 and nano-CuO using the same alga have also been published (Aruoja et al. 2008). The IC50 values for nano and bulk TiO_2 were respectively 5.8 and 35.9 mg TiO_2/L. Both forms aggregate, but the greater toxicity of the nano form was largely due to coverage of the algal cells by nano-aggregates whereas uncovered cells were present with bulk TiO_2. Due to their high specific surface area, NMs can also adsorb nutrients, limiting growth. The respective IC50 values for ionic Cu^{2+} and nano and bulk CuO were 0.02, 0.71 and 11.6 mg/L (Aruoja et al. 2008). Toxicity was largely explained by the greater concentration of soluble copper released from the nano form. These findings make a strong case for regulating metal NMs differently to the equivalent micron-sized particles, except in the extreme cases where solubility nullifies the differences.

Although micron-sized materials are generally less toxic than their equivalent NM or the aggregated form (Romer et al. 2013), this is not always the case and pelagic and benthic organisms may be subject to different concentrations and forms of the NMs. Furthermore, the model system used to test toxicity can influence conclusions about relative toxicity. For example, *in vivo* models such as aquatic invertebrates appear less effective at differentiating toxicity than *in vitro* models such as human cell lines (S.N. Luoma, UC Davis, Davis, CA, USA, unpublished manuscript). Nanoscience has also created the opportunity to study some traditional chemicals in their bulk powder form, some of which have long been regarded as poorly soluble or of low/negligible toxicity to aquatic life. These studies have shown toxic effects from materials previously thought to be of a negligible hazard. For example, bulk forms of TiO_2 reduce embryo viability in the unexposed F1 generation offspring from exposed adult zebrafish (Ramsden et al. 2013). Moreover, the effect was similar to the nano form used in the experiment.

This discussion also raises a more general question related to controls. For instance, does the ‘free’ coating behave differently to the surface-bound coating? Are dissolved ion controls such as silver, suitable, since it is known that they precipitate and form new, smaller and more bioavailable NMs (Merrifield et al. 2017a). The role of controls in nanotoxicology requires detailed, technical study and thought and is certainly not trivial.

BIOACCUMULATION AND TOXICITY OF NANOMATERIALS TO SOIL ORGANISMS

Bioaccumulation and toxicity of metal and metal oxide nanomaterials in soils

Studies examining the toxicity and bioavailability of NMs to terrestrial organisms in soil-based media have increased in number over the past 8-10 years. However, studies of this nature remain uncommon, compared to studies examining aquatic ecotoxicology of NMs or studies examining the toxicity of NMs to terrestrial organisms in artificial exposure settings such as hydroponics. Early work in this area focused on as-manufactured NMs and includes reports of bioaccumulation of CuO and Ag NMs by earthworms (Unrine et al. 2010; Shoults-Wilson et al. 2011) and low bioavailability of CeO_2 NMs to corn (Birbaium et al. 2010), Fe_3O_4 NMs to pumpkin (Zhu et al. 2008) and TiO_2 and ZnO NMs to wheat (Du et al. 2011). More recently, studies have reported toxicity of FeO NMs to clover (Feng et al. 2013), alteration of nutrient localization in corn exposed to CeO_2 NMs (Zhao et al. 2015) and accumulation of CeO_2 NMs in soybean root nodules (Priester et al. 2012).

The strong interaction of most NMs with soils in terms of heteroaggregation with soil minerals and DOM raises doubts as to the environmental relevance of ecotoxicological thresholds developed using pristine NMs in non-soil media (Unrine et al. 2012a; Holden et al. 2016). Similarly effects of surface coatings, size and shape of pristine NMs on toxicity to soil organisms determined in non-soil media will also have little environmental relevance due to the above reactions. More longer-term studies are needed to produce chronic toxicity data, as reactions of NMs in soils may take time to fully express (Diez-Ortiz et al. 2015).

Although early work with as-manufactured and relatively exotic NMs has provided useful information regarding their hazard, recent soil-based nanotoxicology research has concentrated on the NMs most likely to enter the terrestrial environment in large quantities and on the toxicity of NM transformation end-products resulting from the chemical conditions associated with realistic discharge pathways. Consequently, this work has increasingly focused on TiO_2 and ZnO NMs, as a result of their relatively high concentrations expected in sludge (540 and 110 mg/kg 85th percentile concentrations in Europe for TiO_2 and ZnO, respectively (Sun 2014)), and Ag NMs as a result of demonstrated biocidal properties and widespread use (Sondi 2004). Based on current NM usage patterns, TiO_2, ZnO and Ag NMs and their transformation end-products are expected to be introduced into terrestrial ecosystems in substantial amounts as a result of land application of biosolids. Recognizing this, exposures examining the toxicity of these NMs are increasingly taking place in biosolid-amended soil.

Sophisticated nanotoxicological studies are being undertaken in greater number, but nevertheless the potential risk posed to terrestrial ecosystems by metal and metal oxide NMs remains unclear for similar reasons to aquatic systems (limited dosimetry, transformation dynamics, and lack of chronic studies). Recent studies demonstrating transgenerational sensitivity in nematodes exposed to Ag and Ag_2S NMs (Schultz et al. 2016) and toxic effects in tomato progeny that were not observed in a parent generation exposed to CeO_2 NMs (Wang, Ebbs et al. 2013), highlight the potential importance of this type of research.

Judy et al. (2015b) reported data from a study examining the effects of amending soil with biosolids containing either bulk/dissolved TiO_2, Ag, and ZnO, nanoparticulate TiO_2, Ag, and ZnO, or containing no added metals (Chen et al. 2015; Judy et al. 2015b). These biosolids were generated using pilot WWTP facilities and subjected the metal input into the waste streams to chemical conditions similar to what they would experience in an actual WWTP facility. In this work, the NM treatment reduced root nodulation in the model legume *Medicago truncatula* relative to the bulk/dissolved control, a finding that was linked to significantly higher zinc bioaccumulation. This work also reported distinct shifts in the microbial communities in soil treated with NMs compared to the bulk/dissolved treatment. However, another study using the same media in a simpler system without plants reported that, while the microbial community in the bulk/dissolved treatment was significantly different from that in the NM treatment, this shift did not result in a significant loss of ecosystem function (Duremkamp 2016).

As with aquatic systems, a wide variety of Ag NMs will largely transform into insoluble Ag_2S NMs in a range of WWTP configurations (Lombi et al. 2013; Ma et al. 2013; Pradas del Real et al. 2016; Wang et al. 2016), although Ag-cysteine and AgCl are also possible transformation end products. Considering that non-labile transformation end products such as AgCl and Ag_2S are expected to be stable in the environment and to have relatively low bioavailability (Lombi et al. 2013; Donner et al. 2015; Doolette et al. 2015, 2016), the risk to terrestrial ecosystems posed by Ag NMs would appear to be low, although uncertainties related to longer term transformations, dosimetry, etc., are also relevant. This conclusion has been further supported by soil-based studies demonstrating that Ag_2S NMs presented minimal hazard to plant-microorganism symbioses (Judy et al. 2015a, 2016), crop plants (Doolette et al. 2015;

Wanget al. 2016), soil microorganisms (Judy et al. 2015b; Doolette et al. 2016a; Moore et al. 2016) and soil invertebrates (Starnes et al. 2015, 2016).

Du et al. (2015) recently reported the effects of CeO_2 NMs (doses of 100 and 400 mg/kg) on wheat grown in the field for 7 months. There were no effects on wheat biomass, grain yield, or bioaccumulation of cerium, but morphological and biochemical effects were observed, with reductions in chlorophyll, delayed flowering and increased catalase and superoxide dismutase activities. More of these types of study are needed, where interactions and transformations of NMs with soil components are captured and integrated over longer exposure periods.

Bioaccumulation and toxicity of carbon nanomaterials in soils

Carbon-based NMs such as fullerenes and CNTs are also commonly used in consumer products. However, the masses of carbon NMs entering WWTPs are relatively low and, unlike silver, evidence suggests that carbon NMs are only likely to be toxic at relatively high concentrations (Nyberg et al. 2008; Garner et al., 2015). Although some studies have reported toxicity at high concentrations in hydroponics (Canas at al. 2009; Hawthorne et al. 2012) and *in vitro* (Jia et al. 2005), the small amount of ecotoxicological work that has taken place in soil-based media has reported little toxicity to soil organisms (Nyberg et al. 2008; Ge et al. 2016). This research includes work reporting low bioavailability of CNTs to earthworms in both artificial and natural soils (Petersen 2008a,b, 2011), and research indicating that fullerenes have no impact of soil microbial communities (Tong 2007; Nyberg et al. 2008). Newer carbon-based NMs, e.g., graphene, have to date received little study but appear also to have relatively low toxicity (Ge et al. 2016).

Toxicity of nanomaterials to other terrestrial biota

There appear to be no published peer-reviewed studies on the ecotoxicity of NMs to reptiles or birds, and this is a concern given that developmental toxicity and disruption of endocrine processes have been identified in amphibians (Mouchet et al. 2008; Nations et al. 2011). The concerns for mammalian wildlife remain. There are no studies on bioaccumulation hazard to small mammals or similar wildlife, and no studies reporting toxicity in an ecological context. Skalska and Strużyńska (2015) reported neurotoxicity to mammals, but the findings were based on rat brain slice, *in vivo* injection, or cell culture studies at high (milligram) doses that have no ecophysiological relevance.

An *in vivo* injection study (Hanini et al. 2016) reported abnormal body temperature and decreasing heart rate in rabbits exposed to $Zn_{0.8}Co_{0.2}Fe_2O_4$ NMs with organ pathology, but there were no metal salt controls or characterization of metal dissolution – the observations might simply be explained by metal toxicity rather than any particle effect. Nonetheless, studies on small mammals that are not laboratory animals remain a critical data gap for environmental hazard assessment purposes.

Trophic transfer of nanomaterials in terrestrial biota

Early work reported that Au NMs could biomagnify in a simulated tobacco (*Nicotiana tabacum*)-tobacco hornworm (*Manduca sexta*) caterpillar food chain (Judy 2011), raising concerns regarding the potential for humans to be exposed to NMs via trophic transfer. This work was followed by other studies that reported trophic transfer of NMs in other simulated food chains including earthworm (*Eisenia fetida*) to bullfrog (*Rana catesbeina)* (Unrine 2012b), zucchini (*Cucurbita pepo*) to cricket (*Acheta domesticus*) (Hawthorne 2014) and lettuce (*Lactuca sativa*) to cricket to darkling beetles (*Tenebrionoidea*) (De la Torre-Roche 2015). While each of these three studies reported trophic transfer, transferred amounts of NMs were small and the biomagnification reported in the tomato-hornworm study was not observed. However, recently biomagnification of NMs was again reported in a terrestrial food chain, with CeO_2 NM accumulated in kidney bean (*Phaseolus vulgaris*) plants biomagnifying in Mexican bean beetles (*Epilachna varivestis*) as well as in consumers of the beetles, spined soldier bugs (*Podisus maculiventris*) (Majumdar 2016). Taken together, these studies indicate that trophic transfer of NMs is likely to occur at least in some small amount in most terrestrial food chains, with certain terrestrial organisms and food chains being particularly susceptible to biomagnification, though the reasons that some organisms are more likely to biomagnify NMs remain unclear.

CELLULAR MECHANISMS OF TOXICITY

Klaine et al. (2008) identified concerns about the mechanisms of toxicity of NMs, which initially centred around oxidative damage to cellular components, such as proteins and nucleic acids, as well as the theoretical hazard of an interruption of energy (Nel et al. 2006, 2009). However, while many studies have incorporated measures of redox cellular responses, interpretation of their role with respect to nanotoxicity remains difficult. On the one hand, redox responses are a part of a wide array of evolutionarily well-embedded cellular defence mechanisms aiming to overcome perturbations and to promote organism survival and health (Jennings 2013). For example, Minghetti and Schirmer (2016) noted that fish gill cells in culture partly recovered over time from a negative impact on metabolic activity and lysosomal membrane integrity brought about by silver NMs, with a concomitant induction of glutathione reductase mRNA. On the other hand, whether such cellular stress responses are a primary reaction of the cells to the NM, or are a secondary response because of other damage by NMs to the cells, remains mostly undecided. It has therefore become clear that it is important to take a more integrated view of cell barriers and responses of cells in order to shed light on the mechanisms of NMs on cells, the functional units of life (Schirmer 2014).

Cellular barriers

Epithelia and endothelia in animals. The integrity of cellular epithelial or endothelial barriers, such as the epithelia of lungs, gills and the intestine, or the endothelia of blood vessels, is crucial for the gate-keeper function of these barriers. Epithelia act as selective environment-organism barriers to air, water or food; they regulate the interaction of an organism with its surrounding environment. Endothelia, on the other hand, serve as selective internal permeability barriers. Finding NMs in different tissues of animals upon exposure (see previous section) demonstrates the potential of NMs to overcome such barriers. Moreover, it is well known that NMs can disrupt epithelia, such as of the intestine and gill (SWCNT: Smith et al. 2007). Understanding the mechanisms underlying the passage or even damage to cellular barriers, however, is difficult to study in vivo, thus, cell culture-based two-compartment systems have been developed to study particle transport and toxicity.

In such a system, cells are cultured on a porous membrane, which separates an upper (apical) and a lower (basolateral) compartment, mimicking the two sides of the barrier *in vivo* (Schirmer 2014). Transport of particles can be followed by measuring their translocation across the cell layer on the porous membrane. Toxicity to the cells is assessed either as a loss in the electrical resistance of the cell layer or as molecular/cellular changes impacting the normal functioning of cells (see below). Studies applying such systems have revealed that NMs are able to use cellular energy-dependent transport mechanisms through the cells. Size, shape and charge have been characterized to be determinants of this, although a clear relationship between particle characteristics and transport behavior in different epithelial models cannot yet be deduced (e.g., Yacobi et al. 2008; Mahler et al. 2012). However, certain behaviors have been determined. Mahler et al. (2012) showed that 50 nm negatively-charged polystyrene particles were passively transported via paracellular routes, while otherwise similar 200 nm particles followed a transcellular energy depend route.

In order to accomplish NM transport studies in cells from non-mammalian species, a recently established fish epithelial barrier system (Minghetti et al. 2017), based on a rainbow trout (*Oncorhynchus mykiss*) intestinal cell line has been used. The RTgutGC cell line (Kawano et al. 2011) was adopted to allow study of particle translocation and toxicity in the intestine of fish (Geppert et al. 2016). The RTgutGC intestinal model forms a leaky epithelium, which is in accordance with the fish intestinal epithelium in vivo (Geppert et al. 2016; Minghetti et al. 2017). It nevertheless formed an effective barrier for polystyrene NPs (50 nm nominal size): after 24 hours of exposure, about 80% of the particles remained in the apical compartment, 9-16% were associated with the cells, leaving less than 10% permeation to the basolateral side. Permeation was temperature- and time-dependent, indicating that active transport mechanisms, such as endocytosis, might be at play (Geppert et al. 2016). This model system thus offers a wealth of opportunities to study NM-affected barrier function in fish. The role of transformations before, during and after transfer needs to be addressed in parallel with such a system.

Cell-based investigations allow mechanisms to be explored under well-defined conditions and in a variety of processes, such as kinetics of transport in difficult to observe or to obtain tissue, are the only ways to derive experimental validation of hypotheses. However, such studies are limited as certain interactions and processes in and between tissues are difficult to replicate *in vitro*. One example with regard to an environment-organism barrier is described by Millaku et al. (2013). They studied the interaction of tungsten nanofibers *in vivo* in the model invertebrate terrestrial isopod, *Porcellio scaber*. Hepatopancreatic cells of this organism were directly exposed to substances in partly digested food and filtered and transported from the stomach into the lumen of the hepatopancreas. In this process, the digestive gland epithelium is subject to physical forces that ensure peristaltic movement. They therefore proposed that muscle contraction during peristalsis may contribute to fibre insertion into cells and impact on the structure of the lining of the digestive tract. Similarly, Bacchetta et al. (2014) observed ZnO NMs in frog embryo enterocytes. NMs were attached to the apical cell membrane or internalized into microvilli. NM-enterocyte interaction led to the induction of oxidative stress and altered structural and functional integrity of the junctions between the cells.

Cell wall as barrier in microorganisms and plants. Even though the cell wall can be envisioned to be an efficient barrier to combat penetration of NMs into cell-wall bearing cells, mechanistic studies to understand the extent and functional properties to convey a protective function toward particle uptake are still rare. Navarro et al. (2008) postulated a sieve function of the cell wall considering that pores across the cell wall are 5-20 nm in size. With improved methodologies, an increasing number of studies show unequivocal uptake of NMs into plants. CeO_2 NPs were discovered in soybean roots and root nodules upon growth in soil spiked with CeO_2 NMs (Priester et al. 2012). In addition, Zhao et al. (2012) found transfer of cerium oxide from soil into roots and shoots of corn plants with higher transfer into shoots in a carbon-poor compared to a carbon-rich soil. The presence of CeO_2 NPs around vascular vessels suggested that the particles found their way to the transport system and moved through the xylem driven by transpiration. In their study with CNTs, Miralles et al. (2012) found adsorption of the tubes to alfalfa and wheat without significant uptake or translocation. More systematic studies are required to resolve the extent to which plant cell walls act as a barrier and for which NMs.

Focussing on the interaction of Ag NPs with unicellular algae, Piccapietra et al. (2012) and Li et al. (2015) confirmed strong interaction of particles with the cell surface of *Chlamydomonas reinhardtii* and *Euglena gracilis*, respectively, but with negligible uptake. The comparatively thick cell wall of algae appears to be a significant barrier to NM uptake.

These findings are in stark contrast to the efficient uptake of the Ag NPs by fish cells (Yu et al. 2017). Thus, NMs may elicit toxicity to cell wall-containing organisms without having to actually enter the cells. One demonstration of this was provided by Bondarenko et al. (2013) for bacteria and Ag NMs. Using six bacterial strains and carefully accounting for extracellular and intracellular silver ion concentrations liberated from the Ag NMs, they showed that direct contact between the bacterial cells and the Ag NMs enhanced the toxicity of the Ag NMs. The mechanism of toxicity in the absence of internalization needs to be addressed. Clearly, more systematic investigations on the barrier function of the cell wall and the ability of plant, bacterial and algal membranes to endocytose or otherwise interact with NMs, are needed.

In a study of the mechanism of chronic toxicity of CeO_2 NMs to the microalga *Pseudokirchneriella subcapitata,* Angel et al. (2015) used hyperspectral imaging to demonstrate the coating of cells with NPs, potentially causing cell damage (Rogers et al., 2010). Coating was absent in the presence of dissolved organic carbon (humic acid), minimizing toxicity. Although CeO_2, like TiO_2 is a source of reactive oxygen species (ROS), the intensity of ROS production was found to be lower and not sufficient to cause oxidative damage (Angel et al. 2015). Previous toxicity studies have demonstrated the role of ROS in the toxicity of TiO_2 to a range of species including algae, cladocerans and fish (Ma et al. 2012; Miller et al. 2012).

Extracellular matrices. Many types of cells secrete molecules that form an extracellular matrix in direct vicinity (the ‘unstirred layer’ in Figure 6) to protect the cells and for specific interactions of cells with their immediate environment. Mucus-secreting cells in external epithelia of organisms provides a layer of mucus that can temporarily protect the exterior. Mucus is a heterogeneous aqueous mixture of glycoproteins, among a number of other molecules, and its composition is highly conserved across biological species (review, Handy and Maunder 2009). Similarly, bacteria, fungi and/or algae forming biofilms in freshwater or soil synthesize a matrix of extracellular polymeric substances. This matrix contains enzymes that hydrolyze dissolved high molecular weight compounds into small biomolecules and only these can subsequently be taken up by microorganisms. Thus, the extracellular matrix has important physiological roles, which need to be considered when elucidating mechanisms of toxicity of NMs.

In order to elucidate if NMs can stimulate mucus secretion, Chen et al. (2011) exposed human bronchial epithelial cells to TiO_2 NMs and indeed found a concentration-dependent enhancement of mucin secretion, i.e., of glycoproteins forming the mucus. Mucin hypersecretion was strongly dependent on the presence of intra- and extracellular calcium. While the authors interpreted their finding as a link to the hypersecretion observed in chronic airway diseases, this example also demonstrate that a NM generally thought to be rather benign can elicit molecular changes with potential long-term consequences. Whether similar mechanisms of altered mucus secretion can be observed in cells of other organisms, for example fish, would be an interesting route to pursue. Indeed, Smith et al. (2007) found increased gill mucus production on aqueous exposure of rainbow trout to single wall carbon nanotubes.

A somewhat increased extracellular matrix production was observed on exposure to functionalized CdSe/ZnS quantum dots of the marine diatom, *Thalassiosira pseudonana* (Zhang et al. 2013). Quantum dot agglomerates were associated with the extracellular matrix which was thought to limit the toxicity of the quantum dots to the diatoms. This is an illustration of the importance of the behaviour of NMs in the extracellular matrix in order to better account for NM bioavailability and resulting protection or toxicity.

Considering that extracellular enzymes of freshwater or soil biofilms provide essential nutrients to microorganisms and play an important role in nutrient cycling. Almost no knowledge about their susceptibility to NM exposure thus far exists. In an elaborate mesocosm study to explore ecosystem effects of Ag NM exposure, Colman et al. (2013) found reduced levels of leucine aminopeptidase (responsible for nitrogen cycling) and alkaline phosphatase (phosphorus cycling) enzyme activity, which was closely linked to reduced microbial biomass. Exploring intact heterotrophic freshwater biofilms on exposure to differently functionalized TiO_2 NPs in the presence or absence of environmentally realistic UV radiation, Schug et al. (2014) found reduced activity of ß-glucosidase (carbon cycling) and leucin aminopeptidase. Exploring other types of freshwater communities, Gil-Allue et al. (2015) and Tlili et al. (2016, 2017) demonstrated distinct impacts of Ag NMs on biochemical functions of the biofilms in periphyton and litter decomposers, respectively. For example, Tlili et al. (2016) found similar reductions in microbial respiration and utilisation of carbon sources in biofilms exposed to Ag NPs and silver nitrate.

Stress responses in cells

Once cell barriers are passed, distribution in the entire organism may ensue and several response mechanisms can be expected to occur. Several recent reviews focus on mechanisms of NM cytotoxicity, intracellular targets and signalling (e.g., Marano et al. 2011; Fröhlich 2013). However, much of this discussion has focussed on mammalian cells. Thus, there are large uncertainties specific non-mammalian structures or functions, such for algae, bacteria and fish (Behra et al. 2013).

Cell membrane and cytoskeleton. Biophysical responses are likely to occur on contact of NMs with cell membranes (Wu et al. 2013). These processes include the disturbance of the cell membrane's phospholipid bilayer based on the NM charge and the size-dependent formation of "holes", i.e., regions of reduced lipid or protein levels, which were shown to be associated with cytotoxicity. Moreover, there were earlier reports on the ability of carbon-based NMs with diameters of around 1 nm to physically block membrane ion channels (Park et al. 2003). Once internalized, NMs may impair the cytoskeleton. For example, Soenen et al. (2010) proposed that high levels of Fe_2O_3 NMs, localized around the cell nucleus, hinder maturation of actin fibres due to steric interactions, which may severely affect cell migration and differentiation. More generally speaking, NMs can physically interact with biomolecules so that both biomolecule structure/function and NM behaviour may change. Concepts and experimental research (Shemetov et al. 2012) about these types of biophysical interactions are most advanced for peptides and proteins. For example, as particles get smaller and approach the size of some proteins, the curvature of the NM increasingly influences how proteins physically bind to the NM and thus change their proper folding. Active centres of enzymes or receptor binding sites might thereby get hidden (i.e., phasing the NM) or protein structure may be distorted in other ways and thus normal functioning be impaired. These phenomena have thus far been studied almost exclusively in the context of NM-mediated drug delivery, imaging or biosensing using human or other mammalian cells and primarily focused on single protein-NM interactions. It is important to perform these types of investigations with proteins or other biomolecules of particular relevance to organisms living in the environment, and with respect to potential long-term consequences of exposure. It has been shown, e.g., by Linse et al. (2007), that NM-protein interactions can lead to so-called amyloid, i.e., aggregated and deposited, proteins. Yu et al. (2016) developed a methodology to identify proteins that bind to silver NMs in living fish gill cells. The obtained list of candidate proteins may guide such future work on specific NM-protein interactions.

Lysosomes. Another important consideration is the charge of the NM. Harush-Frenkel et al. (2008) demonstrated by means of a polylactic acid-based model NM that negatively-charged particles preferentially localize in the lysosomes while positively-charged particles escape this route and are more dominantly found in the cytosol. The vast majority of environmentally relevant NMs tested thus far are negatively-charged and a variety of them have indeed been found co-localized with lysosomes (e.g., Minghetti et al. 2016; Yue et al. 2016). Accumulation in lysosomes may render these organelles particularly susceptible due to locally increased exposure. Yue et al. (2015) and Minghetti et al. (2016) demonstrated that lysosomal membrane integrity in a fish gill and intestinal cell line was more susceptible to exposure to Ag NMs (but not to silver ions) than metabolic activity or cell membrane integrity. Based on such findings, autophagy and lysosomal dysfunction have been proposed as a major route of NM toxicity (Stern et al. 2012). Autophagy is an evolutionarily conserved stress response for maintaining cellular homeostasis. Using Au NMs, Ma et al. (2011) demonstrated that NMs may cause autophagosome accumulation by interrupting the autophagic flux, specifically by impairing fusion with lysosomes and lysosome degradation capacity. Similar to amyloidosis, defects in lysosome function may have serious long-term consequences for organism functioning (Stern et al. 2012). Moreover, in the short-term, the acidic pH of lysosomes may affect the properties of NM, for instance through dissolution or desorption of metals. Xia et al. (2008), for example, demonstrated dissolution of ZnO NMs in lysosomes.

Mitochondria. With their central role in ATP production and apoptosis, i.e., programmed cell death, mitochondria are susceptible to NM exposure and, indeed, damage to mitochondria has been found on exposure of cells to different particles (e.g., Fröhlich et al. 2013). However, in contrast to lysosomes, mitochondria do not seem to be specifically targeted by NMs. Rather, their susceptibility appears most closely related to their propensity to oxidative stress, which can be caused by interference by NMs, or constituents thereof, with the mitochondrial electron transport chain. With regard to exposure of organisms in the environment, it would be particularly interesting to study if NMs impact apoptosis as part of the normal development of many organ systems. Indeed, developmental malformations of the zebrafish embryo heart, which is the first organ to form and function in these embryos, were found on exposure to quantum dots (King-Heiden et al. 2009) and silica NMs (Duan et al. 2013). Whether these malformations were initiated by interference with normal apoptotic events was not specifically explored but appears to be particularly relevant with regard to potential delayed or long-term effects in organisms exposed to NMs during sensitive windows of development. Along these lines, Kim et al. (2013) determined that exposure of zebrafish embryos to Au NPs, which were surface functionalized with a cationic ligand, caused smaller, mal-pigmented eyes. This defect was explained by a significant increase in cell death in the eye, which in turn correlated with an increase of the apoptosis-signalling genes p53 and bax. Disruption of proper eye development progressed into altered swimming behaviour and reduced axon growth.

Nucleus. Translocation of NMs into the nucleus is, in principle, possible, although numerous reports, studying different non-dividing vertebrate cell lines and a variety of NMs in

≥50 nm range (Bastian et al. 2009; Kühnel et al. 2009; Busch et al. 2011; Yue et al. 2016), showed that NMs were detectable in the cytoplasm but not the nucleus. To allow bidirectional transport between the cytoplasm and the nucleus, the nuclear membrane contains specialized channels, called nuclear core complexes. As described by Paine et al. (1975) for the amphibian oocyte nuclear envelop, these allow ions and small molecules to diffuse through aqueous channels with a diameter of ~9 nm size. Accordingly, small particles, specifically quantum dots, have been shown to exploit the cells' active transport machinery and enter the nucleus via the nuclear pore complex (Nabiev et al. 2007). Molecules or particles larger than this, such as larger NM or viruses, need to be selectively transported into the nucleus by "tagging" them with a signal, i.e., an amino acid "tag", called nuclear localization sequences.

Panté and Kann (2002) demonstrated that Au NMs, coated with a nuclear protein and a receptor for its nuclear localization sequence, were able to move through the nuclear pore complex up to a total diameter of the particles (meaning including the protein coating) of ~39 nm. Their study stresses the size- and signal-dependent translocation of molecules or particles into the nucleus under conditions in which cells do not divide. During cell division, however, nuclear envelope breakdown occurs which allows mixing of cytoplasmic and nuclear compartments and transport of particles of ~100 nm (Lenart et al. 2003). Indeed, Chen and Mikecz (2005) reported on the translocation of up to 70 nm large silica particles into different, dividing mammalian cells. Translocation of the particles into the nucleus induced inhibition of gene expression and formation of protein aggregates, which resemble sub-nuclear pathologies also seen in neurodegenerative diseases and systemic autoimmune responses. Moreover, damage to DNA, i.e., genotoxicity, has been variously reported, for example, as micronuclei formation in mammalian cells by tungsten carbide (Kühnel et al. 2012) or silver (Asharani et al. 2009) NMs.

Yet, genotoxicity by itself cannot be taken as indication that NMs have indeed entered the nucleus.

Among the mechanisms proposed for apparent NM-elicited genotoxicity are the production of ROS and mechanical interference. Both may be elicited without the NM having to enter the nucleus, e.g., by oxidative stress and increased ROS production in the cytosol eventually leading to oxidative DNA damage or by interference of NMs with actin filaments or microtubules during cell division (Gonzalez et al. 2008). In support of the cytosolic ROS production as initial insult to DNA damage, Wang et al. (2012) resolved DNA damage from CuO NM exposure of a human lung cell line to the following time-dependent series of events: oxidative stress associated with mitochondria occurring after two hours of exposure, followed by enhanced transcript abundance of the stress response-sensitive protein kinases p38 and p53 at four hours, and irreversible DNA damage starting to be measurable after eight hours of exposure. On the other hand, for quantum dots small enough to diffuse through the nuclear complex pore, targeting of histone proteins, which play an important role in cell cycle regulation and tumour growth, has been demonstrated (Nabiev et al. 2007). In summary, while damage of DNA by NM exposure has been demonstrated also in the absence of proven transport into nuclei, the mechanisms of impacting on DNA integrity and other functional or structural features of the nucleus, either directly or indirectly, warrant further investigations.

Caveat for sub-lethal exposure: stimulatory hormetic effects

Hormesis is a biological response to a low dose of a toxicant whereby a beneficial effect may be observed (*e.g.,* improved cell fitness, stress tolerance, growth or longevity). This poorly understood phenomenon appears to be related to the activation of global cell repair mechanisms that overcompensate for the exposure to the toxicant (Calabrese et al. 2001). Hormesis has been shown to occur in human and mammalian cells exposed to NMs including nanotubes, quantum dots, zinc oxide, and silver (Lavicoli et al. 2014). Moreover, bacterial exposure to sub-lethal concentrations of antimicrobial silver nanoparticles can induce various adaptation mechanisms that have a counterproductive hormetic effect on the intended microbial control, such as stimulated biofilm formation and increased antibiotic resistance (Yang and Alvarez 2015). Transcriptomic analysis revealed significant upregulation of quorum sensing and lipopolysaccharide (LPS) biosynthesis genes that enhance biofilm formation, possibly as a defense mechanism (Xiu et al. 2014). A hormetic effect (i.e., faster growth) was also been observed for poplar trees and *Arabidopsis thaliana* (Wang, Koo et al. 2013), which precludes generalizations about phytotoxicity. More discussion about hormetic effects can be found in the supplemental information.

CONCLUSIONS

The developments in the field since 2008 are large and important and the emerging consensus is encouraging, with developments in metrology, transformations, bioavailability nd mechanisms of toxicity being very pronounced. Nanoscience and nanotechnology are important social and economic drivers and will hopefully lead to improvements in society and well-being. The potential negative consequences to the environment have been relatively openly studied and discussed and this healthy transparent research environment has led to a general trust in the technology and its implementation. Much of the earlier research on the environmental implications of nanotechnology raised significant concerns based on reductionist experiments conducted under exposure conditions designed to elicit a response and discern toxicity mechanisms (e.g., unrealistically high doses, simple matrices). Recent experiments conducted under more realistic exposure conditions have found that the interactions of NMs with environmental matrices can, but do not necessarily, decrease their reactivity, bioavailability and toxicity, which has suggested a more limited environmental risk in many cases. Despite the lack of major problems associated with NMs in the environment, there are very substantial data and knowledge gaps, including fundamental data on environmental concentrations. For instance, uncertainties in dosimetry remain, although analytical advances are being made currently, and these uncertainties make understanding the dose-response relationship challenging. In addition, the nanotechnology industry continues to grow, such that discharges and environmental burdens are rapidly increasing. Finally, nanotechnology innovation continues apace, with the development of more sophisticated and useful nano-enabled materials and products, which are physico-chemically and biologically active. A good example in the terrestrial environment is the increased use of NMs for pesticide and fertilizer release and delivery and the potential for non-targetted delivery of these and other chemicals. These products are often proprietary and the nature of NMs used in nanofertilizers and nanopesticides is often unclear, though applications employing Ag, CuO and ZnO NMs are known. Trade secrecy around proprietary materials again means that understanding hazard and risk are made much more difficult to accurately quantify. These newer NMs are likely to be more persistent and stable, more dispersed, smaller and multi-functional, which underscores the need to understand their potential unintended impacts. These uncertainties and rapid changes mean that there is a potential risk to the environment and to human health from environmental exposure which should not be ignored; greater integration of technology innovation and risk assessment is needed to ensure the benefits of the technology are gained with a minimization of their possible negative effects. Continued scientific input is required to fill in our knowledge gaps and to judiciously apply the precautionary principle. The appropriate goal of these efforts is to help protect environmental and human health while ensuring the long-term sustainability of the nanotechnology industry.

Finally, and separate from a pragmatic assessment of risk, continued understanding of the environment, and how we affect it, is an important undertaking in its own right and can be justified solely on this basis. A better understanding of the science around NM fate, behaviour and effects is appropriate and should be undertaken, even in the absence of any impact on regulation, risk assessment and innovation.

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Figure 1. General structure of the material-flow model. The model's principle is to track ENM flows throughout the entire life cycle: ENM production; incorporation into products; ENM release from products during use; transport and fate of ENM between and within STP, WIP, landfill and recycling processes (technosphere); transfer from technosphere to air, soil, water and sediments (ecosphere); and transport within environmental compartments. The amounts of ENM in the compartments provide the basis for calculating the overall environmental concentrations of ENM (from Sun et al., 2014).

Figure 2. Modeled and analytical concentrations of NMs in surface waters. The green boxes show the range (and the arithmetic mean on the log scale) of modeled results. Yellow boxes measured concentrations and the orange boxes combine measurements and modelling (from Gottschalk et al., 2013) (for sources of data see the original reference).

Figure 3. Concentration-dependent transformations to NP diameter and number concentration in a moderately hard water (MHW): A) particle diameter verses time for concentrations of 4.4×10^7, 1×10^5 and 1×10^4 particles/mL. B) % change in particle diameter of NPs in MHW after 48 h for 6 concentrations (from Merrifield et al., 2017a). C) particle number concentration verses time for concentrations of 4.4×10^7, 1×10^5 and 1×10^4 particles mL^{-1} and D) the % change in particle diameter of NPs in MHW after 48 h for all 6 concentrations (4.4×10^7, 2.2×10^7, 2.5×10^5 and 1×10^5, 5×10^4 and 1×10^4 particles/mL) (From Merrifield et al., 2017b)

Figure 4. Conceptual diagram of the major transformations which NMs might undergo in the environment (modified from Lowry et al., 2012).

Figure 5. The main fate-determining processes for natural colloids and NMs in soils: 1. Colloid generation. 2. ENM leaching from biosolids. 3. Homoaggregation. 4. Fragmentation. 5.

Sedimentation. 6. Heteroaggregation. 7. Size exclusion. 8. Straining. 9. Deposition. 10. Convective transport (reproduced with permission from Cornelis et al., 2014).

Figure 6. An idealised diagram of an epithelium (freshwater fish gill) showing the mechanisms of uptake for electrolytes, toxic metal ions (Me^+), and electroneutral diffusion of some small organo-metals (CH_3-Me), compared to nanoparticles (NPs, filled circles). Modified from Handy et al. (2008b). The substances in the bulk solution (i.e., the external environment) must diffuse into an unstirred layer (USL) comprising of water/mucus secretions and microbial biofilm, prior to transfer into the epithelium itself. The upper portion of the diagram shows electrolytes and toxic metals ions which diffuse into the USL, and may bind to strands of mucus (mostly polyanionic) where the exclusion of free anions like Cl^- from the mucus layer contributes to the Donnan potential at the apical surface. Electrolytes and toxic metal ions usually move through the cell using ion transport pathways (Na^+ transporters are illustrated here). NPs will diffuse into the USL, albeit at a slower rate than smaller molecules or solutes, and may be influenced by humic substances (HS). Cationic NPs will bind to strands of mucus by electrostatic attraction, but regardless of surface charge, may also become entangled in the mucoproteins (steric hindrance) to prevent uptake by the epithelial cells. NPs are too large to be taken up by ion or other transporters on the cell membranes, although diffusion is known for small lipophilic NPs. The Ca^{2+} and Mg^{2+} rich environment in the tight junctions suggest that NPs would aggregate rather than diffuse through the paracellular route. In addition, some nanometals may release free metal ion (Me^+) by dissolution of ions into the bulk solution. In contrast, nanomaterials can also show surface adsorption of metals, and this is likely to be faster in the higher ionic strength of the USL. Diffusion of charged NPs into the USL will be affected by the Donnan and

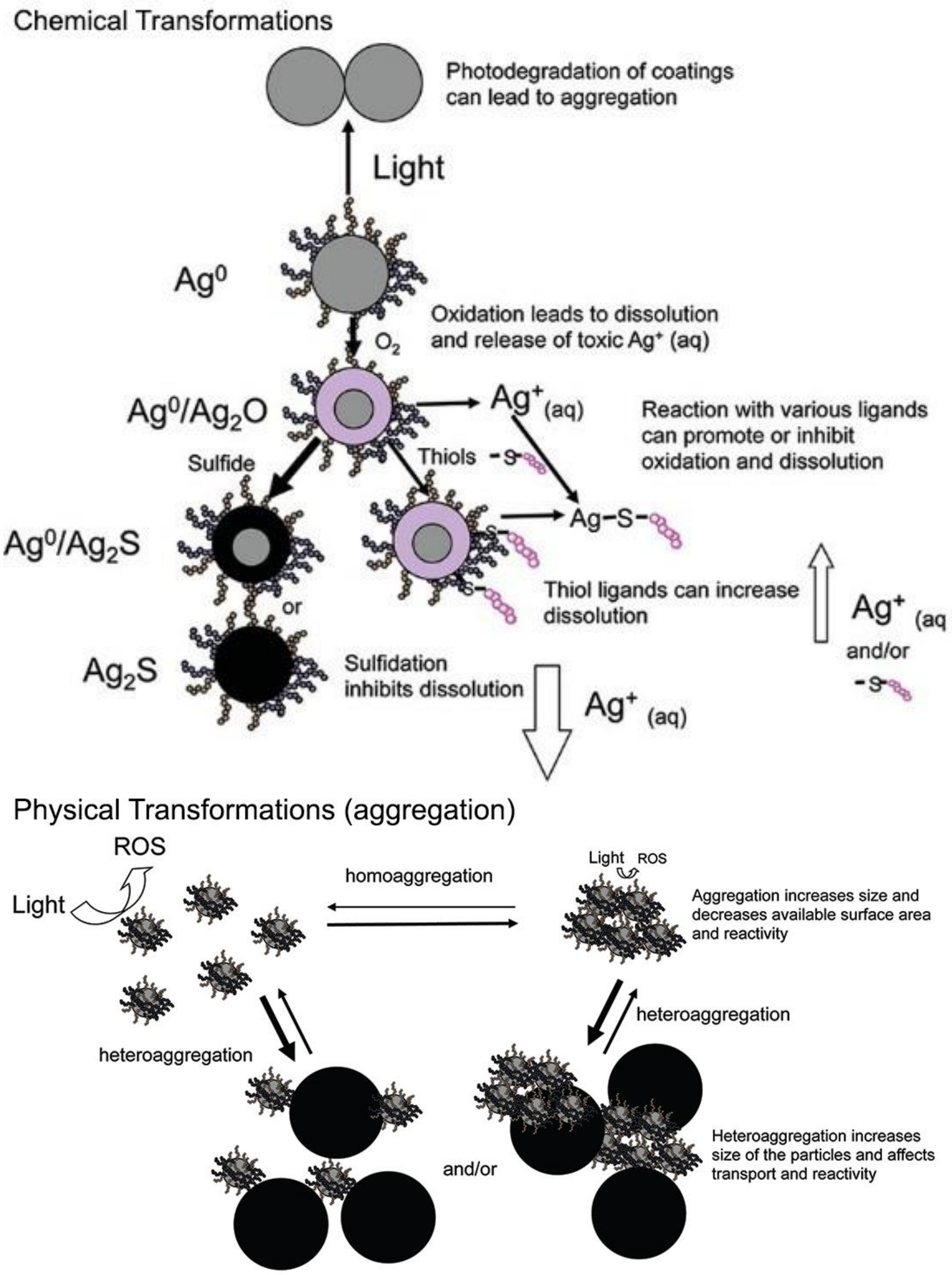
transepithelial potentials, in a similar way to other charged substances. NP uptake through vesicular transport has been pharmacologically confirmed for some ENMs.

Figure 7. Concentration-response curves and calculated log-logistic fits in a 21-day reproduction test with *D. magna* obtained for 14, 20 and 29 nm CeO_2 NPs and CeO_2 bulk material. Concentration is expressed as mass (A) and as surface area (B) (from van Hoecke et al., 2009).

Figure 8. Comparison of the toxicities of micrometer and nanosized ZnO, CeO_2, and Ag and their ionic equivalents Zn^{2+}, Ce^{3+}, and Ag^{+} to the freshwater alga *Pseudokirchneriella subcapitata* in a synthetic soft water (toxicity values are IC50s, the concentrations causing a 50% growth inhibition in μg/L) (respectively from Franklin et al. (2007); Rogers et al. (2008), and Angel et al. (2013); Reproduced from Batley et al. (2013) with permission).

Table 1. Mean chronic HC5 values for engineered NPs in freshwaters based of species sensitivity distributions of chronic and converted acute data (from Garner et al., 2015)

Nanomaterial	HC5
Uncoated nano Ag	1.2 µg/L
PVP-coated nano Ag	0.7 µg/L
Nano CuO	40 µg/L
Nano CeO_2	0.8 mg/L
Nano TiO_2	2 mg/L
CNTs	4.8 mg/L
Nano C60	0.2 mg/L


Chemical Transformations
Photodegradation of coatings can lead to aggregation
Light
Ag^0
Oxidation leads to dissolution and release of toxic Ag^+ (aq)
O_2
Ag^0/Ag_2O
$Ag^+_{(aq)}$
Reaction with various ligands can promote or inhibit oxidation and dissolution
Sulfide
Thiols
-S
Ag-S-
Ag^0/Ag_2S
or
S-
S-
Thiol ligands can increase dissolution
$Ag^+_{(aq}$
and/or
-S
Ag_2S
Sulfidation inhibits dissolution
$Ag^+_{(aq)}$
Physical Transformations (aggregation)
ROS
Light
homoaggregation
Light
ROS
Aggregation increases size and decreases available surface area and reactivity
heteroaggregation
heteroaggregation
and/or
Heteroaggregation increases size of the particles and affects transport and reactivity

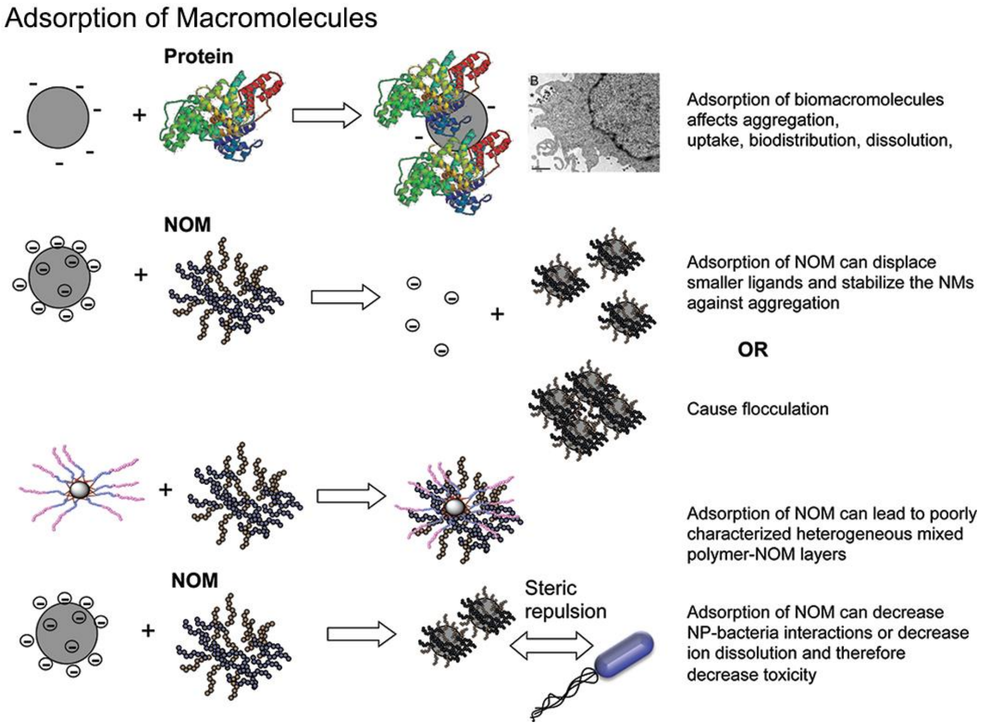
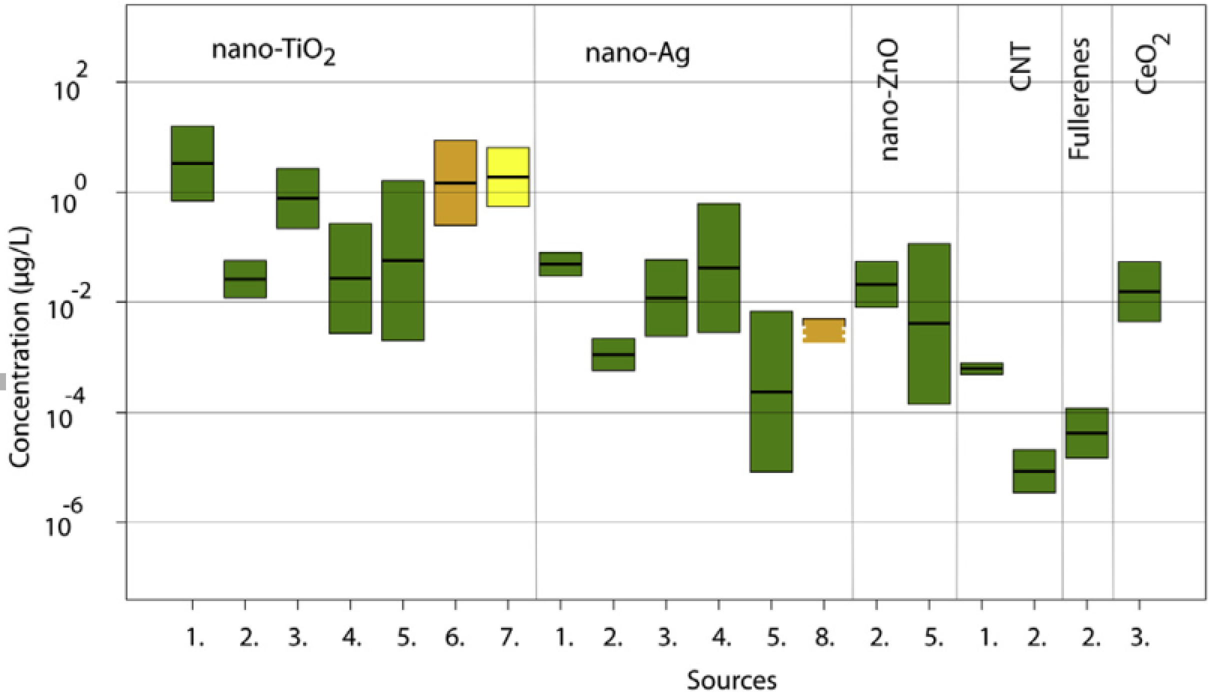

Adsorption of Macromolecules
Protein
Adsorption of biomacromolecules affects aggregation, uptake, biodistribution, dissolution,
NOM
Adsorption of NOM can displace smaller ligands and stabilize the NMs against aggregation
OR
Cause flocculation
Adsorption of NOM can lead to poorly characterized heterogeneous mixed polymer-NOM layers
NOM
Steric repulsion
Adsorption of NOM can decrease NP-bacteria interactions or decrease ion dissolution and therefore decrease toxicity

Figure 1.


nano-TiO2
nano-Ag
nano-ZnO
CNT
Fullerenes
CeO2
10^2
10^0
10^-2
10^-4
10^-6
Concentration (µg/L)
1.
2.
3.
4.
5.
6.
7.
1.
2.
3.
4.
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1.
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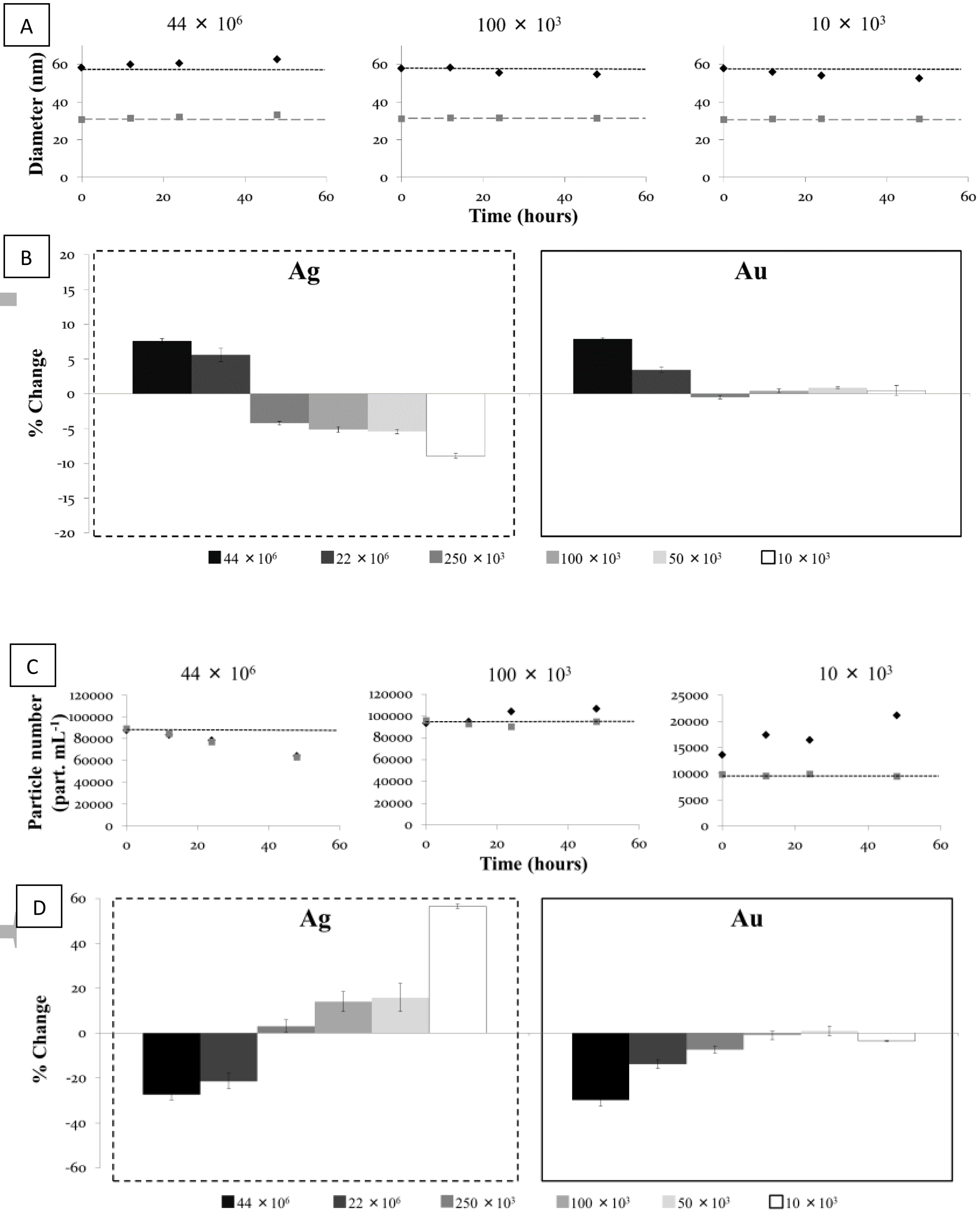
Sources

Figure 2.


A
44 × 10⁶
100 × 10³
10 × 10³
Diameter (nm)
0
20
40
60
Time (hours)
B
Ag
Au
% Change
20
15
10
5
0
-5
-10
-15
-20
44 × 10⁶
22 × 10⁶
250 × 10³
100 × 10³
50 × 10³
10 × 10³
C
44 × 10⁶
100 × 10³
10 × 10³
Particle number (part. mL⁻¹)
120000
100000
80000
60000
40000
20000
0
25000
20000
15000
10000
5000
Time (hours)
D
Ag
Au
% Change
60
40
20
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-20
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-60
44 × 10⁶
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Figure 3.

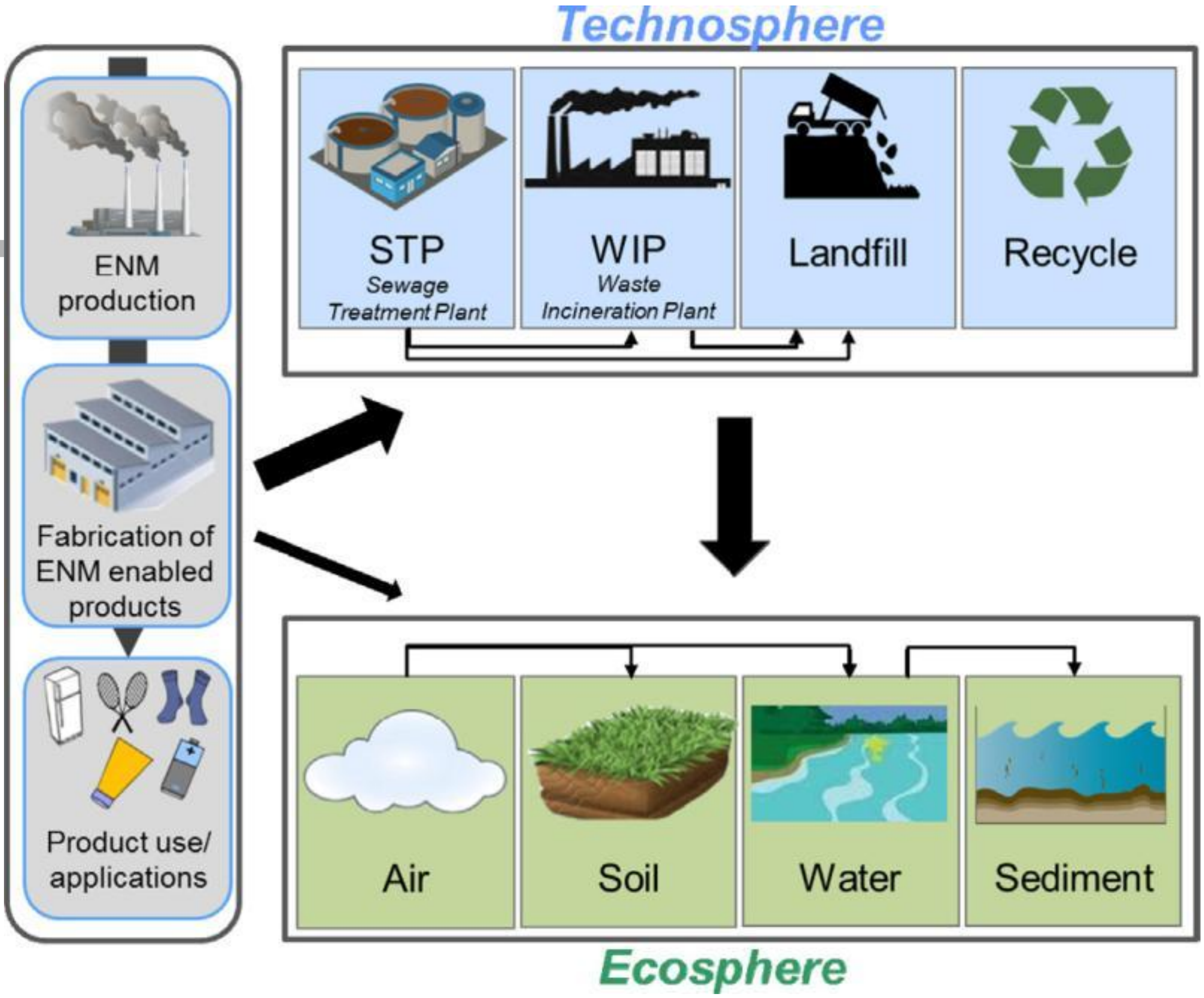
Technosphere
ENM production
Fabrication of ENM enabled products
Product use/ applications
STP
Sewage Treatment Plant
WIP
Waste Incineration Plant
Landfill
Recycle
Air
Soil
Water
Sediment
Ecosphere

Figure 4.

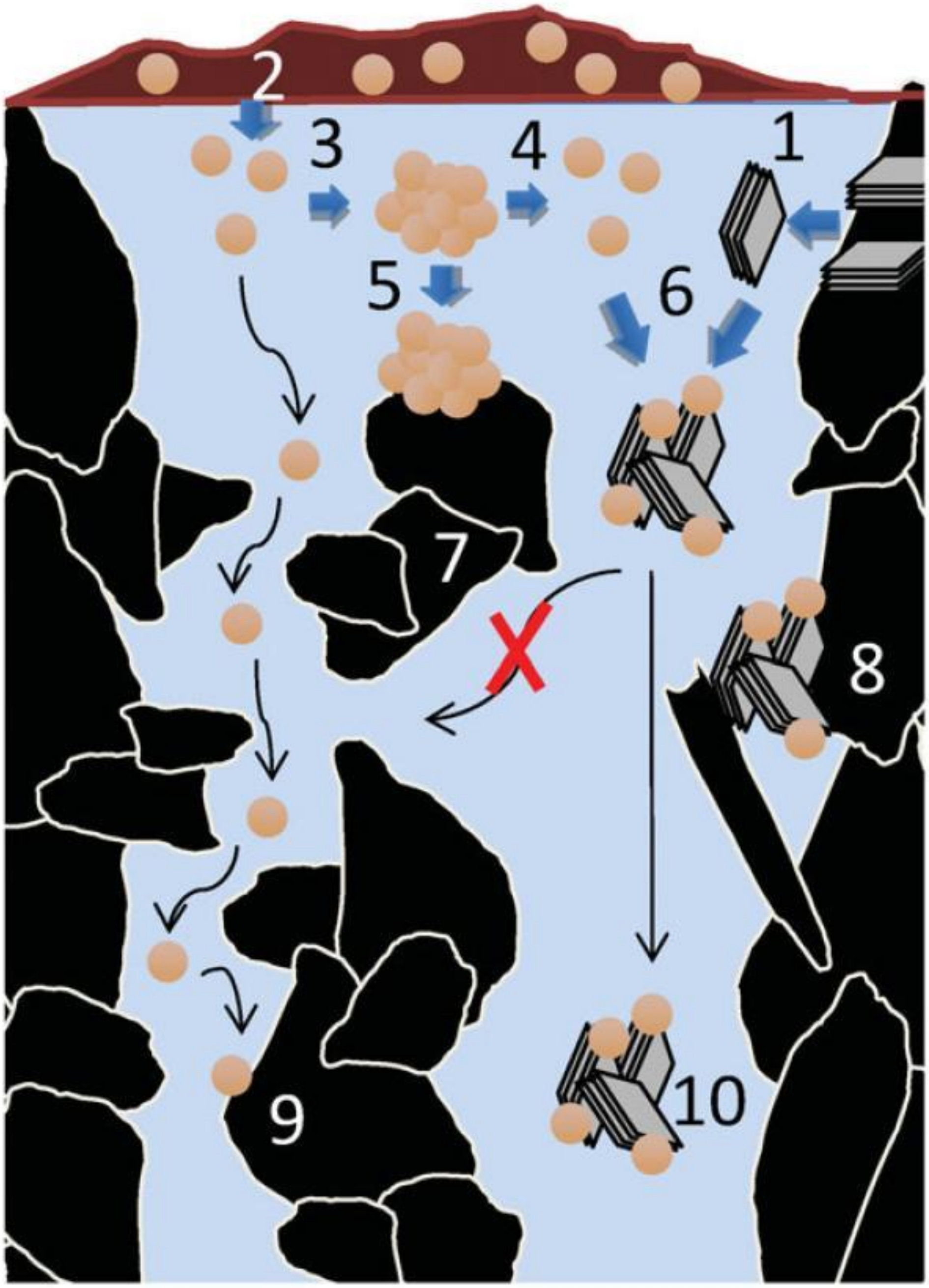

Figure 5.

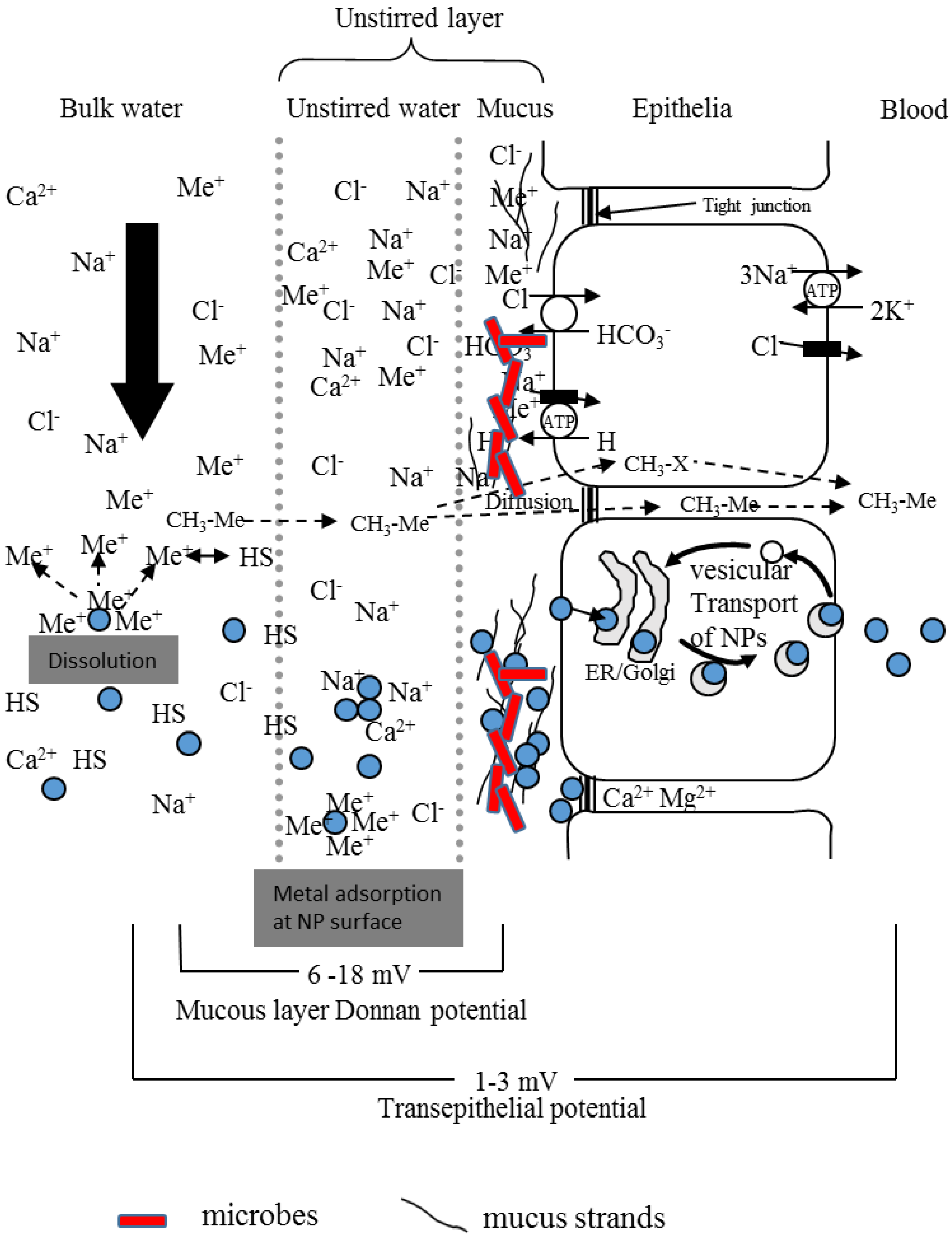
Unstirred layer
Bulk water
Unstirred water
Mucus
Epithelia
Blood
Tight junction
3Na+
ATP
2K+
Cl-
HCO3-
H
Na+
Me+
Ca2+
CH3-X
CH3-Me
Diffusion
Me+ ↔ HS
Dissolution
vesicular Transport of NPs
ER/Golgi
Ca2+ Mg2+
Metal adsorption at NP surface
6 -18 mV
Mucous layer Donnan potential
1-3 mV
Transepithelial potential
microbes
mucus strands

Figure 6.

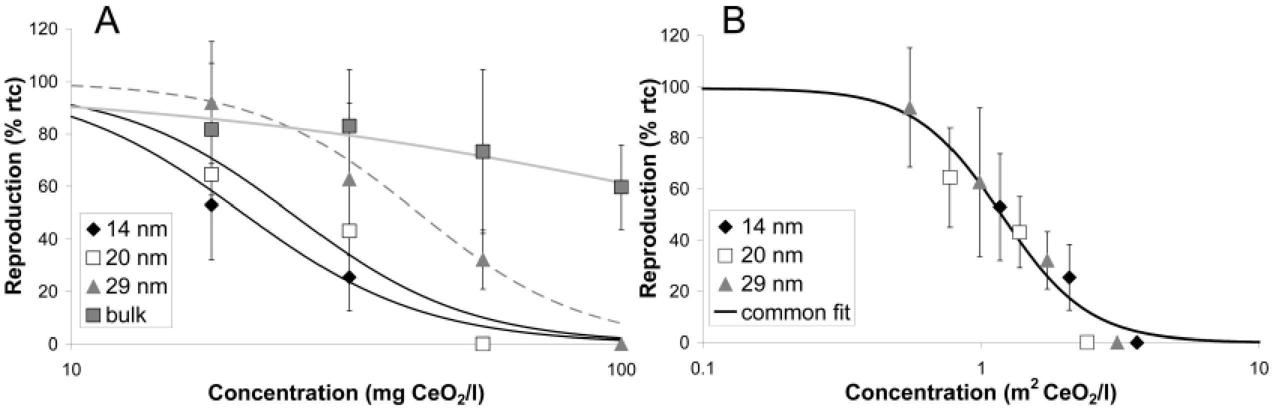
A
Reproduction (% rtc)
120
100
80
60
40
20
0
10
100
Concentration (mg CeO2/l)
◆ 14 nm
□ 20 nm
▲ 29 nm
■ bulk
B
Reproduction (% rtc)
0.1
1
10
Concentration (m2 CeO2/l)
◆ 14 nm
□ 20 nm
▲ 29 nm
— common fit

Figure 7.

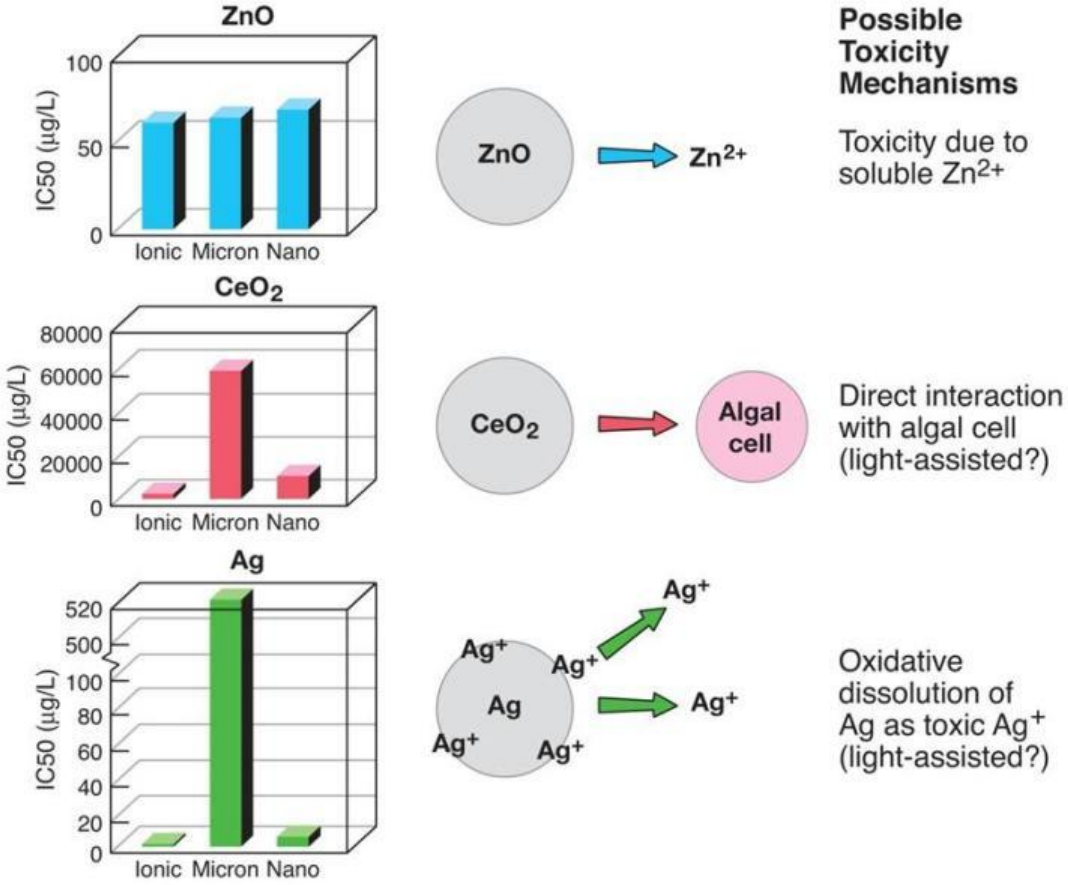
ZnO
100
50
0
IC50 (µg/L)
Ionic
Micron
Nano
CeO2
80000
60000
40000
20000
0
IC50 (µg/L)
Ionic
Micron
Nano
Ag
520
500
100
80
60
40
20
0
IC50 (µg/L)
Ionic
Micron
Nano
ZnO
Zn2+
CeO2
Algal cell
Ag
Ag+
Ag+
Ag+
Ag+
Ag+
Ag+
Possible Toxicity Mechanisms
Toxicity due to soluble Zn2+
Direct interaction with algal cell (light-assisted?)
Oxidative dissolution of Ag as toxic Ag+ (light-assisted?)

Figure 8.