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REVIEW ARTICLE

Review: Do engineered nanoparticles pose a significant threat to the aquatic environment?

T. M. Scown, R. van Aerle, and C. R. Tyler

Ecotoxicology and Aquatic Biology Research Group, Hatherly Laboratories, University of Exeter, Exeter, United Kingdom

Abstract

Nanotechnology is a rapidly growing industry of global economic importance, exploiting the novel characteristics of materials manufactured at the nanoscale. The properties of engineered nanoparticles (ENPs) that make them useful in a wide range of industrial applications, however, have led to concerns regarding their potential impact on human and environmental health. The aquatic environment is particularly at risk of exposure to ENPs, as it acts as a sink for most environmental contaminants. This paper critically evaluates what is currently known about sources and discharge of ENPs to the aquatic environment and how the physicochemical characteristics of ENPs affect their fate and behaviour and thus availability for uptake into aquatic organisms, and assesses reported toxicological effects. Having reviewed the ecotoxicological information, the conclusion is that whilst there are data indicating some nanoparticles have the potential to induce harm in exposed aquatic organisms, there is insufficient evidence for harm, for known/modelled environmental concentrations for almost all ENPs considered. This conclusion, however, must be balanced by the fact that there are significant gaps in our understanding on the fate and behaviour of ENPs in the aquatic environment. Greater confidence in the assessments on ENP impacts in aquatic systems to enable effective comparisons across studies urgently requires more standardised approaches for ENP hazard identification, and critically, more thorough characterisations on the exposed particles. There is also an urgent need for the advancement of tools and techniques that can accurately quantify and visualise uptake of nanoparticles into biological tissues.

Keywords: *Behaviour; fish; toxicology; uptake*

Contents

Address for Correspondence: C. R. Tyler, Ecotoxicology and Aquatic Biology Research Group, Hatherly Laboratories, University of, Exeter, Prince of Wales Road, Exeter, EX4 4PS, United Kingdom. E-mail: c.r.tyler@exeter.ac.uk

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Introduction

Worldwide investment in the nanotechnology industry is currently in the US$ billions and the sale of so-called 'nano-enabled' products is projected to reach US$ trillions, emphasising the global economic importance of the industry (Lux Research Inc., 2009; Schmidt, 2009). Global funding into nanotechnology research and development in 2008 reached $18.2 billion, led by the United States and Japan (Lux Research Inc., 2009) and in the Seventh Framework Programme (2009–2013) the European Union (EU) will be investing approximately €600 million annually to nanotechnology research funding (Technology Transfer Centre, Institute of Nanotechnology, 2007). In the UK alone, there are already over 600 micro- and nanotechnology companies (Nanotechnology Knowledge Transfer Network, 2009).

The diversity of potential applications means that nanotechnology will inevitably yield considerable benefits to society in terms of general consumer products and health care. However, as the industry grows and the number of nanoparticle types and applications increase, so does the likelihood that they will be released into the environment, and in significant quantities. Due to their extremely small size and unusual physical properties, the behaviour of engineered nanoparticles (ENPs) in the environment, their uptake, distribution, and effects within the bodies of living organisms is likely to be different when compared to conventional xenobiotics. In recent years, this realisation has generated considerable concern from both government bodies and scientists regarding the possible negative impacts nanotechnology may have on human and environmental health. Investigation of possible effects in humans has started to receive significant attention, but less effort has been directed towards the possible environmental implications (Colvin, 2003; Dowling et al., 2004; Klaine et al., 2008; Oberdörster et al., 2005; Owen and Handy, 2007; Williams et al., 2005).

This review paper assesses, critically, what is currently known about the potential impact of ENPs on the aquatic environment, covering what is known regarding the likely sources, (predicted) concentrations, fate and behaviour in aquatic systems, their bioavailability and toxicity to aquatic organisms, and features of nanoparticles that may affect their toxicity. The paper concludes with a perspective on future research needs for the development of appropriate risk assessment strategies for ENPs to protect the aquatic environment and more harmonised approaches for effectively advancing our understanding on the (eco)toxicity of ENPs.

Sources and routes of entry of nanoparticles to the aquatic environment

The fate and behaviour of both natural and nanoparticulate materials generated from anthropogenic activity in the environment has been the subject of study for many years (Klaine et al., 2008; Lead and Wilkinson, 2006; Peters et al., 1997; Sioutas et al., 2005). Until recently, however, little work has been conducted on ENPs in the environment and consequently little is known about the concentrations of ENPs in air, soils, or water, or on their transport and fate. Modelling studies have been undertaken to start to address this shortfall and estimate the likely load of ENPs in various environmental compartments (Boxall et al., 2008; Mueller and Nowack, 2008). In this work, a model proposed by Boxall and colleagues (2008) included assessments on exposure routes, and established an inventory of consumer products containing ENPs and their concentrations in the United Kingdom. Predicted exposure concentrations for TiO_2 ENPs were 7 mg m^{-3} for persons applying sunscreen (via inhalation) and atmospheric exposure levels to CeO_2 derived from diesel vehicle emissions were 6×10^{-7} mg m^{-3}. Another model developed by Mueller and Nowack (2008) focused on estimating the concentrations of silver and TiO_2 ENPs and carbon nanotubes into the various environmental compartments in Switzerland. Their model considered global production volume, production volume in product categories, release of particles from products, and flow coefficients of ENPs between the different environmental compartments as input parameters. From that work, predicted atmospheric concentrations of ENPs released through use of sprays and cleaning agents and abrasion of nanoparticle containing products were 1.7×10^{-3}, 1.5×10^{-3}, and 1.5×10^{-3} μg m^{-3} for silver ENPs, TiO_2 ENPs, and carbon

nanotubes, respectively. Direct comparisons between the estimates for exposures to TiO_2 (the common study particle) derived from the two models cannot be drawn, as the exposure routes considered differed. As yet, there are no measured atmospheric concentrations available for any of these ENPs. Air contains large numbers of nano-sized particles (1–30,000 particles cm^{-1}) (Oberdörster, 2001) and as a consequence detecting a relatively low number of ENPs in the air against a high background level of other particles is challenging and reliable methods to do so are not yet available.

Considering sources of ENPs to soils, direct routes of entry are via degradation of products containing ENPs in landfill, through accidental spills from factories, and runoff from road surfaces (Dowling et al., 2004), but there are no studies that have quantified these inputs. Entry for some ENPs into the soil can also occur via direct application, when ENPs are used for the remediation of soils contaminated with heavy metal ions, polychlorinated hydrocarbons, pesticides, and radionuclides (Zhang, 2003). This technology has already progressed to full-scale commercial use for zero-valent iron nanoparticles (Tratnyek and Johnson, 2006) with applications in the form of slurries, with concentrations in the order of grams per litre (Zhang, 2003), or injected into the soil to form a colloidal reactive barriers (Giasuddin et al., 2007). Major sources of silver, TiO_2, and carbon nanotube ENPs to soils are predicted to be via run-off through use of paints and cleaning agents and sprays. Release and subsequent deposition of airborne ENPs may offer a further route of entry into soils. In the Swiss studies conducted by Mueller and Nowack (2008), predicted soil concentrations for silver ENPs, TiO_2 ENPs, and carbon nanotubes were 0.02, 0.4, and 0.01 μg kg^{-1} of soil, respectively.

Surface waters receive pollutants from atmospheric deposition, leaching from soil and through direct inputs, such as wastewater discharges, and all of these provide routes for ENP entry. Surface water bodies can also import water from groundwater reservoirs (Schaller and Fan, 2009), transporting with it pollutants, including ENPs (Caruso and Dawson, 2009). Given these diverse input sources, the aquatic environment is highly susceptible to contamination with certain ENPs. Although methods for detecting and characterizing ENPs in natural waters, involving field flow fractionation coupled to inductively coupled plasma mass spectrometry, are being developed (Dubascoux et al., 2008; Hassellöv and Stolpe, 2007), they are still in their infancy and again, as for the air and soil, information on levels of ENPs in aquatic environments is scarce. In the model by Mueller and Nowack (2008) estimated concentrations of silver and TiO_2 nanoparticles and carbon nanotubes in freshwaters were 0.3, 0.7, and 0.0005 μg L^{-1}, respectively. In a scenario of high emission of TiO_2, however, the model predicted levels up to 16 μg L^{-1}. The major sources of TiO_2 into surface water systems are predicted to derive from cosmetics and coatings, disposed paints and sprays, and abrasion of ENP-containing metals and plastics via run-off and wastewater treatment works (WWTWs). A major source of silver nanoparticles is from the washing of fabrics that have been impregnated with silver as an antimicrobial agent. A study by Benn and Westerhoff (2008) found that socks contained up to 1360 μg silver per gram of material, and that both colloidal and ionic silver leached out into the washing water at concentrations of up to 1.3 mg L^{-1} after successive 24-hour immersions. Wastewater and effluents from factories producing raw nanoparticles and nano-enabled products are likely to be a major point source for contamination into the aquatic environment, but studies of this nature have not been forthcoming. In a study by Limbach et al. (2008) a model WWTW plant was used to illustrate that the majority of cerium oxide (CeO_2) nanoparticles adhered to the clearing sludge that was retained within the plant; however, up to 6 wt % of the particles were found in the exit stream. Surface charge and addition of stabilizing surfactants, used routinely in nanoparticle-derived products, caused a significant increase in the levels of cerium oxide found in the treated effluent. This demonstrates that passage through WWTWs cannot guarantee the removal of ENPs from the discharged wastewater.

Kaegi et al. (2008) have provided the only published measurements of ENPs (TiO_2 nanoparticles) entering the aquatic environment via runoff, in this case derived from the detachment from new and aged facade paints via natural weathering. Concentrations of titanium from nanoparticles in the runoff collected from directly beneath newly painted and aged facades were approximately 550 and 300 μg L^{-1}, respectively. In that study, measured concentrations of TiO_2 in samples of urban runoff at a point of entry into a stream was approximately 300 μg L^{-1}. The principal findings from this work are in agreement with the model proposed by Mueller and Nowack (2008) that suggested weathering of paints containing ENPs could be responsible for significant discharges of ENPs into the aquatic environment.

Behaviour of ENPs in the aquatic environment

The behaviour of naturally occurring nanoparticulate and colloidal matter in natural waters and soils has been studied for many years. Colloids are usually defined as material with one dimension between 1 nm and 1 μm and in natural aquatic systems, are a complex aquatic mixture including viruses and bacteria, natural organic matter (NOM) such as humic acids (HAs), protein and polysaccharide exudates from microbes, and inorganic matter such as oxides of iron, manganese, aluminium, and silicon (Klaine et al., 2008; Lead and Wilkinson, 2006). ENPs entering aquatic systems will thus become components of these colloids and their subsequent behaviour and transport will depend both on physicochemical characteristics of the aqueous media and interactions with other colloidal components.

The stability of colloidal suspensions is determined by the interaction between attractive and repulsive forces, which are governed by surface charges of the colloidal material. These interactions are detailed by the DLVO theory (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948), which describes the forces between charged surfaces interacting in a liquid medium. Here, the effects of van der Waals attraction and the

electrostatic repulsion due to a double layer of counter-ions that surround insoluble particles in a liquid suspension are combined. Colloids carry an electrical charge, which produces a force of mutual electrostatic repulsion between adjacent particles. If the charge is high enough, the colloids will remain discrete, and are stabilised in suspension. Reducing or eliminating the charge causes the colloids to agglomerate and settle out of suspension or form interconnected matrices.

Colloids therefore have a propensity to adsorb to particulate matter and to aggregate into particles that may be >1 μm in size, leading to sedimentation (Klaine et al., 2008). Recently, a number of studies have emerged where the physicochemical factors relating to the aquatic media have been investigated to determine their role in the formation of aggregates and the size of ENP aggregates that form. Dose-dependent increases in ENP aggregate formation have consistently been found to be associated with increases in cation concentration in the medium (0.1–100 mM) (Domingos et al., 2009; Fang et al., 2009; Wang et al., 2008b). Aggregate formation has also been found to be dependent on the concentration of dissolved organic carbon (Fang et al., 2009), humic acid (Baalousha et al., 2008), and fulvic acid (Domingos et al., 2009) as well as the pH of the aquatic medium (Baalousha et al., 2008; Domingos et al., 2009; Fang et al., 2009).

These findings have major implications in terms of exposure of aquatic organisms as aggregation and sedimentation of ENPs reduces the likelihood of transport within the water column (Baalousha et al., 2008). This suggests lower transport of ENPs in cation-rich marine and estuarine environments (Keller et al., 2010) and thus sediment-dwelling and benthic organisms may be more prone to exposure than pelagic species (Johnston et al., 2010). Alterations in these conditions, however, may favour the stabilisation of ENPs in the water column, giving them the potential for uptake by aquatic organisms and transport within water systems. General models for predicting this behaviour have yet to be developed (Baalousha et al., 2008).

The physicochemical characteristics of the ENPs themselves are also key elements in determining the behaviour of ENPs, and thus bioavailability to organisms, in the aquatic environment. The zeta potential on the surface of the ENPs has been shown to influence aggregation behaviour, with values closer to zero point charge (0 mV) leading to increased aggregation (Fang et al., 2009). The presence of functional groups and coatings on the surfaces of ENPs are also likely to influence how ENPs interact with each other and other components of the aquatic medium, and thus play a part in determining their stability.

The interaction of nanoparticles with organic matter, such as humic and fulvic acids in particular, is now receiving considerable interest, in order to better understand how these interactions might affect both the stability ENPs in aquatic media, but also their ability to bind and act as co-transporters of other pollutants. Colloidal material from natural waters has been found to be coated by films of organic material and since particle surface charges and force interactions between particles are dominated by adsorbed layers, this has important implications for understanding mechanisms by which colloids might bind trace elements and pollutants (Lead et al., 2005).

It has been shown that adsorption of HA to various metal oxide nanoparticles (TiO_2, aluminium oxide [Al_2O_3], and zinc oxide [ZnO]) can result in a decrease in particle zeta potential, suggesting that HA-coated nano-oxides could be more easily dispersed and suspended and more stable in solution than uncoated ones because of their enhanced electrostatic repulsion (Yang et al., 2009). A number of studies have also shown that various ENP types with applied coatings of HAs showed enhanced sorption for organic chemicals such as polycyclic aromatic hydrocarbons (Hu et al., 2008; Wang et al., 2008a; Yang and Xing, 2009).

Advancing our understanding on the ecotoxicology of ENPs urgently requires a better understanding on the characteristics and behaviour of ENPs in aquatic systems and their interaction with other particles in natural waters. In turn this will allow for a better assessment of the nature of the ENPs to which study organisms are being exposed and their likely bioavailability.

Toxicological effects of nanoparticles

The unusual properties that materials possess at the nanoscale may result in effects within the environment and interactions with living organisms that are exaggerated or unexpected compared with their bulkier counterparts. In addition, their small size means they may be able to bypass barriers that prohibit the entry of other xenobiotics, allowing them entry to cells of living organisms, through membranes and junctions between cells. This, coupled with their enhanced reactivity, may mean ENPs have the potential to induce adverse cellular effects and cause harm to living organisms.

The link between mesothelioma and asbestos exposure (Elmes et al., 1965; Fowler et al., 1964) prompted much research to elucidate the effects of exposure to ultrafine particulate matter on human respiratory health (Peters et al., 1997; Sioutas et al., 2005). Many studies conducted in rodent models have now established that ultrafine particles, now synonymous with nanoparticles, are capable of inducing adverse effects in the lungs (Donaldson et al., 1990; Driscoll et al., 1991, 1995; Kusaka et al., 1990; Lam et al., 1985) and this has set a precedent for toxicity studies using ENPs. As a consequence, until very recently, the majority of nanotoxicology studies conducted have been inhalation-based studies in terrestrial vertebrates, with comparatively less attention paid to exposure of organisms living in other environmental compartments. Now, however, studies are emerging exploring the exposure effects of ENPs on aquatic organisms, investigating potential routes of uptake, translocation, fate, and effects in the body, as well as how the characteristics of the ENPs and the surrounding exposure medium affect uptake and effect. The next section in this review provides a critical analysis on the reported findings for exposure effects of ENPs on aquatic organisms, including microbes, algae, invertebrates (*Daphnia magna* and *Ceriodaphnia dubia*), and predominantly fish.

Effects of nanoparticles in microbes and algae

Bacterial populations account for a large proportion of the primary production and carbon flux within the aquatic environment (Cole, 1999). They therefore play an important role in regulation of key processes within these systems, and disruption to these populations and their activities is likely to impact other organisms that share their environments.

There is some evidence to suggest that a number of different carbon-based nanoparticle types demonstrate antibacterial activity. Suspensions of fullerene (C_{60}) in water prepared by a variety of methods have been shown to have antibacterial effects to *Bacillus subtilis* (Lyon et al., 2006) at concentrations of between 0.1 and 1 mg L^{-1} and *Escherichia coli* at 140 μM (Brunet et al., 2009) and the antibacterial activity has been demonstrated to be caused by the production of reactive oxygen species (ROS) (Brunet et al., 2009). Exposure to C_{60} suspensions has also been shown to cause changes to bacterial diversity in soil-dwelling bacterial populations at concentrations of 5–10 mg kg^{-1} soil (Johansen et al., 2008). Interaction of C_{60} with soil and natural organic matter (e.g. humic acid) has been demonstrated to reduce its antibacterial activities, suggesting that the presence of abiotic and biotic material in natural aquatic systems may limit the potential impacts of C_{60} on microbial activity (Li et al., 2008).

Carbon nanotubes have also been shown to exhibit antimicrobial activity (Kang et al., 2007; Simon-Deckers et al., 2009), with membrane damage resulting from direct contact with single-walled nanotubes as the likely mechanism for cell death (Kang et al., 2007). It has also been shown that sensitivity to carbon nanotubes is dependent on the strain of bacteria. Exposure to both unpurified and purified multiwalled carbon nanotubes at 100 mg L^{-1} caused a 50% reduction in survival of *E. coli* but no change to the survival of cultures of *Cupriavidus metallidurans* CH34 (Simon-Deckers et al., 2009).

The antibacterial properties of various metal oxide nanoparticles such as TiO_2, ZnO, CeO_2, and Al_2O_3 are well established. Toxicity of metal oxide nanoparticles to bacteria has been suggested to be dependent on chemical composition, size, surface charge, and shape (Jones et al., 2008; Simon-Deckers et al., 2009; Zhang et al., 2007a), ability to generate reactive oxygen species (Adams et al., 2006; Verran et al., 2007) or cause oxidative stress (Thill et al., 2006), and their photocatalytic activity (Adams et al., 2006; Jones et al., 2008; Kuhn et al., 2003).

A considerable amount of work has been done on the effects of silver ENPs on bacterial populations given that silver has known antibacterial properties, and the increasing use of silver ENPs in industrial applications and consumer products (Chen and Schluesener, 2008). Like metal oxide nanoparticles, the antibacterial effects of silver nanoparticles have been correlated with production of reactive oxygen species (Choi and Hu, 2008) as well as with the presence of Ag^+ ions on the surface of the particles (Lok et al., 2007). Differences in susceptibility according to the strain of bacteria have also been demonstrated (Jayesh et al., 2008), with nitrifying bacteria shown as particularly sensitive (Choi et al., 2008). Contrasting with this a study examining the effects of silver nanoparticles in estuarine sediments found no evidence in changes in bacterial diversity as a result of exposure (Bradford et al., 2009).

Algal populations also play an important role as primary producers in the aquatic environment. The majority of studies on algae have focussed on establishing toxicity dose-response relationships. Titanium dioxide nanoparticles have been shown be toxic to *Pseudokirchneriella subcapitata* at concentrations of between 1 and 5 mg L^{-1} (Aruoja et al., 2009; Hall et al., 2009) but only at 44 mg L^{-1} for *Desmodesmus subspicatus* (Hund-Rinke and Simon, 2006). In other studies at comparable exposure concentrations, however, an absence of any algal toxicity for TiO_2 exposure has been reported (Griffitt et al., 2008; Velzeboer et al., 2008). Collectively these studies not only suggest potentially differential sensitivities of algal species to TiO_2 exposure, but also illustrate differences in exposure regimes and TiO_2 nanoparticle types may profoundly influence their toxicity to algae.

Dissolved metal ions arising from various nanoparticle types, rather than the nanoparticles themselves, has been implicated as the cause of toxicity in studies conducted on *P. subcapitata* (Aruoja et al., 2009; Franklin et al., 2007; Griffitt et al., 2008) and *Chlamydomonas reinhardtii* (Navarro et al., 2008). Nevertheless, Navarro and colleagues established that not all the toxicity they observed in an exposure of *C. reinhardtii* to silver nanoparticles could be attributed to Ag^+.

These studies on exposures of microorganisms and algae to nanoparticles suggest that many nanoparticle types have the potential to have adverse effects and, for microorganisms, to affect population composition. Such effects could clearly in turn have implications for higher organisms living in those environments.

Effects of nanoparticles in aquatic invertebrates

Daphnid organisms are filter feeders and are thus especially susceptible to many xenobiotics compared with other pelagic aquatic animals. Daphnids are, in turn, used routinely as bioindicators for pollutants in aquatic systems and for aquatic toxicity testing. Exposure of daphnids, and other aquatic invertebrates, to carbon-based nanoparticles has been associated with a number of detrimental effects. These effects have often been linked to the chemical nature of the nanoparticles but sometimes also the preparation method for the nanoparticles. As an example, fullerenes prepared by either sonication of the exposure medium or filtered in tetrahydrofuran (THF) with subsequent evaporation of the THF have been found to cause significant mortality in exposed *Daphnia magna*. Filtered fullerenes in THF, however, were shown to be markedly more toxic, causing 100% mortality at 800 ppb, compared with sonicated fullerenes in aqueous solutions, where there was a 65% mortality at the highest tested dose of 9 ppm. Total removal of the THF from the exposure medium was not demonstrated, so it is possible that some of the toxicity observed

in the filtered fullerene may have been attributable to THF (Lovern and Klaper, 2006). In another study with *D. magna*, by the same research group, exposure to fullerene dispersed in THF affected heart rate, and exposure to fullerene/THF or a water-soluble fullerene ($C_{60}H_xC_{70}H_x$) induced alterations in behaviours and this was associated with increased risk for predation and reproductive decline. In this case the elimination of THF from the exposure medium was demonstrated, suggesting the biological effects seen were in fact due to the fullerene compounds and not THF (Lovern et al., 2007).

Fullerene suspensions (prepared by stirring) have also been found to cause a delay in moulting and a reduced number of offspring at exposure concentrations of 2.5 and 5 ppm, respectively, after a 21-day exposure (Oberdörster et al., 2006). Exposures of the estuarine meiobenthic copepod *Amphiascus tenuiremis* to fractions of 'as prepared' single-walled carbon nanotubes (SWCNTs) was shown to cause increased mortality, reduced fertilisation rates, and reduced moulting success (Templeton et al., 2006), but none of these effects were shown for exposure to purified SWCNTs, suggesting that effects were due to the preparation method and not ENPs.

Considering metal oxide–based ENPs, exposures of daphnids to TiO_2 nanoparticles have shown varied results with respect to toxicity, which, like the carbon-based nanoparticles, tends to vary with both the preparation method for the TiO_2 exposure and the physicochemical characteristics of the particles themselves. In one study, sonicated TiO_2 nanoparticles were shown to cause a 9% mortality in *Daphnia magna* at 500 ppm whereas TiO_2 filtered in THF had an LC_{50} of 5.5 ppm and caused 100% mortality at 10 ppm (Lovern and Klaper, 2006). In another study, 25-nm TiO_2 nanoparticles (NPs) comprised predominantly of anatase TiO_2 crystals and 100-nm 100% anatase TiO_2 were both found to cause immobilisation of exposed daphnia, although the 25-nm NPs were markedly more potent at comparable concentrations (Hund-Rinke and Simon, 2006). Illumination of the exposure vessels containing these daphnids at 250 W increased immobilisation rates (73% for the 25-nm particles [2.5 mg L^{-1}] and 30% for the 100-nm TiO_2 particles [1.5 mg L^{-1}]) (Hund-Rinke and Simon, 2006). Contrasting with these findings, other studies with *Daphnia magna* have shown little effect of exposure to TiO_2 nanoparticles. Examples include exposure to 2 ppm 30-nm TiO_2 particles caused no change in either a suite of behaviours or heart rate (Lovern et al., 2007), and exposures to 7-nm and 20-nm TiO_2 particles at 1 mg ml^{-1} caused no effects on reproduction or mortality (Lee et al., 2009). In the same study, exposures to cerium oxide nanoparticles (15 nm and 30 nm) were found to induce DNA strand breaks in *D. magna*. Parallel studies with those conducted in *D. Magna* and adopting identical exposure concentrations found no effect on reproduction, growth, or mortality in adult/larval aquatic midges *Chironomus riparius* (Lee et al., 2009). A recent study on the marine polychaete *Arenicola marina* showed uptake of TiO_2 particles (25 nm) from the sediment via the alimentary canal, but provided no evidence for transfer across the gut wall (Galloway et al., 2009). Limited work has been carried out on the effects of other nanoparticle types in aquatic invertebrates; however, an exposure of *Ceriodaphnia dubia* to quantum dots showed no mortality for concentrations up to 110 ppb (Bouldin et al., 2008). These authors did, however, show transfer of quantum dots to *C. dubia* from dosed algae, illustrating the potential for trophic transfer (Bouldin et al., 2008).

In summary effects assessments for ENPs on microbes, algae, and aquatic invertebrates vary widely, even for a single ENP type. Although there is some evidence for adverse effects, there has been a lack of consistency in these observations, which may derive from differences in the materials used in the different laboratories conducting these studies (generally there has been a lack of comprehensive characterisation data to make direct comparisons of the exposure scenarios), and throughout, effects reported occur at concentrations exceeding anything that is likely to occur in natural waters.

Effects of nanomaterials in fish

Fish are widely used as sentinels for chemical exposure and effects in the aquatic environment and in chemical testing guidelines, and therefore, not surprisingly, have been adopted for assessing ENPs in the aquatic environment. Potential routes of uptake for ENPs in fish include absorption/uptake from the water, or sediment for demersal species, via the gill or skin epithelia, or as a result of dietary exposure and drinking or via the gut epithelia (Handy et al., 2008).

Carbon nanomaterials

One of the first in vivo exposure studies of fish to ENPs was with fullerenes. Exposing juvenile largemouth bass *Micropterus salmoides* (Oberdörster, 2004) was reported to induce oxidative stress in the brain. In that study, however, THF was used as a dispersion solvent and the amount of residual THF in the exposure water, which could complicate the biological effects analysis, was not quantified. Considering the solvent issue further, Henry et al. (2007) undertook an exposure of larval zebrafish, to fullerenes dispersed in THF, with parallel controls of THF that was subsequently evaporated off prior to the fish exposures. They showed that a THF oxidation product, γ-butyrolactone, was produced that had an LC_{50} of 47 ppm, suggesting that the effects observed in fish exposed to fullerene prepared in this way may be attributable to this degradation product, rather than the fullerenes. In another study where zebrafish were exposed to fullerenes prepared in solvents (benzene, acetone, and THF), effects reported included delays in embryonic and larval development, decreased survival and larval hatch rates and pericardial oedema (Zhu et al., 2007), but again the solvent preparation method may have contributed to the effects seen. Exposure of fathead minnow to fullerenes prepared by a long-term stirring method (solvent free) found no effects on a suite of cytochrome P450 (CYP) enzymes (CYP1A, CYP2K1, CYP2M1) in the livers of exposed fish (Oberdorster et al., 2006). Shinohara et al. (2009) showed that there was no uptake of fullerenes (4.5 mg L^{-1}), prepared solvent free, into the brains of exposed European carp *Cyprinus carpio*, but lipid peroxidation was

induced when the fullerene preparation was applied directly to a carp brain homogenates in vitro. Chronic exposure (32 days) of goldfish *Carassius auratus* to a solvent-free preparation of fullerenes (0.04–1.0 mg L^{-1}) resulted in significant induction of antioxidant enzymes superoxide dismutase and catalase and depletion of GSH in the gills and liver. Lipid peroxidation was increased only in the liver and there was an inhibitory effect on fish growth (Zhu et al., 2008b). It is difficult to harmonise the findings from the studies undertaken on fullerenes, again because of differences in particle preparation, exposure methods, and limited particle characterisations. Some of the most comprehensive in vitro studies, undertaken by Zhu et al. (2008b), suggest that oxidative stress is a likely effect in some biological compartments, but whether fullerene uptake in vivo into any body tissue is sufficient to induce harmful effects has not been proven for any exposure. Also, again without exception, the exposure regimes far exceed anything that is likely to be found in the natural environment, even in the most polluted waters.

There are now a series of studies that have investigated the biological effects of carbon nanotubes in zebrafish embryos. SWCNTs and double-walled carbon nanotubes (DWCNTs) have been shown caused hatching delay at concentrations over 120 and 240 mg L^{-1}, respectively; however, even at these huge exposure concentrations there were no effects on embryo morphology and 99% of the embryos hatched by 72 hour post fertilisation (hpf). The delay to hatching was attributed to trace levels of residual cobalt and nickel catalysts in the CNTs. Carbon black nanoparticles were similarly found not to affect hatching at similar exposure concentrations (Cheng et al., 2007). Exposure of the larvae of the amphibian *Xenopus laevis* to DWCNTs at very high concentrations found no genotoxicity and the acute toxicity seen at all concentrations was related to physical blockages of the gills and digestive tract (Mouchet et al., 2008).

In contrast with the above studies, an exposure of juvenile rainbow trout *Oncorhynchus mykiss* via the water to SWCNTs has been reported to induce a dose-dependent increases in mucus secretion, oedema, altered mucocytes, and hyperplasia in the gills as well as an increase in Na^+,K^+-ATPase activity in this tissue at concentrations of 0.1, 0.25, or 0.5 mg L^{-1} (Smith et al., 2007). Thiobarbituric acid reactive substances (TBARS) were decreased in the gill, brain, and liver and increases in glutathione (GSH) were noted in the gills and livers, suggesting evidence of lipid peroxidation and oxidative stress in these tissues (Smith et al., 2007).

Studies on fish embryos with MWCNTs have shown dose-dependent effects on mortality and hatching success and tissue level effects, including deformation of the notochord, bradycardia, slowed blood flow, and apoptosis (Asharani et al., 2008). At an exposure of 200 μg MWCNT mL^{-1} there was 100% mortality. Microinjection of fluorescently labeled MWCNTs into zebrafish embryos at the 1-cell stage found no effects either on larval development or on adult reproduction; however, the survival of the second generation larvae to 14 days post hatch was 50% lower by than in control fish.

The results of these studies suggest that some carbon-based nanoparticles, such as fullerenes and nanotubes, have the capacity to induce toxicity to aquatic vertebrates, both as a function of their chemistry by inducing oxidative stress and lipid peroxidation and as a result of their aggregation causing physical blockages (Mouchet et al., 2008), but not at concentrations likely to be found in most (if not, any) aquatic environment.

Metal/metal oxide nanoparticles

Exposures to TiO_2 nanoparticles have so far found them to be relatively non-toxic to fish. Studies by Griffitt et al. (2008) found no effects in zebrafish embryos or in adults exposed to 30 nm TiO_2 (20% rutile and 80% anatase crystals) at concentrations up to 10 mg L^{-1} and 1000 μg L^{-1}, respectively. Other studies have reached similar conclusions, showing a lack of toxicity of TiO_2 to zebrafish embryos and larvae (Zhu et al., 2008). Hall et al. (2009) recently calculated an LC_{50} for TiO_2 nanoparticles greater than 500 mg L^{-1} in fathead minnow *Pimephales promelas*.

Contrasting with these collective findings, Federici et al. (2007) reported that a 14-day semi-static exposure of rainbow trout to comparable concentrations of TiO_2 nanoparticles reported in the studies above resulted in gill oedema and thickening of the gill lamellae, as well as decreases in Na^+,K^+-ATPase activity in the gills and intestine. Concentration-dependent increases of TBARS in the gills, intestine, and brain were reported along with increases in GSH in the gills, suggesting evidence of oxidative stress, although a depletion of GSH was observed in the liver (Federici et al., 2007). This study reported transient and marginal increases in the content of liver and spleen TiO_2, but this was complicated by the fact that levels were close to the detection limits for the analytical approach used, an issue of key concern for all studies of this nature on TiO_2. In another study that exposed rainbow trout to TiO_2 via the water, at concentrations up to 5000 μg L^{-1}, found no uptake into a wide range of tissues (gills, liver, blood, brain, gut, skin), or any evidence for biological effects (Johnston et al., 2010). Moger et al. (2008) used coherent anti-Stokes Raman scattering (CARS) as an imaging technique to demonstrate that the gills are capable of taking up TiO_2 from the water, albeit at an extremely low rate, isolating TiO_2 within gill cells. TiO_2 nanoparticles have also been measured in the gills and gut of carp exposed simultaneously to TiO_2 with heavy metals (arsenic and cadmium), but the heavy metals are likely to have affected the uptake of TiO_2 (Sun et al., 2009; Zhang et al., 2007b). In oral exposures of rainbow trout to TiO_2 nanoparticles (up to 100 mg kg^{-1}) no accumulation of titanium in the blood, brain, gills, skin, liver, or gall bladder was observed and there were no significant effects on growth, haematological parameters or TBARS in the gill, intestine, or liver across the studies combined (Handy et al., 2008; Johnston et al., 2010). Overall, there are no convincing data that exposure to TiO_2, via the water or diet, at realistic exposure concentrations have obvious health implications for fish.

Silver nanoparticles

Exposure of zebrafish embryos to silver nanoparticles has been shown to induce dose-dependent increases in mortality and cause hatching delays, deformations of the notochord (including expression of Sel N1, a gene associated with notochord development), slow blood flow, and induce pericardial oedema and cardiac arrhythmia (for particles between 5 and 20 nm (Asharani et al., 2008b; Yeo and Kang, 2008). In their work Asharani et al. capped the silver particles with starch or bovine serum albumin (BSA) to aid dispersion.

Unlike the aggregates of carbon nanotubes, which appear unable to pass through chorion pores, silver nanoparticles of 5 and 46 nm have been shown to be transported in and out of chorion pore channels by Brownian diffusion (Lee et al., 2007). Indeed, Asharani et al. (2008b) used transmission electron microscopy (TEM) to show the presence of silver nanoparticles in the brain, heart, yolk, and blood in silver nanoparticle–exposed zebrafish embryos. An issue of uncertainty with respect to the toxicity of silver nanoparticles via aqueous expose is whether it is the particles themselves that are toxic, the silver ions they release, or a combination of both. Yeo and Kang (2008) in their work confirmed the presence of silver ions (Ag^+) in the exposure media to which they attributed the detrimental effects on the zebrafish embryos. In another study, silver ions were found to be over 300 times more toxic to zebrafish fry (on a mass basis) compared with silver nanoparticles (Griffitt et al., 2008). A very recent study applying global gene expression in the gills found a different response between nanoparticle-exposed fish and fish exposed to soluble silver ions, suggesting that the biological effects of exposure to silver nanoparticles may not be driven solely by the release of silver ion (Griffitt et al., 2009). It does appear, therefore, that silver nanoparticles can induce harm in exposed fish, but whether this is a function of their release of dissolved silver, or there is a true and direct nano-toxicity effect is still uncertain.

Copper nanoparticles

Exposure of zebrafish via the water to copper nanoparticles (80 nm) has been found to induce gill damage and cause dose-dependent decreases in Na^+,K^+-ATPase activity (0.25–1.5 mg L^{-1}), with a 48-hour LC_{50} value of 1.5 mg L^{-1} (Griffitt et al., 2007). These researchers observed a rapid aggregation and subsequent settling of the particles, with 50–60% of the particles sedimenting out from the water column. Although some dissolution of the copper occurred, it was not sufficient to explain the mortality observed. A further exposure of zebrafish to either 100 μg L^{-1} copper nanoparticles or to the corresponding concentration of copper ions released due to dissolution showed that the nanoparticles produced greater proliferation or hypertrophy of epithelial cells and differing gene expression patterns in the gills than seen with soluble copper (Griffitt et al., 2007).

Ceria nanoparticles

An exposure of zebrafish to cerium dioxide nanoparticles via the water (semi-static for concentrations of 0.5–5 mg L^{-1}) provided no evidence for uptake into the brain, gills, or skin, or any obvious biological effects (Johnston et al., 2010).

Aluminium nanoparticles

In a single study on zebrafish exposed to aluminium nanoparticles (static water for 72 hours), up to concentrations up to 500 g L^{-1}, there was evidence of gut-ingested nanoparticles and a reduced gill ATPase activity, indicating compromised gill function (Barber et al., 2005). Uptake of metal/metal oxide nanoparticles via the gut for exposures via the water has also been reported for studies on ceria in zebrafish (Johnston et al., 2010), where fish were observed to ingest aggregated cerium particles from the bottom of the tank. These findings highlight the need for care when considering/reporting uptake routes for ENPs dosed via the water.

Studies with other nanoparticle types

Various other nanoparticles have been adopted in fish exposure studies to investigate the importance of size in particle uptake. In studies with embryonic medaka (*Oryzias latipes*), fluorescent latex nanoparticles were found to adsorb to the chorion of eggs and accumulate in oil droplets, enter the yolk and the gallbladder of the embryos, and uptake was found to vary according to particle size. Salinity was found to influence the increase in toxicity-associated mortality, but this may have been as a function of the embryos experiencing overt stress due to high salt concentrations, rather than an effect directly on the nanoparticle toxicity. In adult see-through medaka exposure to these fluorescent latex nanoparticles resulted in an accumulation in the gills and intestine, although they were also detected in the testis, liver, blood, and brain (indicating that they are able to cross the blood-brain barrier; Kashiwada, 2006). Surface coatings have been shown to influence toxicity of quantum dots in zebrafish embryos and also to influence the stability of the quantum dots in the exposure media. The toxicity observed was characteristic of that observed with exposure to cadmium (core material of quantum dots) and correlated weakly with metallothionein expression; however, not all toxicity observed could be explained simply by release of cadmium (King-Heiden et al., 2009).

Exposure studies of aquatic organisms to date have demonstrated that ENPs may induce a wide range of biological effects. Both carbon-based and metallic nanoparticles have been shown to exhibit toxicity in fish embryos characterised by developmental abnormalities and mortality, whereas TiO_2 nanoparticles have shown limited toxicity to either embryonic or adult fish. In a number of studies, the dissolution of metal ions from nanoparticles into the exposure media, or the presence of metal impurities, has been implicated as important factor in the toxicity of ENPs in aquatic exposure systems. Drawing commonalities between the aquatic exposure studies reported upon at this stage, however, is complicated by the lack of standardisation in exposure methods and nanoparticle preparations and the lack of ENP characterisation data in some studies. The importance of both characterisation of nanomaterials in exposure studies and the issue of standardisation between studies is beginning to be recognised.

Standardised approaches for nanoparticle effects assessments

In order to conduct comparative analyses of exposure studies, the development of standardised test methods is imperative. Currently, developing knowledge on the ecotoxciology of nanoparticles is complicated by the fact there is an almost unlimited variety of nanoparticles available that can be modified by a variety of coatings or functionalisations to make them suited to a required purpose. This, coupled with the numerous methods used for preparing nanoparticles for biological exposure experiments, such as the use of solvents or biologically compatible capping agents that aid dispersion, means finding commonalities between existing exposure studies and drawing firm conclusions on biological effects, relative potencies, etc., presents a considerable challenge, to say the very least. This will continue to be the case into the future, especially without standardised testing approaches. Efforts are underway to promote cooperation between research groups around the world in establishing data sets covering environmental toxicity and fate, materials characterisation and physical-chemical property and safety endpoints, as well as standard environmental health and safety test guidelines (European Commission, 2007; OECD, 2009).

Ideally test systems are needed that can inform upon hazards for a wide range of species. Many more studies have examined the toxicity of ENPs in mammals and this information can be used to inform (or help direct) studies applied to other vertebrates, to maximise meaningful hazard identification of ENPs, supported by the fact that similarities have been found for exposure effects in cell models, mammalian models, and aquatic organisms. Exposure of various fish species to metal, metal oxide, and carbon-based nanoparticles has often been characterised by oxidative stress and the induction of lipid peroxidation, as in many mammalian, including in vitro, studies (Gurr et al., 2005; Park et al., 2008; Shwe et al., 2006; Stoeger et al., 2006; Warheit et al., 2004). In both fish and mammals carbon-based nanoparticles have been shown to induce developmental abnormalities in embryos (Tsuchiya et al., 1996). In both mammals and fish (although studies are limited for fish) ENPs translocated around the body most commonly partition to the liver, kidney, and brain (Cagle et al., 1999; Li et al., 2002; Oberdörster et al., 2002, 2004; Olmedo et al., 2008; Sugibayashi et al., 2008).

Of course when considering the potential for read-across for effects from one species/organism to another in the environment, it is important to realise that the characteristics and behaviour of ENPs are profoundly affected by the type and composition of the exposure medium. The physicochemical nature of a particular nanoparticle taken up by a terrestrial organism exposed via the air may, therefore, be very different from the nature of that same particle when exposed via water to an aquatic organism. Similarly, the behaviour of a nanoparticle exposed to cells in vitro in cell culture media may be different to its behaviour in water. Differing routes of exposure between terrestrial and aquatic organisms may also mean that the target organs for ENP exposure are different. Furthermore, direct comparison between perceived major target organs, e.g. lungs in mammals and gills in fish, is difficult, as although their primary function is the same, they differ markedly in their structure.

Dose metrics and particle features affecting toxicity

In the development of appropriate tests for ENPs, it is essential that standardised dose metrics (i.e. the measure of a dose) are established to allow for comparisons between studies and to elucidate relative toxicities of ENP types. The use of parts or mass per unit volume or molar concentrations, as used in exposures to conventional xenobiotic compounds, may not be appropriate in the case for exposures to ENPs. A review by Oberdörster (1996) brought this issue to light after examining findings from chronic inhalation studies. Large concentration ranges from a few milligrams per cubic meter up to 250 mg m^{-3} of insoluble, low-cytotoxicity ultrafine particles were found to induce similar adverse effects, including impaired lung clearance, chronic pulmonary inflammation, pulmonary fibrosis, and lung tumours, within the lungs of study animals. This suggested that the particles inhaled differed significantly in their toxicity, and the response seen was governed by factors other than simply mass per unit volume dose. Widespread discussions suggest that assessments on particle uptake into cells may better explain the responses seen in exposure to nanoparticles (Wittmaack, 2007).

Size and surface area

Size, and therefore surface area, have been implicated as key factors influencing the toxicity of nanoparticles. Inhalation studies in rodents and in vitro studies have in general indicated that smaller nanoparticles generate greater inflammatory responses than larger particles (Brown et al., 2001; Inoue et al., 2009; Singh et al., 2004). Although size and surface area are related, size may, however, not be an accurate dose metric. Instillation studies in mouse and rat models have shown that although smaller-sized nanoparticles cause a greater inflammatory response than larger-sized particles on a mass basis, the level of inflammatory response was dependent on the total surface area of particles instilled (Brown et al., 2001; Oberdörster, 2000; Stoeger et al., 2006; Yamamoto et al., 2006). In vitro studies with mammalian cells have also shown that the size of a particle, and therefore likely its surface area, may play a role in mediating response, demonstrating that smaller-sized particles are more toxic (Karlsson et al., 2009; Renwick et al., 2004; Tamura et al., 2004) than larger-sized particles and there is a size-dependent generation of ROS (Cagle et al., 1999; Choi and Hu, 2008).

In non-mammalian systems, particle size has been indicated to affect its relative toxicity. The size of a number of different nanoparticle types, including TiO_2 and polystyrene, has been shown to affect their toxicity to bacteria and mammalian cell lines (Mayer et al., 2009; Singh et al., 2004; Verran

et al., 2007), although other factors such as ability to generate free radicals (Verran et al., 2007), particle surface charge (Mayer et al., 2009), and particle-cell interaction (Singh et al., 2004) have been shown to be involved also.

In exposure studies with fish, association of particles in the tissues was found to depend on both the size of the nanoparticle and the tissue examined. In rainbow trout, 10-nm silver particles were found to associate more readily with gill tissue, whereas particles of 600–1600 nm were more readily taken up into the liver (Scown et al., 2009). Size may also potentially be a factor in determining particle transport in biological systems. Kreyling et al. showed smaller-sized iridium nanoparticles were more readily transported from the lung epithelium to extrapulmonary organs compared with larger nanoparticles (Kreyling et al., 2002); however, another study showed there was no size-related difference in the transport of TiO_2 particles in a tracheal explants (Churg et al., 1998).

Surface chemistry

Several studies by Warheit et al. have indicated that neither size nor surface area may be accurate dose metrics and that the surface chemistry of a nanoparticle is the dominant factor in determining toxicity. Rat lungs instilled with nanoscale and bulk TiO_2 particles showed no differences in pulmonary effects (Warheit et al., 2006), but it was subsequently demonstrated that exposure to nanoparticulate TiO_2 composed of both anatase and rutile crystal types caused cytotoxicity, whereas exposure to micro- and nano-sized rutile TiO_2 particles produced only transient inflammation. This suggested differing crystal structure type or surface reactivity between particles were responsible for the differences in effects (Warheit et al., 2007b). Crystal phase–dependent toxicity was similarly demonstrated in human dermal fibroblasts and human lung epithelial cells where anatase TiO_2 was found to be 100 times more toxic than rutile TiO_2 at the same mass dose (Sayes et al., 2006). In agreement with this, a further study found that intra-tracheal exposures of rats to mined and synthetic quartz particles of varying sizes produced effects that were correlated with surface activity such as haemolytic potential (Warheit et al., 2007a).

Functional groups

Chemical functionalisation of the surface of nanoparticles, particularly carbon-based nanoparticles, not surprisingly, has been shown to alter toxicity in vitro. Increasing derivatisation of the surface of fullerenes has been shown to be associated with a decrease in toxic effect in two human cell lines, with a pristine fullerene being around 3 orders of magnitude more toxic than a polyhydroxylated fullerene (Sayes et al., 2004). Different mechanisms of toxicity have also been observed between functionalised and non-functionalised fullerenes, where pristine fullerenes induced ROS causing cell death and functionalised fullerenes caused cell death by apoptosis (Isakovic et al., 2006). Functionalisation of the surface of carbon nanotubes was found to increase their cytotoxicity (Magrez et al., 2006).

Coatings

The addition of surface coatings to ENPs can aid their dispersion in aquatic media or make them more biologically compatible, or aid their embedding into inert matrices to improve their function, but this also has implications for their potential to induce biological effects.

In an exposure of iron-doped silica and pure iron oxide nanoparticles to human lung epithelial cells, the pure iron oxide particles produced a weaker induction of ROS than the iron-doped silica despite containing 20–100 times more iron. The authors suggested this was due to increased catalytic activity of the transition metal sites on the surface of the iron-doped silica compared to in the pure iron oxide nanoparticles (Limbach et al., 2007). Partial oxidation or modification of nano-sized zero-valent iron with a polyaspartate surface coating, however, was found to decrease the toxicity to cultured rodent microglia and neurons (Phenrat et al., 2009).

The surface coating on quantum dots has been found to influence the interaction of nanoparticles with cells and subsequent toxic effect. Organic-coated quantum dots have been found to be the more toxic to murine macrophages than uncoated particles. Carboxylated quantum dots were found to be readily taken up by these murine cells but had little impact on cell viability. Amine-poly(ethylene glycol) (PEG) quantum dots showed slower uptake compared with other coating types tested (Clift et al., 2008).

Coating type has also been demonstrated to influence cytotoxicity for gold nanoparticles. Triphenylphosphine-stabilised gold particles have been shown to exhibit size-dependent toxicity in a range of human cell lines (Pan et al., 2007); however, PEG-stabilised gold particles showed no obvious cytotoxicity in human cervical cancer cells despite uptake into the cytoplasm and nuclei (Gu et al., 2009). In contrast, however, one study has shown that the inflammatory effects of TiO_2 nanoparticles in lung epithelial cells were not altered by modification of the particle surface by methylation, and related the dose-dependent toxicity observed to the surface area of particles applied (Singh et al., 2004).

Charge and aggregation

Modification of ENPs by the addition of functional groups or coating is likely to alter the charge on the nanoparticle. The stability and aggregation behaviour of ENPs within aquatic media is determined by both by physicochemical properties of the media, and the charge on the surface of the ENPs. Charge and aggregation are, therefore, likely to be key factors in determining the exposure and uptake of nanoparticles in aquatic organisms.

The degree of aggregation of nanoparticles has been shown in some cases to affect toxicity in vitro, for example, rope-like agglomerates of SWCNTs were found to be more cytotoxic to human mesothelioma cells by mass than asbestos, whereas dispersed SWCNTs produced a lower toxic response than asbestos (Wick et al., 2007). The uptake of cerium oxide nanoparticles into human lung fibroblasts was found to be correlated with relative particle size as a result of the aggregation behaviour. Larger particles were found to agglomerate

more slowly than smaller particles, enabling them to penetrate cells more efficiently before sedimentation occurred (Limbach et al., 2005).

Charge in itself has been implicated as a factor influencing ENP transport and toxicity. Wax nanoparticles with neutral charge and low concentration of anionic nanoparticles applied to an in situ rat brain perfusion had no effect on the blood-brain barrier integrity; however, high concentrations of anionic nanoparticles and cationic nanoparticles caused disruption to the blood-brain barrier, with uptake of anionic nanoparticles higher than either neutral or cationic nanoparticles at the same concentrations (Lockman et al., 2004). Both size and surface charge of polystyrene nanoparticles have been shown to affect haemocompatibility, with negatively charged particles larger than 60 nm in diameter being less toxic than smaller-sized particles (Mayer et al., 2009).

Particle chemistry and solubility

Many factors relating to the physical chemistry of the nanoparticles have been shown to be important in determining their effects on biological systems. Nanoparticles of different chemical compositions of course can, and have been demonstrated to, have differing biological effects (Griffitt et al., 2008; Renwick et al., 2004); however, other chemical factors such as the ability of a nanoparticle to generate reactive species (Sayes et al., 2006; Verran et al., 2007; Xia et al., 2006) and the oxidation state of the nanoparticle (Wörle-Knirsch et al., 2007) have also been demonstrated to affect toxicity.

The solubility of the nanoparticle may also be a significant factor in determining toxicity. The toxicity of a range of seven oxide nanoparticle types to a rodent fibroblast cell line was attributed to the surface and shape of the particle and its composition and degree of solubility (Brunner et al., 2006). Furthermore, works by Gagné et al. (2008) and Griffitt et al. (2007, 2009) have shown that the toxicity of both copper and silver nanoparticles to zebrafish can be attributed in part by dissolution of ions in the exposure media.

Navarro et al. (2008) examined the contribution to toxicity of silver ions from silver nanoparticles in *Chlamydomonas reinhardtii* using cysteine to bind free silver ions. Around 1% of the silver present in the silver nanoparticle exposure media was in the form of silver ions and based on total silver concentration, the toxicity was 18 times higher for silver nitrate than for silver nanoparticles. However, when toxicity was compared as a function of silver ion concentration, the silver nanoparticles were more toxic, but this higher toxicity could not be explained by the level of silver ions present. They postulated that interaction of the nanoparticles with the algae influenced the toxicity of the particle by mediating the release of silver ions at the surface of the algal cells.

Shape

The shape of the particle itself may also play an important role in determining its toxicity. Cytotoxicity studies on a murine macrophage cell line tested a range of different nanoparticle types using crysotile asbestos as a positive control. Carbon nanotube aggregates were found to have a similar cytotoxicity index to asbestos, which the authors suggested may be due to the physical similarities between the two particle types (Soto et al., 2005). A similar study also found MWCNTs to cause injury to plasma membranes of mouse macrophages that was similar to damage caused by asbestos (Hirano et al., 2008) and in vivo studies have shown that mice exposed to MWCNTs via inhalation and intraperitoneal injection can cause asbestos-like pathogenic responses (Poland et al., 2008) and induction of mesothelioma (Takagi et al., 2008) and granulomas (Warheit et al., 2004).

Photochemistry

The presence of ultraviolet (UV) light may influence the toxicity of some nanoparticles. A number of metal oxide nanoparticles exhibit photocatalytic activity when exposed to UV light, resulting in the generation of reactive oxygen species in aquatic media, with obvious implications for aquatic organisms.

Increased growth inhibition of *Bacillus subtilis* and *Escherichia coli* cultures was observed in cultures illuminated by sunlight in the presence of TiO_2 and ZnO, compared with those kept in the dark (Adams et al., 2006), and damage to cell structure characterised by a decrease in cellular stiffness was seen in human skin fibroblasts exposed to TiO_2 nanoparticles in the presence of UVA radiation (Vileno et al., 2007).

Genotoxic effects of UV-illuminated TiO_2 nanoparticles have also been reported. The hydroxylation of guanine bases in calf thymus DNA was found to be dependent on the intensity of UVA radiation and the concentration of TiO_2 in the exposure media (Wamer et al., 1997) and in rainbow trout gonadal tissue cells, the presence of UVA radiation in combination with TiO_2 was found to significantly increase toxicity and the number of DNA strand breaks compared to exposure with TiO_2 nanoparticles alone (Vevers and Jha, 2008).

Preparation methods

The method by which the nanoparticles are prepared can also have a profound effect on their biological effects. Unpurified carbon nanotubes often contain significant levels of residual metal catalysts, which have been implicated as the cause of toxic responses in vivo (Cheng et al., 2007; Murray et al., 2009). The methods of preparation of nanoparticles in exposure media, such as the use of solvents like THF to aid the dispersion of hydrophobic carbon-based nanoparticles (Henry et al., 2007) and sonication of fullerenes (Oberdörster et al., 2006), have also been found to influence their toxicity.

Presence of other compounds

The presence of other compounds within the exposure media may also influence the uptake and effects of nanoparticles on cells and biological systems. As the behaviour of nanoparticles in aquatic media is governed by the surface characteristics of the particle, adsorption of other compounds to the surfaces of nanoparticles is likely to have a profound effect on their behaviour within the media and their interaction with biological material.

It has been demonstrated that in biological fluids the adsorption of proteins to the surfaces of nanoparticles occurs, resulting in a protein 'coronas' (Cedervall et al., 2007; Wasado, 2008) and the presence or absence of proteins on the surface of SWCNTs and silica nanoparticles has been found to affect their toxicity.

Albumin-coated SWCNTs were found to inhibit the induction of cyclooxygenase-2 (Cox-2) by lipopolysaccharide in a macrophage-like model cell line, but this anti-inflammatory response was inhibited by treatment of SWCNTs with a non-ionic surfactant that inhibited the absorption of albumin. The profile of proteins adsorbed onto amorphous silica nanoparticles was, however, qualitatively different from those adsorbed to SWCNTs, and a reduction in toxicity was seen when adsorption of proteins to the silica was prevented by the addition of the surfactant (Dutta et al., 2007).

Adsorption of other xenobiotic compounds to the surfaces of nanoparticles may also occur. It has been shown that arsenate and cadmium readily adsorb to the surface of TiO_2 nanoparticles and that uptake of arsenate and cadmium to the tissues of carp is significantly enhanced in the presence of TiO_2 nanoparticles (Sun et al., 2007, 2009; Zhang et al., 2007b); however, it is not yet known how the presence of these adsorbed metal ions may affect the uptake or toxicity of TiO_2 or other nanoparticles to which metal ions might adsorb.

Environmental parameters

In addition to characteristics of the particles in their raw state, the characteristics of the exposure media will influence the behaviour and interaction of nanoparticles with cells and organisms. The pH and composition and concentration of ions in the media are likely to affect the charge on a particle and therefore its aggregation behaviour and likelihood of interaction with cells. The presence of NOM such as humic and fulvic substances and other organic matter that may adsorb to the surfaces of nanoparticles will also be likely to alter their uptake and behaviour in biological tissues.

In summary, a variety of parameters relating to a particle's physical characteristics and composition have been shown to influence the toxicity of different nanoparticles. These parameters, however, are still poorly understood, with many studies producing contradictory results, making predicting effects difficult. In reality, a combination of many particle characteristics, combined with the characteristics of the exposure medium and the behaviour of a particular particle in that exposure medium, must all be considered in nanoparticle exposures, whether in vitro, in vivo, or in nature, and what may be an appropriate dose metric for one nanoparticle type may not necessarily be appropriate for another.

A number of reviews have highlighted the importance of accurate and comprehensive characterisation of nanoparticles and the experimental or environmental media when conducting exposure experiments in order to more fully understand the observed effects (Hassellöv et al., 2008; Powers et al., 2007; Warheit, 2008). Recently, a new initiative called the Minimum Information on Nanoparticle Characterization (MINChar Initiative, 2010) has been created to help develop recommendations for characterisation of nanoparticles used in toxicology studies and to encourage the research community to adopt these recommendations to raise the quality of nanotoxicology research. A Letter to the Editor published in the *Journal of Food Science* (Card and Magnuson, 2009), citing MINChar as a source, proposed a set of nine characterisation parameters for studies on food-related nanomaterials. These included agglomeration and/or aggregation, chemical composition, crystal structure/crystallinity, particle size/size distribution, purity, shape, surface area, surface charge and surface chemistry (including composition and reactivity). These authors are not alone in calling for rigorous characterisation of nanomaterials used in toxicity studies, and we strongly support the adoption of these recommendations for aquatic toxicology studies.

The future

In order to advance significantly our understanding on the ecotoxicology of nanoparticles, there are many issues that need urgent attention. Considering the aquatic environment, studies need to more thoroughly characterise nanoparticles and their behaviour in aquatic media, as this will allow for better effect comparisons across studies. Establishment of routes of uptake, uptake mechanisms, target organs, and potential toxic effects are all needed to start to address the level (or not, as the case may be) of the problem of ENP discharges into the aquatic environment.

A prominent challenge for nanoparticle uptake studies is the development of systems to allow for accurate tracing and quantifying nanoparticle uptake in the tissues, cells, and sub-cellular components of cells in bodies of exposed organisms. Presently, inductively coupled plasma optical emission spectrometry (ICP-OES) and/or inductively coupled plasma mass spectrometry (ICP-MS) are employed to measure and trace metal and metal oxide nanoparticles in tissues; however, there are inherent disadvantages to using these methods. Preparation of samples for ICP-OES/ICP-MS analysis involves acid digestion of the tissue samples, therefore preventing further histological work or toxicity assays for that tissue. Some metal oxides, e.g. TiO_2, are difficult to digest, resulting in low recovery rates, and high background levels of other metals in animal tissue (e.g. zinc) may prevent measurement of low levels of uptake. Also, ICP-OES/ICP-MS does not give information about the form of the metal present in the sample before preparation. This is a particular issue for metallic nanoparticles that may undergo some dissolution in water, as the proportion of dissolved ions released from the nanoparticles into the media cannot be distinguished using this method without prior filtration, so the relative importance of nanoparticles and/or ions in terms of uptake and mediating toxic response cannot be determined.

Methods presently used to visualise particles in tissues include confocal microscopy, transmission electron microscopy (TEM), and coherent anti-Stokes Raman scattering (CARS) microscopy, all of which have advantages and disadvantages. Whilst TEM has sufficient resolution to vizualise

individual particles, samples require extensive preparation, which may alter the position of particles within the cells, and the resolution is limited to two dimensions. Confocal microscopy requires less invasive sample preparation but relies on either auto-fluorescence of the particle or the use of fluorescent tags or coatings to trace the particles and these have the potential to significantly alter particle behaviour. CARS microscopy allows for three-dimensional visualisation of a variety of metal oxide nanoparticles in intact biological tissue and requires little sample preparation (Moger et al., 2008); however, it is limited in its ability to resolve sub-cellular structures and it is not yet known if it can detect non-metallic nanoparticles.

All of these methods are limited for their capacity in making quantitative measurements of tissue burden, but the use of stable isotopes (elemental forms of differing neutron number) offers a practical method for both tracing nanoparticles and making quantitative measurements of nanoparticles in tissues (Gulson and Wong, 2006). The use of stable isotopes is particularly useful for tracing uptake of elements with a high natural tissue background level and have been used effectively to measure dermal absorption of zinc oxide (Gulson et al., 2008). Their use to date, however, has been limited due to availability of nano-sized stable isotopic compounds and their high cost. Radio-labelled nanoparticles also provide a good method for quantitative uptake tracing of nanoparticles in biological tissues and have been used to measure uptake of fullerene derivatives and identify target organs in exposed mice and rabbits (Li et al., 2002). As with stable isotopes, however, there is limited availability of radio-labelled nanoparticles and, with respect to exposures of fish, there would be safety concerns surrounding the use of large volumes of radioisotope-contaminated water. For aqueous exposures, a major issue is whether or not to employ chemical dispersants or solvents in order to achieve monodispersed suspensions of nanoparticles. Understanding whether nanoparticles in their 'raw' state are capable of eliciting toxic responses as a result of waterborne exposure is still a pressing concern, and the use of such agents, as well as changing the fate and behaviour of ENPs in the water column, may also change their uptake and distribution within the tissues or introduce compounding factors such as inherent toxicity or mixture effects. That said, many industrial and consumer-based nanoparticle preparations are likely to employ the use of coatings, dispersants, or solvents to achieve monodisperse suspensions, and thus it could be argued that little raw ENPs will enter the aquatic environment. From this perspective it could be argued that coated particles are of more immediate environmental relevance. There is as yet very little information in the literature regarding present or projected loads of ENPs in aquatic systems, however, or in what forms or preparations (i.e. powders, suspensions, coated/uncoated, etc.) they are likely to be released into the environment. At this stage, it is therefore difficult to design exposures with direct environmental relevance.

It is clear that the differences in the way ENPs behave in aquatic media compared to conventional xenobiotics requires adopting different approaches for aquatic exposures to ENPs. In aquatic exposures involving conventional xenobiotic compounds, organisms are exposed to test chemicals dissolved in the water or in a solvent, which is then added to the water. The use of flow-through systems provides a constant replacement of water and test chemical, which allows for the maintenance of optimal water chemistry conditions, good homogeneity of the chemical in experimental tanks, and a good match between nominal dosing concentrations and actual water concentrations for the duration of the exposure. Due to the propensity of many ENP types to aggregate and settle out of suspension in aquatic media, the use of flow-through systems for exposure of aquatic organisms to nanoparticles is impractical. Semi-static exposures with frequent water changes are adequate, but not ideal, and due to the less than optimal water quality and frequent disturbances, organisms may incur increased levels of stress that potentially make them more susceptible to uptake of compounds in the water column.

Aggregation behaviour of nanoparticles in water also means that nominal dosing concentrations will not necessarily reflect the concentrations that are bioavailable to organisms (indeed they are unlikely to do so). Similarly, measurements of primary particle size will not reflect the sizes of particles to which aquatic organisms are necessarily exposed and, in addition, the possible interactions of ENPs with NOM present in the water such as mucus exudates, faecal material, and other material of biological origin mean that ascertaining exactly what the organisms are being exposed to in terms of particle composition is difficult. How much of the nanoparticle remains suspended in the water column and how much settles into the benthic zone will determine not only the bioavailability of the nanoparticles to aquatic organisms, but also the type of organisms likely to be exposed and is also likely to determine the route and mechanism of uptake into the bodies of aquatic organisms.

It is clear, therefore, from the increasing number of studies in the literature that understanding what organisms are exposed to during an aquatic exposure to ENPs is an important issue for the progression of aquatic nanotoxicology. An essential element of future aquatic nanotoxicity studies is rigorous characterisation of ENPs, experimental exposure media, and characterisation of the ENPs in the media to gain a better understanding of the aggregation behaviour of the nanoparticles and what is bioavailable to the organisms or cells during the exposure. Such characterisation should involve measurements of the physicochemical characteristics of the nanoparticles, such as composition (including levels of impurities), size, shape, charge, and applied coating, as well as measurements of ionic composition, pH, concentration of NOM, and temperature of the exposure media. The behaviour of nanoparticles in the exposure media should also be examined, including measurement of the dissolution of ions from nanoparticles into the media, the size distribution of aggregates that form, the concentration of nanoparticles

remaining in suspension, and the charge on the aggregates. The formation of coatings on ENPs and ENP aggregates through interactions of nanoparticles with organic elements in the exposure media (e.g. fulvic and humic substances) should also be studied, as these are likely to have an impact on the aggregation and uptake behaviour.

To undertake all of the above requires a multi-disciplinary approach involving many different analytical techniques and expertise across diverse scientific disciplines. Nevertheless, such an approach is essential if we are to fully comprehend the likely bioavailability of nanoparticles to aquatic organisms, elucidate routes of exposure and uptake mechanisms, and make predictions as to their potential environmental impact. It will also give us a better understanding of the way nanoparticles are likely to behave and be transported in the bodies of organisms if they are taken up.

Due to the increasing numbers of different types of nanoparticles in existence and the multitude of ways in which they can be modified for their desired use, there are important practical considerations for those working to understand ENP toxicology and ecotoxicology. Although it is beginning to be understood that the characteristics of aquatic media surrounding a nanoparticle has a large impact on its behaviour, testing every single nanoparticle type with every given surface modification in every aquatic test medium would be neither a time nor cost-effective approach to tackling the problem. As highlighted above, significant efforts should therefore be directed towards developing standard aquatic test methodologies for examining ENP toxicity, investigating the relative toxicities of nanoparticles in their raw state as well as nanoparticles that have been dispersed using a set of standard solvent/surfactants. Nanoparticles of different size ranges should be investigated as well as corresponding bulk particles in order to elucidate the relationship between particle size and toxicity. Once these standard methods are in place, 'classic' toxicity characteristics between certain classes of nanoparticles (e.g. metal oxides, carbon nanotubes, or fullerene-based ENPs) can begin to be identified, so that predictive modelling approaches can be developed.

In certain instances, lack of standard terminology in nanoparticle-based exposure studies has the potential to cause misunderstandings. The terms 'suspension' and 'solution' and 'colloid', for example, are often used interchangeably to mean the dispersion of nanoparticles within aquatic media; however, they can have differing connotations and meanings within different scientific fields. As the involvement of multi-disciplinary groups is a fundamental aspect of the progression of nanotoxicology, establishment of standard terminology is therefore essential to ensure effective communication between these groups.

At present we know very little about the actual amounts of ENPs in the aquatic environment. We cannot rely on the modelled predictions reported upon in this review and need more empirical data. The lack of environmental measurements is due in part to the fact that for most ENPs, current environmental levels are still likely to be extremely low, but also because of the lack of the techniques and equipment needed to make such measurements. For ecotoxicologists, this means devising environmentally relevant exposure scenarios for ENPs is not yet possible, and is largely based on educated guess work. Increasing our knowledge on the environmental burden will require the further development of techniques to trace, measure, and visualise the nanoparticles as well as differentiate between ENPs and naturally occurring nanoparticles that may be similar in chemical composition. This again reinforces the importance of collaboration between scientific fields with differing specialities.

The current literature strongly suggests that many ENP types have the potential to cause adverse effects in a wide variety of aquatic organisms, from bacteria to fish and other vertebrates. The reality, however, is that overall their hazard potential is unclear. Advancing our knowledge on particle (eco)toxicology requires a greater understanding on the physicochemistry of individual ENPs, more detailed studies on their behaviour in, and interaction with, components of natural systems that will very likely affect their bioavailability to exposed organisms, and also (and importantly) the development of standardised approaches for both ENP characterisation and (eco)toxicological testing. Furthermore, in order to more accurately assess likely exposures and ecotoxicological effects, some 'laboratory' studies should seek to replicate likely (and measured) environmental conditions. A further major shortfall is the lack of empirical data regarding the actual loading of ENPs in aquatic environments, and this needs to be addressed rather than relying simply on modelled predictions. Of these needs identified, we would especially highlight the importance of the development of robust methods for identifying and quantifying ENPs in the natural environment, for understanding their physicochemical nature, and for establishing how they behave and move in ecological systems. Without this information, studies on the ecotoxicology of ENPs are, and will continue to be, significantly compromised.

Declaration of interest

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