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Bioavailability of Nanoscale Metal Oxides TiO_2, CeO_2, and ZnO to Fish

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Nanoparticles (NPs) are reported to be a potential environmental health hazard. For organisms living in the aquatic environment, there is uncertainty on exposure because of a lack of understanding and data regarding the fate, behavior, and bioavailability of the nanomaterials in the water column. This paper reports on a series of integrative biological and physicochemical studies on the uptake of unmodified commercial nanoscale metal oxides, zinc oxide (ZnO), cerium dioxide (CeO_2), and titanium dioxide (TiO_2), from the water and diet to determine their potential ecotoxicological impacts on fish as a function of concentration. Particle characterizations were performed and tissue concentrations were measured by a wide range of analytical methods. Definitive uptake from the water column and localization of TiO_2 NPs in gills was demonstrated for the first time by use of coherent anti-Stokes Raman scattering (CARS) microscopy. Significant uptake of nanomaterials was found only for cerium in the liver of zebrafish exposed via the water and ionic titanium in the gut of trout exposed via the diet. For the aqueous exposures undertaken, formation of large NP aggregates (up to 3 μm) occurred and it is likely that this resulted in limited bioavailability of the unmodified metal oxide NPs in fish.

Introduction

Nanotechnology shows great promise in solving many of today's problems in medicine, energy production, and environmental sustainability, due to the unique properties that many particles possess when manufactured at the nanometer scale. Widespread use of nanotechnology, therefore, is inevitable and will increase rapidly in the near future. Metal oxides, including titanium dioxide (TiO_2), cerium dioxide (CeO_2), and zinc oxide (ZnO), are a class of manufactured nanoparticles (NPs) that are among the first nanoscale materials to be used in commercial and industrial products. TiO_2 and ZnO are currently used in cosmetics and sunscreens (*1, 2*) and CeO_2 is used as a fuel additive to enhance combustion efficiency (*2, 3*). These compounds also show great potential for use in solar-driven energy production, as catalysts in various industrial applications, and as groundwater and soil remediation agents (*2*). Due to their diverse applications, human and environmental exposures are likely to increase substantially in the near to midterm future.

Despite their potential for widespread use, current information on the toxicity of many of these new compounds in either human or animal models is limited (*4*−*6*). In mammalian models, routes of exposure examined include inhalation (*7*−*12*), oral administration (TiO_2 NPs) (*13*), and adsorption via the skin (microfine ZnO and TiO_2) (*14*). Where toxicity has been demonstrated, a common finding has been the incidence of an inflammatory response (*7, 10, 15*−*19*). In addition, several studies have indicated the capacity of TiO_2 and other metal oxides to induce oxidative stress in various cell types (*13, 17*−*21*). Long-term toxicity has also been indicated through in vitro studies with the induction of DNA damage (*22, 23*) and apoptosis (*24*). In contrast, a few studies have also shown positive biological effects of metal oxide NPs, primarily through the protection of cells against damage by free radicals and reactive oxide species (ROS), in particular CeO_2 (*25, 26*). Despite the potential for effects, an accurate exposure model for these compounds in the environment has yet to be produced and questions of bioavailability remain.

Studies on the fate and effects of NPs in the aquatic environment have been focused on carbon-based compounds (*4, 27, 28*). A few studies have so far investigated the effects of exposures to metal oxide nanoparticles in aquatic organisms. Work on the water flea, *Daphnia magna*, has indicated the importance of the colloidal behavior and mode of preparation of TiO_2 NPs to resultant toxicity; there was an LC_{50} of 6 mg L^{-1} for exposure via water to filtered TiO_2, whereas the mortality rate for sonicated TiO_2 did not differ from controls (*29*). In the gills of fish (rainbow trout, *Oncorhynchus mykiss*), exposure to TiO_2 NPs has been reported to decrease Na^+/K^+-ATPase activity, induce edema and thickening of the lamellae, and result in increased levels of glutathione (*30*). These studies, however, did not demonstrate active uptake of TiO_2 from the water column into fish tissues, and therefore these effects cannot be positively correlated with measured exposure levels. Recently, nanoscale TiO_2 was shown to have low toxicity (<10 mg L^{-1}) in zebrafish (*31*). In order to determine the ecotoxicological potential of nanoscale metal oxides, such as TiO_2, in the aquatic environment, it is crucial to determine the actual bioavailability and therefore, the chemical fate of these molecules in the environmental compartment and in an animal model, with consideration to environmentally relevant exposure conditions.

The purpose of this study was to determine the fate of well-characterized metal oxide NPs, specifically zinc oxide, cerium oxide, and especially titanium dioxide, in the aquatic environment and in quantified exposure assessments to determine their bioavailability to fish following exposure via

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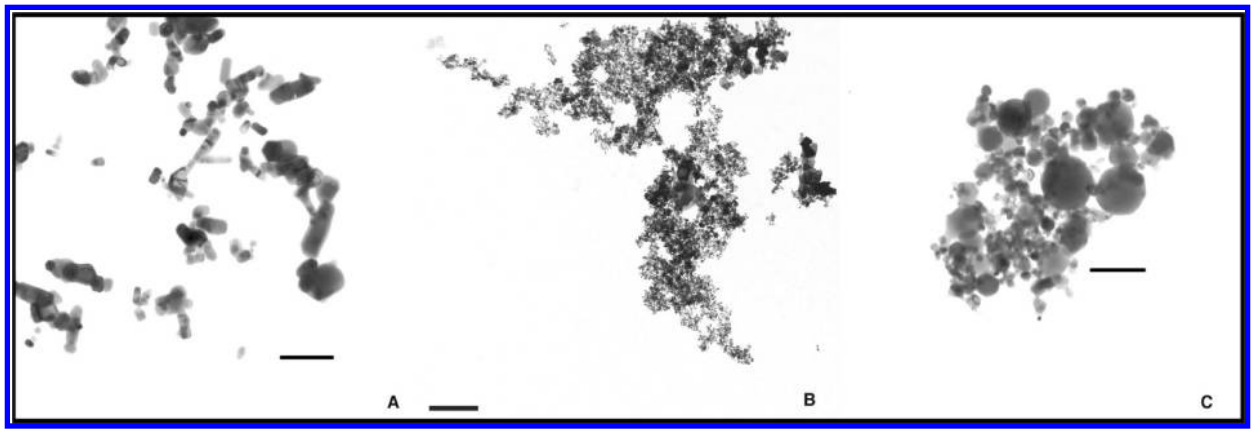

FIGURE 1. TEM micrographs of nanoparticle suspensions: (A) zinc oxide, (B) cerium dioxide, and (C) titanium dioxide. Scale bars represent 200 nm.

the water or diet without the use of a solvent vehicle or prior modification of the NP surface.

Materials and Methods

A series of exposure studies was undertaken with zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*), exposing them to various sonicated metal oxide NPs either via the water column under semistatic conditions, for between 24 h and 14 days, or via an oral dose by incorporation into feed pellets over a 21-day period (see Figure S1 in Supporting Information for details on exposure regimes). Exposure via the water avoided the use of dispersants, to allow investigation of the core NP alone without the possibility of mixture effects. Gill, liver, skin, brain, gut, blood, and kidney were analyzed for zinc, cerium, or titanium content with inductively coupled plasma mass spectrometry (ICP-MS) or optical emission spectroscopy (ICP-OES).

Nanochemicals and Exposures. NPs were characterized for particle size (mean ± SE, in nanometers), particle number and mass concentration, particle shape, qualitative aggregation, and ζ potential by transmission electron microscopy (TEM), ICP-MS, a dynamic light scattering (DLS) particle sizer (Malvern Instruments zetasizer), and coherent anti-Stokes Raman scattering (CARS) multiphoton microscopy. Stock suspensions of NPs were diluted to 250 μg L^{-1} and aliquots (10 μL) were dropped onto copper 200 hexagonal mesh grids and examined in a JEOL 100S transmission electron microscope at 80 kV. Water and tissue samples were also characterized by TEM and environmental scanning electron microscopy (ESEM) with energy-dispersive X-ray analysis (EDX) elemental analysis (XL-30 FEG ESEM) fitted with an Oxford Inca 300 EDS system). Stock suspensions of the uncoated ReagentPlus ZnO nanopowder (>99.9%, nominal size <100 nm) and CeO_2 (>99.9%, nominal size <25 nm), and TiO_2 (>99.9%, nominal size <100 nm) powders (both Sigma−Aldrich, U.K.) were produced by suspending 2.5 g L^{-1} powder in ultrapure water and sonicating for 30 min in a Decon F51006 ultrasonic bath to break up particle aggregates prior to direct dosing in 60 L aquaria with zebrafish (n = 30 per treatment) or trout (n = 8 per treatment) or incorporation into feed for oral dose experiments. All tanks were replicated and nominal NP concentrations for the aqueous exposures were 50, 500, or 5000 μg L^{-1}. Control tanks included bulk zinc, cerium, or titanium oxides, as well as ionic titanium (titanium metal standard solution, catalog no. J/8330/05, Fisher Scientific U.K.) to determine whether size and form of particle suspension had an effect on uptake in fish (Figure S1 in Supporting Information). The contribution of soluble ions to the exposures in this experiment is not known; however, ZnO is the most important to consider as it is the most soluble of the NPs used in this study. Franklin et al. (*32*) have shown the soluble fraction of ZnO nanoparticles could reach 16 mg L^{-1} at equilibrium and a pH of 7.5−7.6. Recently, we have demonstrated that the solubility of Ti and Ce from NPs is <10 μg L^{-1} (Lead et al., unpublished data).

Water and Tissue Samples. To determine NP exposure levels in the tank, water samples (3 mL) were digested in concentrated acid (3 mL of HCl for ZnO, 4 mL of HNO_3^- for CeO_2 and TiO_2) boiled in a Gerhardt Kjeldatherm digester before being reconstituted into 10 mL of nitric acid (10% for ICP-OES, 2% for ICP-MS). CeO_2- and ZnO-exposed water and fish tissue sample analysis was carried out on a Vista-MPX charge-coupled device (CCD) simultaneous ICP-OES. Zinc (ICP multielement standard IV, Merck) and cerium (ICP standard Ce, VWR) standards were used. Analysis of TiO_2 and quality control of exposed water and fish tissue samples were carried out on a Thermo Elemental PlasmaQuad PQ2 + STE, under clean-room conditions, at the Natural Environmental Research Council's ICP facility at Kingston University in Kingston-upon-Thames, U.K. ICP standard Ti (VWR) was used for these analyses. Tissue samples were prepared similarly to water samples with the addition of 1−3 mL of hydrogen peroxide to the concentrated acid to aid tissue digestion.

Coherent Anti-Stokes Raman Scattering (CARS) Microscopy is a multiphoton imaging technique that derives contrast from molecular vibrations within a sample. It provides noninvasive, label-free, three-dimensional imaging of biological structures at depths of up to several hundred micrometers with subcellular resolution. Metal oxides produce strong CARS signals, due to two-photon electronic resonance of the semiconductor band gap; a property that has been used to localize metal oxide NPs within the secondary gill lamellae at the cellular level (*33*). CARS microscopy was performed on a custom-built imaging system (further details of the CARS setup can be found in Supporting Information). Rainbow trout gill tissue was excised, gently rinsed in ice-cold trout Ringer's solution, and fixed in an ice-cold solution of 3% glutaraldehyde/2.5% paraformaldehyde. The forward CARS signal was collected by an air condenser (NA = 0.55) and directed onto a red-sensitive photomultiplier tube (R3896, Hamamatsu) via a mirror and collimating lenses. The epi-CARS signal was collected by use of the objective lens and separated from the pump and Stokes beams by a long-wave pass dichroic mirror (z850rdc-xr, Chroma Technologies) and directed onto a second R3896 photomultiplier tube at the rear microscope port. Three-dimensional data were acquired by taking a series of 2D images in the $x-y$ plane each separated by an increment in the z-direction.

Results

TEM images of stock NPs (Figure 1) indicated that the ZnO particles were rod-shaped with a low aspect ratio, while the CeO_2 particles were irregular but roughly symmetrical and the TiO_2 particles were spherical. The ZnO remained largely dispersed under these conditions, while the other NPs formed larger aggregates, up to 1 mm in the longest axis, but these aggregates were rarely spherical. The CeO_2 aggregates appeared more tightly cohered, possibly fused, compared to the TiO_2 aggregates. ZnO NPs had an average size of 68.7 ± 3.35 nm ($n = 100$). CeO_2 NPs had an average size of 10.2 ± 0.78 nm ($n = 100$), and TiO_2 NPs had an average size of 34.2 ± 1.73 nm ($n = 100$).

Analysis of water samples from tanks dosed with NPs by ICP showed decreasing concentrations of all metal oxides in experimental tanks over time, in both the presence and absence of fish (see Figure S6 in Supporting Information). This was likely due to the formation of large aggregates that precipitated out of solution. Aggregate formation was concentration-dependent and varied with the type of water used in the exposures. As shown in Figure 2A, the hydrodynamic diameter of TiO_2 measured by DLS was in good agreement with the TEM results (*34*), with small aggregates present of about 25 nm. These measured *z*-average diameters did not vary with NP concentration in the ultrapure Milli-Q water (MQ). However, concentration increased in reverse osmosis water (RO, low but detectable salt concentrations; details in Supporting Information) that was used in the trout exposures, and synthetic water (SY, high added salt concentrations; details in Supporting Information) that was used in zebrafish exposures. This tendency to aggregate can be explained by the reduction in the ζ potential and charge screening by the cations present in the RO and, especially, the SY waters (Figure 2B). Hydrodynamic diameters of particles from TiO_2 exposure tanks (Figure 2A) and ESEM images with EDX elemental analysis on filtered samples (Figure 3) clearly demonstrated the formation of large aggregates in the exposure water. Particle-size analysis on filtered exposure water indicated that the majority of the particles that the fish were exposed to had a hydrodynamic diameter greater than 450 nm (Figure 2A,C). While measurements of aggregate sizes over 1 μm by DLS may not give an accurate indication of aggregate size (Figure 2C), the data are still useful in demonstrating the nature of this aggregation behavior. Addition of ionic titanium to the exposure medium resulted in the production of a white precipitate, suggesting that not all Ti in the tank was in ionic form.

Analysis of tissues from rainbow trout exposed to TiO_2 NPs showed no significant uptake at any of the exposure concentrations (Table 1). There was an increase of Ti concentrations in gill tissues of fish in the positive controls that were exposed to ionic titanium via the water column. Significantly higher levels of TiO_2 were found in the guts of fish fed with medium and high doses of TiO_2. Analysis of the tissues of zebrafish exposed to ZnO NPs via the water showed there was no significant uptake of zinc in any of the four tissues (gill, liver, brain, and kidney) analyzed at either exposure concentration adopted in this study (500 or 5000 $\mu g\ L^{-1}$; Figure S7 in Supporting Information). Analysis of zebrafish tissues exposed to CeO_2 NPs showed significant uptake (Mann−Whitney, $p < 0.0001$) of cerium in the livers of fish exposed to 500 $\mu g\ CeO_2\ L^{-1}$ but no significant uptake in fish exposed to 5000 $\mu g\ L^{-1}$ CeO_2 (Figure S8 in Supporting Information). It is not clear whether this represented uptake into the liver or contamination of the sampled liver tissues with gut tissues, as these tissues are closely interconnected in the zebrafish (see discussion). There was no significant uptake into any of the other tissues analyzed.

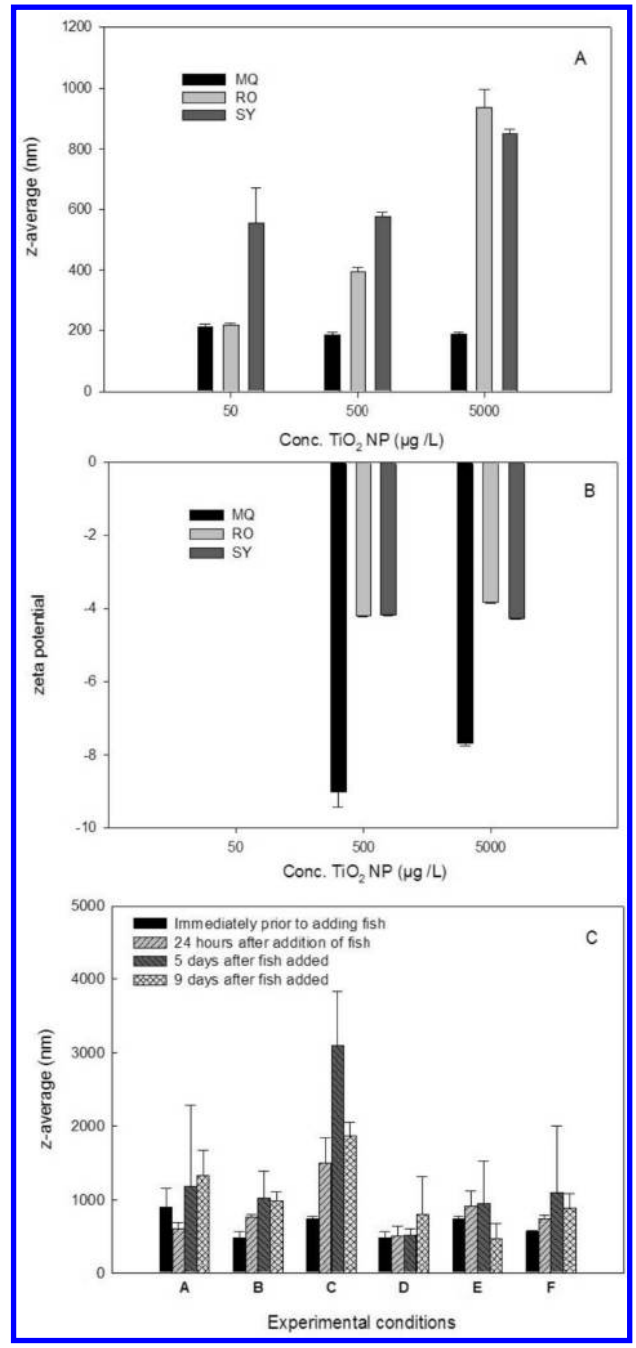

FIGURE 2. Hydrodynamic diameter and ζ potential of nanoscale titanium dioxide under different water conditions and exposure regimes. (A) Particle size vs concentration and water type. MQ, Milli-Q ultrapure water; RO, reverse osmosis-treated city water; SY, synthetic water (containing high ion concentrations; details in Supporting Information). (B) ζ Potential under different water conditions. (C) *z*-Average data from dynamic light scattering (DLS) analysis of the fish tank waters according to experimental conditions: just prior to adding fish; 24 h after fish were added; and 5 and 9 days after fish were added. Group A, control, fish added, no nanoparticles (NPs); group B, control, no fish, 5000 $\mu g\ L^{-1}$ TiO_2 NPs; group C, control, no fish, 5000 $\mu g\ L^{-1}$ TiO_2; group D, fish added, 500 $\mu g\ L^{-1}$ TiO_2 NPs; group E, fish added, 5000 mg L^{-1} TiO_2 NPs; group F, fish added, 5000 $\mu g\ L^{-1}$ bulk TiO_2.

CARS imaging of rainbow trout gill tissues clearly showed large aggregates of TiO_2 (up to 3 μm) on the surface of the gill epithelium following 24−96-h exposures (Figures 4 and 5). NPs were detected in several samples of gill tissue on the surfaces of the primary or secondary lamellae. One sample

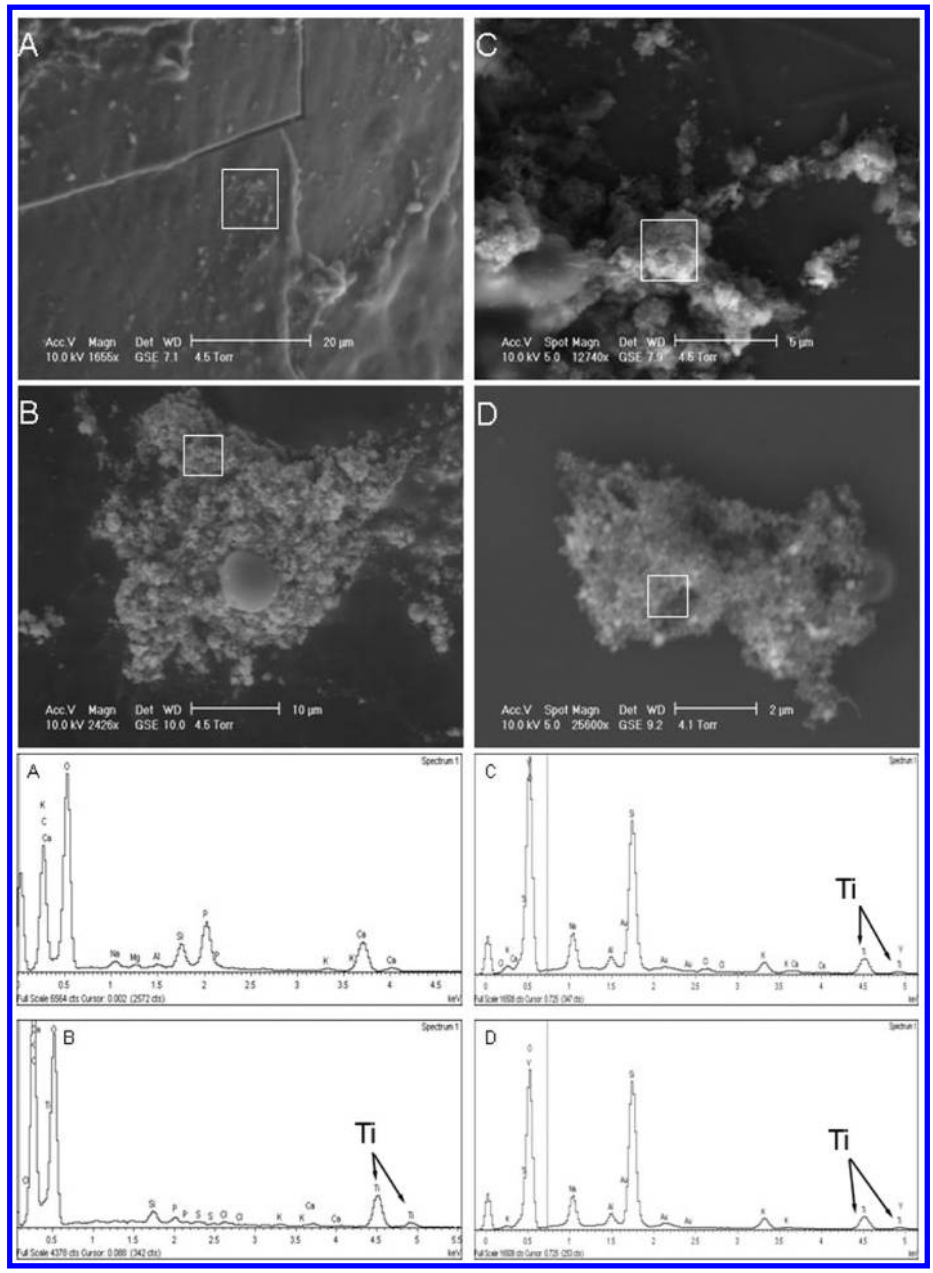

FIGURE 3. Environmental scanning electron micrographs (ESEM) of water samples and the corresponding EDX spectrum analysis (white square) at day 9 for water samples taken from tanks containing (A) fish with no particles, (B) fish with 5000 μg L^{-1} bulk TiO_2, (C) fish with 5000 μg L^{-1} TiO_2 NPs, and (D) fish with 500 μg L^{-1} TiO_2 NPs. Images were analyzed at 4.5 Torr, 10 kV, 80% humidity at 4 °C.

analyzed showed the presence of several NPs in the marginal channel in the outer tip of the secondary lamellae, following a 14-day exposure (Figure 5).

Discussion

The purpose of this extensive series of exposure studies was to determine whether uptake of unmodified metal oxide NPs could be detected in fish tissues following exposure via the water column (and diet) without the use of a solvent vehicle or prior modification of the NP surface. The chemical fate and bioavailability of the metal oxide NPs (zinc oxide, cerium oxide, and titanium dioxide) in the aquatic environment was determined through a comprehensive evaluation of uptake into fish with full characterization of the NPs under a wide variety of exposure conditions.

Our results show little or no measurable uptake of TiO_2 or other metal oxides in fish tissues, as determined by ICP-MS/ICP-OES, following short-term exposures in the water column across all treatment groups, up to a nominal exposure concentration of 5000 μg L^{-1}, or following a 21-day feeding exposure up to 300 mg g^{-1} TiO_2 NPs in the food. However, ESEM/EDX elemental analyses of filtered water samples (450 nm, 100 nm, and ultrafiltered at 1 kDa) coupled with CARS imaging of gill tissue shows that limited uptake can occur directly from the water column and across the epithelial membrane in the gill. It is clearly the case that, under these conditions, NP behavior such as aggregation and association with biological material results in reduced bioavailability of unmodified metal oxides and therefore limits the uptake of these compounds into fish.

Our data emphasize the importance of understanding the fate and behavior of NPs in aquatic systems in order to determine their likely bioavailability to organisms, such as fish. In particular, for an assessment of the ecotoxicological

TABLE 1. Concentrations of Zinc, Cerium, and Titanium in Tissues of Fish[a]

	Water Exposure				
tissue	**control**	**500 μg/L nano**	**5000 μg/L nano**	**5000 μg/L bulk**	**5000 μg/L ionic**
			Zinc Oxide		
gill	0.45 ± 0.05	0.51 ± 0.09	0.53 ± 0.06		
liver	0.36 ± 0.07	0.36 ± 0.08	0.40 ± 0.09		
brain	0.33 ± 0.03	0.34 ± 0.04	0.39 ± 0.06		
skin	1.14 ± 0.09	1.03 ± 0.09	0.91 ± 0.08		
			Cerium Oxide		
gill	nd	nd	nd		
liver	0.03 ± 0.03	1.35 ± 0.58*	1.01 ± 0.59		
brain	nd	nd	nd		
skin	nd	nd	nd		
			Titanium Dioxide		
gill	nd	nd	nd	0.01 ± 0.01	0.32 ± 0.06*
liver	nd	nd	0.88 ± 0.27	nd	0.03 ± 0.02
brain	0.24 ± 0.04	0.20 ± 0.01	0.19 ± 0.04	nd	nd
skin	nd	nd	nd	nd	nd
blood					
gut	nd	0.16 ± 0.06	0.39 ± 0.08	0.10 ± 0.017	0.75 ± 0.066*

	Oral Exposure		
tissue	**control**	**low dose**	**high dose**
		Titanium Dioxide	
gill	nd	0.02 ± 0.01	0.15 ± 0.04
liver	nd	nd	nd
brain	nd	nd	nd
skin	nd	nd	nd
blood	nd	nd	nd
gut	0.11 ± 0.01	0.36 ± 0.03*	1.49 ± 0.14*

[a] Values are given as milligrams per gram dry weight. Fish were exposed via tank water or diet to various concentrations and preparations of zinc oxide, cerium oxide, titanium dioxide NPs, and bulk particles and ionic titanium. Values represent means ± SE; an asterisk indicates a value significantly different; nd = not detected; $n = 16$.

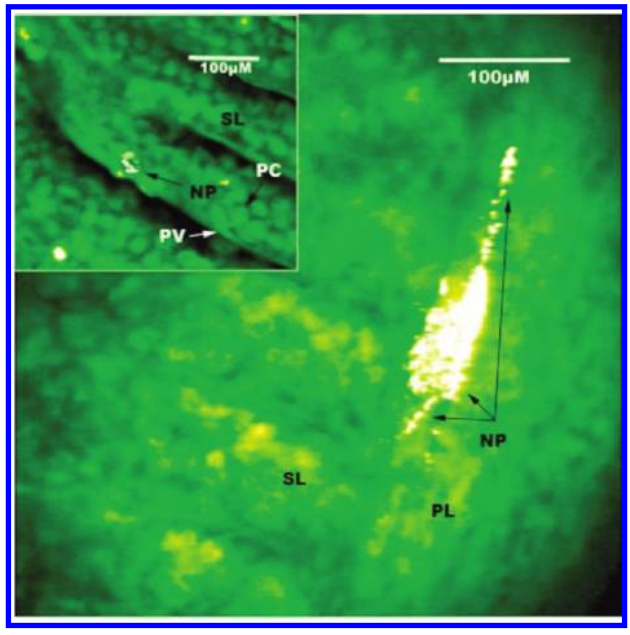

FIGURE 4. CARS image of TiO_2 nanoparticles on a section of the primary lamellae (main panel) and three-dimensional projection showing a nanoaggregate on the secondary lamellae (inset). PL, primary lamellae; SL, secondary lamellae; PC, pillar cell; PV, pavement cell (epithelium); NP with arrow, TiO_2 nanoparticles.

potential of any compound, it is crucial to understand the concentration and form of NPs that aquatic organisms such as fish will be exposed to, as this will influence the route of exposure and likely target organs, should any uptake occur. Such information will also help identify the most appropriate testing strategies for identification of potential environmental hazards.

Under laboratory conditions, it is often difficult to achieve a stable monodispersed suspension of NPs without the use of chemical dispersants or surfactants (*35*−*37*). Although dispersants within experimental systems can help to form more stable colloidal solutions and facilitate the exposure of aquatic organisms to nanometer-sized particles, as opposed to micrometer-sized aggregates of NPs, their use can be controversial in ecotoxicological experiments, as they can be inherently toxic and introduce the possibility of interactive mixture effects, thus complicating any analyses and conclusions drawn (*38*). Our adopted approach, without the use of a solvent or prior functionalization, provided more environmentally relevant conditions but is nevertheless, a simplistic paradigm, especially with regard to the high exposure concentrations adopted. Furthermore, natural organic macromolecules (NOM) are likely to have a significant impact on the partitioning of metal oxide nanoparticles into the aqueous and sediment phases in natural systems and thus on their availability to pelagic fish. Future studies will need to consider exposures to reduced NP concentrations and the addition of organic or colloidal material to determine how these ecologically important variables may affect colloidal/particle stability and bioavailability. Additionally, many nanoparticles incorporated into consumer products are likely to be modified through addition of coatings or chemical adducts or use of surfactants to improve their function. Such modifications will affect the behavior of NPs

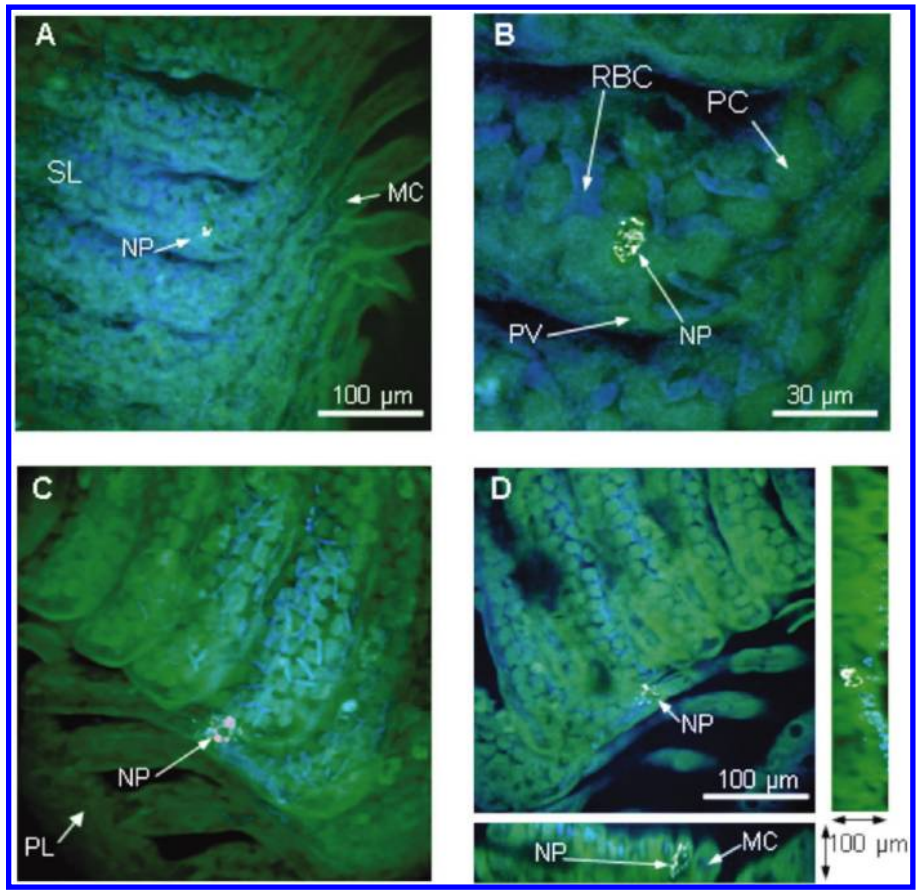

FIGURE 5. CARS images of gill tissue of rainbow trout, *Oncorhynchus mykiss*, following a waterborne exposure to TiO_2 nanoparticles (NPs). The cellular structure of the primary (PL) and secondary (SL) gill lamellae, composed of pillar cells (PC) and pavement cells (PV), was obtained by epidetection of the CH_2 vibration (shown in green). The red blood cells are effectively separated from the lamellae cells by forward detection of the CH_2 vibration (shown in blue) (*33*). (A) Gill tissue following a 28-day exposure. An aggregate of NPs can be seen occupying the space between the pillar cells. (B) The same NP aggregate under a 3× increase in magnification. (C) Projection of a 300 × 100 µm 3D data set of gill tissue following a 14-day exposure. A cluster of NPs can be seen in the region of the marginal channel (MC). (D) Multiplanar view of the same exposure. The two adjacent subpanels specifically locate the NPs inside the tissue near the surface of the marginal channel (MC).

in aquatic systems and thus are an important consideration for future investigations.

In general, unmodified NPs are not highly dispersible in water and in most cases will exist in the aquatic compartment as a colloidal suspension, have a propensity to flocculate into aggregates up to several micrometers in diameter, and tend to precipitate out of solution. This tendency may be reversed or delayed by the presence of NOM (*31*), although at present no studies have investigated the effect of NOM on NP bioavailability to fish. An exception to this may be ZnO, which is partially soluble in water [and produces significant amounts of free Zn^{2+} cations, up to 16 mg L^{-1} at equilibrium (pH 7.5−7.65) (*32*)] and it is therefore likely that some bioavailable free Zn^{2+} was present in the exposure medium in our studies. In this study, no significant uptake of zinc in fish tissues was observed for concentrations in the water spanning 500−5000 µg L^{-1} with ICP-OES as a quantification technique (see Figure S7 in Supporting Information). It is not known whether this represented a true lack of uptake of Zn^{2+} or ZnO NPs or the result of the measurement of Zn being masked by high background levels of Zn in fish tissues observed between 0.3 and 1.1 mg g^{-1} dry weight (see Figure S7 in Supporting Information).

In our experiments, ESEM analysis and measurement of the hydrodynamic diameters of NPs in water indicated that metal oxide NPs formed large aggregates and precipitated out of solution, especially in the presence of fish. This is most likely due to active mucus production, as a consequence of a response of the fish to irritation induced by the NPs, and formation of mucus−NP complexes. Ti was found as particulates in exudates from the fish, at the bottom of the tank (Figure 3; see also Figures S2, S4, and S5 in Supporting Information). This aggregation decreases the bioavailability of the NPs to pelagic fish, both by reducing the concentration in the water column and by increasing the size of the particles that come into contact with the epithelial surface of the gill or presumably, the gut, thus rendering the NPs less likely to diffuse across boundary layers or through membranes. Therefore, predictions of the environmental behavior and impacts of NPs based on results derived from laboratory-based exposures need careful consideration of the water chemistry and whether it is representative of ecologically relevant natural waters and exposure conditions.

The high degree of particle aggregation and flocculation of metal oxide NPs in solution that was seen in our studies suggests that the oral route may be a more likely source of exposure for metal oxide NPs to organisms in the aquatic environment. Thus, in the wild, significant exposures to metal oxide NPs are more likely to occur for benthic or pelagic fish feeding on aggregated NPs that have sunk to the river bottom or seabed or for filter-feeding animals that actively collect particles from the water column, rather than for pelagic, non-filter-feeding species living higher in the water column. This is still a hypothesis, however, and requires further testing.

Our results indicate that the likelihood is low for unmodified metal oxide NPs to enter the fish via the water

column or via the oral route, albeit with the limitations of the experimental system we used when compared with the more complex exposure dynamics for natural waters. In particular, the lack of strong evidence of substantial concentrations of NPs in the gill tissue, which is the most important port of entry for many dissolved compounds (*39*), implies that NPs are unlikely to enter the fish via the gills at toxic concentrations under relevant environmental conditions.

Our CARS analysis has confirmed, for the first time, entry of TiO_2 nanoparticles into the marginal channel of the gill of rainbow trout via the water column in the absence of artificial dispersants or prior functionalization, following a 14-day aqueous exposure. This bioimaging technique demonstrated that although bioavailability is limited, small amounts of unmodified metal oxide NP uptake in fish does occur (perhaps largely below the limits of most conventional methods of detection). Although individual NPs are too small to be resolved by CARS microscopy, the signal obtained is sufficient to provide the location of NPs within biological tissues. This method has advantages over TEM, which is limited to two dimensions and requires fixation, which can alter the position of the NPs (*26*). Our results provide an accurate location of NPs in intact gill tissue and show a clear signal for TiO_2 NPs in the marginal channel across the epithelial membrane (Figure 5).

At this time, we are not able to specify whether the signal represents internal colocalization of NPs or an aggregation process that occurs within the cell once uptake has occurred. Pinocytosis of some NPs across membranes has been demonstrated in cultured HEp2 2B cells (*40*) and isolated Kupffer cells (*41*). NPs have also been shown to be taken up by a murine macrophage line (*42*). In order to build a greater understanding of the bioavailability of metal oxide NPs to fish in the aquatic environment, we require more information on the mechanisms of translocation from the water column and an understanding of local surface charge characteristics of nanoaggregates in contact with the gill or gut epithelium under environmentally relevant conditions. CARS imagery shows that, upon coming into contact with fish gills, nanoaggregates are likely to adhere to mucus on the gill surface and remain bound for short periods, as has been shown for mucal clearance of bacteria from rainbow trout gill (*43*). It is interesting to speculate about the possibility that mucus production in fish may have evolved in an environment rich in natural aquatic colloids as an important natural defense mechanism against nanoparticulates.

Taken together, our results indicate that unmodified, manufactured metal oxide NPs, in the absence of NOM, are likely to have low bioavailability in high-cation environments. This would indicate that, for many nonbenthic fish, metal oxide NPs are unlikely to be a major ecotoxicological hazard. However, this needs to be considered against the context of a general lack of knowledge of the fate, behavior, and bioavailability of these types of particles in natural systems and suggests a need for longer-term and more environmentally realistic NP exposure regimes to fully determine the transport capabilities of NPs in the aquatic environment.

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Supporting Information Available

Details of exposure regimes, CARS photon emission microscopy setup, and background results of water chemistry measurements that support the conclusions in this paper. This material is available free of charge via the Internet at http://pubs.acs.org.

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