Uptake and Effects of Nanoparticles in Fish

Submitted by

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to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences, *September 2009*.

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

.....(Tessa Scown)

ABSTRACT

Nanotechnology is a rapidly growing industry of global economic importance, with new technologies exploiting the novel characteristics of materials manufactured at the nanoscale being developed for use within the biomedical, electronic, energy production and environmental sectors. The unusual properties of engineered nanomaterials (ENMs) that make them useful in such applications have led to concerns regarding their potential impact on the environment. The aquatic environment is particularly at risk of exposure to ENPs, yet, there is currently little known about their behaviour in aquatic systems, their capacity to be taken up by aquatic organisms or their potential toxic effects. The studies that were conducted during this work sought to investigate the ecotoxicology of a range of metal and metal oxide nanoparticles using fish as a vertebrate model.

In order to gain a better understanding of the uptake and effects of ENMs in fish, rainbow trout (*Oncorhynchus mykiss*) were exposed to nanoparticulate (34 nm) and bulk (>100 nm) titanium dioxide particles *via* the water column (500 and 5000 μg L^{-1}), and to titanium dioxide nanoparticles *via* the diet (0.1 and 1 mg g^{-1} food) and *via* intravenous injection (1.3 mg kg^{-1} body weight). Uptake of titanium dioxide into the tissues of trout after waterborne and dietary exposure was found to be very low, suggesting limited bioavailability of the nanoparticles to the fish, although small amounts of uptake of titanium dioxide across the gill epithelial membrane were observed using coherent anti-stokes Raman scattering. Intravenously injected titanium dioxide accumulated and was retained in the kidneys for up to 21 days, but no adverse effect on kidney function was detected.

Silver nanoparticles are already in widespread use in a variety of consumer products such as wound dressings, food containers, sock fabrics and paints, principally for their antimicrobial activity. Despite its growing commercialisation, there is little known about the environmental effects of the use of nanoparticulate silver in these products. In order to investigate these potential effects, rainbow trout were also exposed to 10 nm, 35 nm and bulk (0.6-1.6 μm) silver particles *via* the water column at concentration of 10 and 100 μg L^{-1}. Uptake of silver in the gills and liver of trout occurred, with smaller

nanoparticles showing a greater propensity for association with gill tissue, but with no significant differences in uptake between particles of different sizes in the liver. No increases in lipid peroxidation were detected in gills, liver or blood plasma of trout, however, expression of *cyp1a2* was significantly up-regulated in exposures to 10 nm silver particles in the gill, suggesting an increase in oxidative metabolism.

In an attempt to develop an effective high through-put *in vitro* screening assay for ENMs, the suitability of isolated rainbow trout primary hepatocytes was examined as a potential model for *in vitro* screening of a range of toxicological endpoints in response to nanoparticles and for studying uptake of nanoparticles into cells. The hepatocytes retained a good level of functionality after culturing as evidenced by vitellogenin production in response to the synthetic oestrogen, 17β-oestradiol. The cultured hepatocytes, however, showed limited responses on exposure to titanium dioxide, zinc oxide, cerium oxide and silver nanoparticles for lipid peroxidation and glutathione-s-transferase activity assays. Furthermore, the hepatocytes were unresponsive to the induction of these biological responses in the positive controls, suggesting they are not a good model for investigating the potential toxic effects of ENMs in terms of these endpoints. Uptake of the nanoparticles into the cells, however, was demonstrated by coherent anti-stokes Raman spectroscopy, indicating that this *in vitro* assay may provide a useful model for studying uptake of ENPs into cells.

The studies conducted in this thesis contribute the science base regarding the bioavailability of ENPs in aquatic media as well as highlighting the importance of characterisation of ENPs in understanding their behaviour, uptake and effects in aquatic systems and in fish.

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"Patience and tenacity of purpose are worth more than twice their weight of cleverness."
Thomas Henry Huxley 1825-1895

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RESEARCH PAPERS AND AUTHOR'S DECLARATION

Research Paper 1. Johnston, B.D.; Scown, T.M.; Moger, J.; Cumberland, S.; Baalousha, M.; Linge, K.; van Aerle, R.; Jarvis, K.; Lead, J.R.; Tyler, C.R. (2010) Bioavailability of nanoscale metal oxides, TiO_2, CeO_2, and ZnO to fish. *Environmental Science and Technology* 44(3): 1144-1151.

Research Paper 2. Scown, T.M.; van Aerle, R.; Johnston, B.D.; Cumberland, S.; Lead, J.R.; Owen, R.; Tyler, C.R. (2009) High doses of intravenously administered titanium dioxide nanoparticles accumulate in the kidneys of rainbow trout but with no observable impairment of renal function. *Toxicological Sciences* 109(2): 372-380.

Research Paper 3. Scown, T.M.; Santos, E.; Johnston, B.D.; Gaiser, B.; Baalousha, M.; Mitov S; Lead, J.R.; Stone, V.; Fernandes, T.; Jepson, M.; van Aerle, R.; Tyler, C.R. (2010) Effects of aqueous exposure to silver nanoparticles of different sizes in rainbow trout. *Toxicological Sciences* (In Press).

Research Paper 4. Scown, T.M.; Goodhead, R.; Johnston, B.D; Moger, J.; van Aerle, R.; Iguchi, T.; Tyler, C.R. (2010) Assessment of cultured fish hepatocytes for studying cellular uptake and (eco)toxicity of nanoparticles. *Environmental Chemistry* 7(1): 36-49.

Statement: I, Tessa Scown, was involved in the following parts of the presented papers: I planned and carried out the exposures and ICP-OES analysis as well as co-writing **paper 1** with Blair Johnston. Kim Jarvis and Katherine Linge assisted with ICP-MS analysis in **paper 1.** I planned and carried out the exposures and ICP-OES analyses in **papers 2 and 3** and played the leading role in writing these papers, receiving valuable input from co-authors. Birgit Gaiser carried out preliminary work relating to **paper 3** with supervision and assistance from Vicki Stone, Teresa Fernandes and Mark Jepson, and Eduarda Santos assisted in the method development and analysis of QPCR work in **paper 3**. I planned and carried out the hepatocyte isolations, exposures, LDH, TBARS and GST assays in **paper 4** and co-wrote **paper 4** with the help of Rhys Goodhead. I was responsible for the TBARS and LDH assay and Rhys Goodhead carried out the ELISA work. Charles Tyler and Ronny van Aerle contributed to both study design and editing of manuscripts for **papers 1-4**. CARS microscopy was carried out by Julian Moger and Blair Johnston in **paper 1** and by Julian Moger and Rhys Goodhead in **paper 4**. Nanoparticle characterization work for **papers 1-4** was carried out by Susan Cumberland, Mohamed Baalousha and Jamie Lead. Svetlin Mitov was involved in characterisation work for **paper 3**.

Published or accepted versions of papers 1, 3 and 4 are included in the appendix.

Other papers – included in the appendix

Handy, R.D.; Henry, T.B.; Scown, T.M.; Johnston, B.D.; Tyler, C.R. (2008) Manufactured nanoparticles: their uptake and effects on fish – a mechanistic analysis *Ecotoxicology* 17(5): 396-409

LIST OF GENERAL ABBREVIATIONS

Ag^+	Silver ion
Al_2O_3	Aluminium oxide
BSA	Bovine serum albumin
C60	Buckminsterfullerene (fullerene)
CARS	Coherent anti-Stokes Raman Scattering
CNT	Carbon nanotube
DLS	Dynamic Light Scattering
DLVO	Derjaguin & Landau, Verwey and Overbeek Theory
DOC	Dissolved organic carbon
DWCNT	Double-walled carbon nanotube
ENMs	Engineered nanomaterials
FA	Fulvic acid
Fe_2O_3	Iron oxide
GM	Genetically modified
GSH	Glutathione
GST	Glutathione-S-transferase
HA	Humic acid
hpf	Hour post fertilization
ICP-OES	Inductively coupled plasma – optical emission spectrometry
ICP-MS	Inductively coupled plasma – mass spectrometry
LC50	Median lethal concentration
LDH	Lactate dehydrogenase
MRI	Magnetic Resonance Imaging
MWCNT	Multi-walled carbon nanotube
NOEC	No observable effect concentration
NOM	Natural organic matter
PEG	Polyethylene glycol
ROS	Reactive oxygen species
STM	Scanning tunnelling microscope
SWCNT	Single-walled carbon nanotube
TBARS	Thiobarbituric acid reactive substances
TEM	Transmission electron microscopy
THF	Tetrahydrofuran
TiO_2	Titanium dioxide
UFPs	Ultrafine particles
UV	Ultraviolet (also UVA and UVB)
V_2O_3 and V_2O_5	Vanadium oxide
VTG	Vitellogenin
WWTWs	Wastewater treatment works
ZnO	Zinc oxide

LIST OF SPECIES

Amphibians

African clawed frog	*Xenopus laevis*

Fish

European carp	*Cyprinus carpio*
Fathead minnow	*Pimephales promelas*
Goldfish	*Carassius auratus*
Largemouth bass	*Micropterus salmoides*
Rainbow trout	*Oncorhynchus mykiss*
See-through medaka	*Oryzias latipes*
Zebrafish	*Danio rerio*

Invertebrates

Amphiascus tenuiremis	benthic estuarine hepacticoid copepod
Daphnia magna	water flea
Ceriodaphnia dubia	water flea
Chironomus riparius	midge, blood worm

Algae

Chlamydomonas reinhardtii

Bacteria

Bacillus subtilis
Escherichia coli

CHAPTER 1

GENERAL INTRODUCTION

1. INTRODUCTION

Nanotechnology is undoubtedly one of the most important technologies of the 21st century. With an estimated $147 billion worth of so-called 'nano-enabled' commercial and consumer products sold in 2007 and a projected value of $3.1 trillion by 2015 (Lux Research 2009), nanotechnology has been referred to as the next industrial revolution (Schmidt, 2009). Levels of funding in nanotechnology research and development in 2008 reached $18.2 billion worldwide, with the United States and Japan leading in this activity (Lux Research 2009). In Europe, the EU Seventh Framework Programme is contributing approximately €600 million per year to 2013 to support research funding opportunities (Government funding, companies 2007). In the UK alone, there are already over 600 micro and nanotechnology companies (The Nanotechnology Knowledge Transfer Network 2009).

Nanotechnology shows great promise in providing solutions to many of today's problems in medicine, energy production, and environmental sustainability. Governments and investors around the world are therefore keen to explore and exploit the exceptional properties of nanomaterials opportunities which exist within nearly every industrial and technological sector.

As this technology grows and the number of nanomaterial types and applications increase, so does the likelihood that they will be released into the environment, and in significant quantities, a fact that has been met with increasing concern over their safety in terms of human health and the environment (Dowling et al., 2004; Williams et al., 2005). The aquatic environment is likely to be particularly vulnerable to contamination with engineered nanomaterials (ENMs) as it acts as a sink for almost all environmental pollutants. Sources to the aquatic environment will include waste water discharges, accidental release from factories, and the degradation and wear of products containing ENMs.

Due to their extremely small size and unique physical properties (described in detail below), the behaviour of ENMs in the environment, their uptake, distribution and effects within the bodies of living organisms are likely to be different when compared to

conventional xenobiotics. To date, however, there is a lack of data and understanding about their environmental fate, bioavailability and biological effects. There is therefore an urgent need to fill the gaps in our understanding and for research and regulatory activities to ensure these compounds do not pose a significant hazard to human and environmental health. This is vital to ensure the sustainability of the industry.

In this introduction, an overview of nanotechnology and the different types of nanomaterial and their uses are provided, followed by a summary of what is currently known about their potential environmental impact and toxicity. The introduction then addresses the physicochemical characteristics of nanomaterials that are likely to affect their fate and behaviour in the environment and in biological systems. Finally, this introduction outlines the main aims and objectives of the research work undertaken.

1.1 NANO–CONCEPT AND HISTORY

Nanotechnology is defined as the "design, characterisation, production and application of structures, devices and systems by controlling shape and size at nanometre scale" (Williams et al., 2005). Materials that have structures or components of their structures that are 100 nm or less in at least one dimension are considered to be nanoscale (Dowling et al., 2004). In principle, therefore, any product using compounds that exist as, or are manufactured to, these specifications can be considered to be examples of nanotechnology. Table 1 provides an illustration of the definition of a nanoscale object compared with cell and sub-cellular structures in the human body.

Table 1. Comparison of sizes of biological molecules and structures in nanometers. Taken from Gupta and Kompella, (2006).

Object	Size (nm)
Carbon atom	0.1
DNA double helix (diameter)	3
Ribosome	10
Virus	100
Bacterium	1,000
Red blood cell	7,000
Human hair (diameter)	80,000

In his talk "There's Plenty of Room at the Bottom" in 1959 presented at an American Physical Society meeting, physicist Richard Feynman discussed concepts relating to the potential development of nanotechnology and described the possibility of a top-down approach to manipulating matter, where precision tools allowed the creation and operation of smaller and smaller sets of precise tools that in turn enabled the manipulation of individual atoms and molecules (Feynman, 1959; Sandhu, 2006).

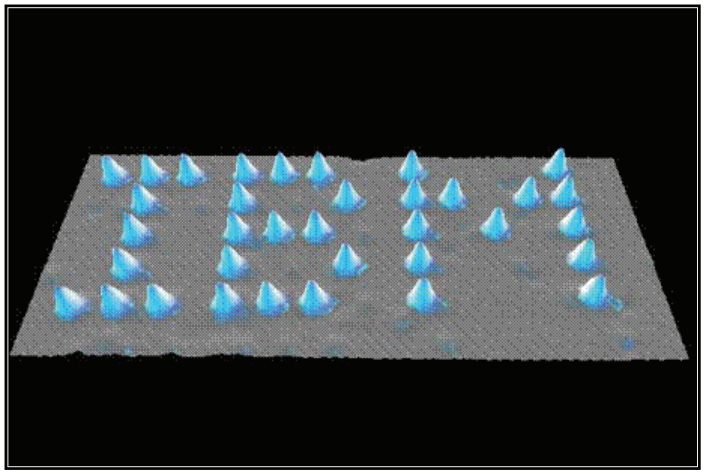
The term nanotechnology was first adopted by Professor Norio Taniguchi of the Tokyo Science University in his paper, "On the Basic Concept of 'Nanotechnology'", presented at a meeting of the Japan Society of Precision Engineering in 1974. He stated in his paper that "Nano-technology mainly consists of the processing of separation, consolidation and deformation of materials by one atom or one molecule" (Sandhu, 2006; Taniguchi, 1974).

These ideas were explored further by Dr K. Eric Drexler who highlighted the potential technological significance of nanotechnology. He proposed that the ability to design protein molecules by a bottom-up approach would open the way to construction of complex molecular machines with important implications for computation technology and manipulation of biological materials (Drexler, 1981).

The advent of cluster science (the study of aggregates of molecules and how their properties contribute to the characteristics of a bulk solid) and the invention of the scanning tunnelling microscope (STM) in the 1980s and later the atomic force microscope, heralded the beginning of practical nanoscience and led to the discovery of fullerenes in 1985 (Kroto et al., 1985). In 1990 IBM researchers Eigler and Schweizer (Eigler and Schweizer, 1990) demonstrated that manipulation of atoms using the STM was possible by creating an IBM logo using xenon atoms (Figure 1).

Since then, nanotechnology has grown into the multi-billion dollar industry, with a seemingly limitless array of particle types being developed with potential applications within all industrial sectors.

Figure 1. IBM Logo created using the scanning tunnelling microscope to move 35 xenon atoms (Eigler and Schweizer, 1990).



1.2 PROPERTIES OF NANOPARTICLES

There are obvious advantages associated with being able to scale down the size of various technologies and products and they include saving space and reducing production and transportation costs. For example, the progressive miniaturisation of computers and their components has enabled the transition from room-sized machines capable of simple calculations to pocket-sized devices that can rapidly perform multiple complex tasks simultaneously.

Nanotechnology, involving the manipulation and creation of molecules to exact specifications, also allows for the creation of novel materials that function more effectively and can perform new roles. This is primarily because various physical qualities set nanoparticles apart from larger materials of the same chemical composition. It is these qualities that have largely fuelled the interest in their potential uses.

An important determining factor in the overall bulk properties of any given particle are the surface characteristics of that particle (Amato, 1989). Due to their small size, nanoparticles have a very large surface area to volume ratio. Therefore an increasing number of the atoms that make up the particle are present on the particle's surface compared with a larger particle of the same chemical composition (Table 2). Nanosized

particles will therefore be chemically more reactive per unit mass than their bulk sized counterparts. This property makes them particularly desirable for use within biomedical applications where dissolution rate is enhanced (Gupta and Kompella, 2006) and within chemical processes as catalysts.

Table 2. Percentage surface molecules in particles of different diameters. Taken from Gupta and Kompella (2006).

Particle Size (nm)	Surface Molecules %
1	100
10	27.1
100	2.97
1,000	0.3
10,000	0.03

Furthermore, as the size of a particle decreases to the nanoscale, the physical properties of the particle are altered, rules of quantum mechanics, which determine the behaviour of matter and light at the atomic and subatomic level, begin to dominate as particles shrink to the nanoscale. This means nanosized particles, particularly at the lower end of the nanoscale, have optical, electrical and magnetic properties that differ substantially from larger particles of the same compounds (Dowling et al., 2004) and it is these factors that lend themselves to new potential applications such as within electronics, data storage and solar cells.

Additional differences between nanoparticulate and bulk forms of a compound may contribute to their potential for practical applications. For example, gold particles have been shown to have altered melting temperatures depending on their size (Buffat and Burrel, 1976). Another example is that of nanoparticulate titanium dioxide (TiO_2) which in its bulk form is bright white in colour, but at the nanoscale is transparent. Nanoparticulate TiO_2 has therefore been utilised as an ultraviolet radiation absorber in transparent sunscreen formulations (Nohynek et al., 2007) and in specialist photocatalytic coatings for glass (Medina-Valtierra et al., 2009).

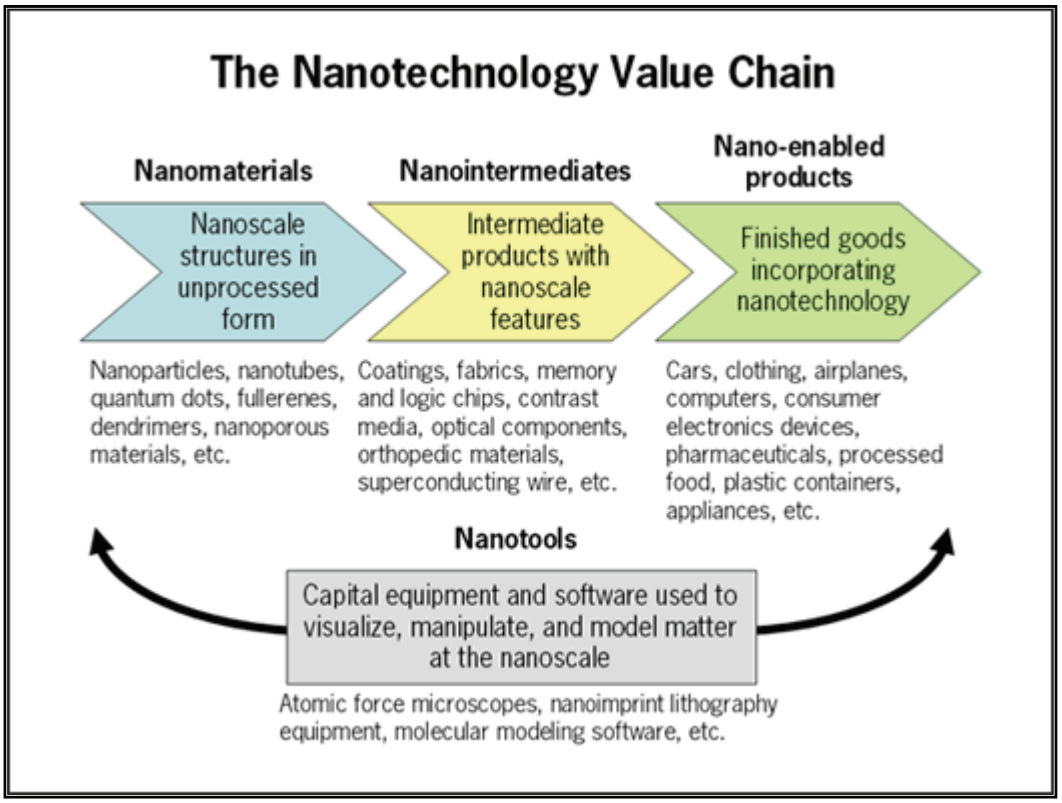
1.3 TYPES OF NANOPARTICLES - NANOMATERIALS TO NANOTECHNOLOGY

The great diversity of engineered nanoparticles means there is huge scope for their use in the innovation of new products as well as in the improvement of existing products. The industrial sectors in which nanomaterials can and are being used are therefore extremely diverse. Improvement and invention of new production pathways has meant that nanoparticles of many different compositions, shapes, sizes and precise particle size distributions, can now be produced using both bottom-up (eg precipitation in liquors, thermal decomposition catalytic growth, arc discharge or laser ablation etc) and top-down (eg atomic layer deposition, dip pen nanolithography, focussed ion beam deposition/ablation) approaches.

Few nanoparticles are used in their raw form - most are incorporated into other materials or components which are then used within consumer products. The Nanotechnology Value Chain (2004) (Figure 2.) provides a useful system for categorizing the stages involved in the production of so-called 'nano-enabled products' and thus provides a clear framework for discussing and highlighting some of the key nanoparticles in production and use.

The following section discusses the materials and devices into which they are being incorporated and the eventual consumer products that contain them, as well as some of the technologies that are in the research and development stage.

Figure 2. The Nanotechnology Value Chain. Taken from 'Sizing Nanotechnology's Value Chain' Lux Research Inc. 2004



1.3.1 NANOMATERIALS

Nanomaterials are the raw engineered, sub-100 nm particles or structures and include nanoparticles, fullerenes and other carbon-based molecules, such as carbon nanotubes, quantum dots, dendrimers, nanoporous materials and biological nanoparticles**.**

1.3.1.1. Nanoparticles

Nanoparticles include ultrafine particles of metals, metal oxides, non-metals and ceramics. Metal nanoparticles and nano-alloys are usually produced *via* reduction or co-reduction of metal salts (Masala and Seshadri, 2004). As electrical conductors, metal nanoparticles naturally are being implemented in electronics (Chow et al., 2009; Lee et al., 2008b; Wang et al., 2008a; Wang et al., 2008b), however their potential extends to

use as catalysts in industry (Du and Wang, 2009; Kim et al., 2009; Sharghi et al., 2009) and in the remediation of polluted groundwater (Morrison et al., 2002; Ponder et al., 2001; Zhang, 2003), within medicine for new pharmaceuticals (Elechiguerra et al., 2005; Sun et al., 2008), drug delivery (Choi et al., 2007), medical imaging (Hacliipanayis et al., 2008; Qu et al., 2008), and use within fuel cells (Du and Wang, 2009) and in chemical sensors (Buso et al., 2008; Guerrini et al., 2009).

Engineered metal oxide nanoparticles such as TiO_2 and zinc oxide (ZnO) are commonly made by hydrolysis of the transition metal ions (Masala and Seshadri, 2004). They are already in widespread use in consumer products, most notably within cosmetics and sunscreen applications (Brezová et al., 2005; Lademann et al., 2000). Much current research is investigating their catalytic properties (Bertelli and Selli, 2006) and their potential use within solar cells (Quintana et al., 2007) and fuel cells for alternative forms of energy production.

Non-metal nanoparticles such as silica can be produced *via* carbon dioxide-laser-induced decomposition of silicon hydride in a gas flow reactor, reduction of silica salts by sodium metal or metal silicides in a nonpolar organic solvent, or by top-down laser ablation and ultrasonic methods. Similar techniques are also used to produce germanium (Masala and Seshadri, 2004).

Silica nanoparticles are currently being investigated for their use in cancer therapy (Hirsch et al., 2003), as substrates for oligonucleotide synthesis (Zhao et al., 2009) and for their anti-biofilm properties (Hetrick et al., 2009).

1.3.1.2. Fullerenes and Carbon-based nanomaterials

The fullerenes are perhaps the best known of the carbon-based nanomaterials. They are allotropes of carbon and take the form of spheres, tubes or sheets formed from 6-membered carbon rings. Buckminsterfullerene (C60) was discovered in 1985 by Kroto and colleagues, shortly followed by other fullerene compounds, which now include carbon nanotubes and graphene sheets, also known as nanoribbons or nanosheets. They

are commonly made using an arc discharge method or by combustion of precursors such as benzene in a flame producing soot (Murayama et al., 2004).

Fullerenes have many prospective uses particularly within the medical and biological fields with potential in drug delivery, gene therapy and the treatment of HIV and neurodegenerative diseases, such as Parkinson's and Alzheimer's (Bosi et al., 2003; Nakamura and Isobe, 2003) through their properties as enzyme inhibitors and free radical scavengers, respectively.

Their versatility is partly due to the ability to chemically modify the fullerene surface by addition of functional groups which present altered biological and pharmacological activity such as increased solubility in aqueous media (Bosi et al., 2003; Sayes et al., 2004). It is also possible to trap other compounds such as metals within the cage of the fullerene (endohedral metallofullerenes) giving them potential as X-ray and magnetic resonance imaging (MRI) contrast agents (Bosi et al., 2003).

Carbon nanotubes may be single or multi-walled. They are lightweight and have high tensile strength and therefore have been incorporated into composite materials that are now used for tennis rackets, golf clubs and bicycle frames (Project on Emerging Nanotechnologies 2008). They also possess good thermal conductivity, so have prospective uses as heat sinks for electronic devices (Kordas et al., 2007). They may be conductors or semi conductors depending on their structure (Collins and Avouris, 2000; Mauter and Elimelech, 2008) and therefore have a host of potential applications in electronic displays (Yotani et al., 2009), electrical components and computer chips (Weitz et al., 2009) and solar cells (Lee et al., 2009b; Mauter and Elimelech, 2008; Yu and Chen, 2009). Their potential for use in hydrogen storage (Collins and Avouris, 2000; Leonard et al., 2009), within environmental sensors (Mauter and Elimelech, 2008; Valentini et al., 2007) and in drug delivery and pharmaceuticals (Klumpp et al., 2006) are also being investigated.

Graphene is formed from a single-atom layer of 6-membered carbon rings. Although it is effectively an unrolled carbon nanotube sheet it has differing electronic and thermal properties giving it potential in hydrogen storage (Geim and Novoselov, 2007; Rao et al.,

2009), conductive plastics, batteries, gas sensors and plasma displays (Geim and Novoselov, 2007).

1.3.1.3. Quantum dots

Quantum dots are semi-conductor nanocrystals commonly made from binary compounds such as cadmium selenide, cadmium sulphide, indium arsenide, and indium phosphide, where there is quantum confinement of both the electron and hole in all three dimensions. This results in an increase in the band gap of the material with increasing crystallite size. This means that as the size of the quantum dot decreases, the optical absorption and emission shift towards the blue end of the spectrum (Bawendi Group, 2009). Quantum dots are commonly prepared using the arrested precipitation technique, by reduction of sulphur or selenium in the presence of potassium borohydride and the desired metal salt, by application of ultrasound irradiation (Masala and Seshadri, 2004) or by pyrolysis of organometallic reagents by injection into a hot coordinating solvent (Masala and Seshadri, 2004; Murray et al., 1993). Potential applications include use in security tagging and anti-counterfeit pigments (Chang et al., 2004), *in vivo* and *in vitro* cellular imaging (Delehanty et al., 2009; Mukherjee et al., 2009), detection of biomolecules (Chan and Nie, 1998; Marin and Merkoci, 2009), tumour targeting (Delehanty et al., 2009), as well as in light emitting diodes in displays (Kang et al., 2008; Walters et al., 2005) and in solar cells (Chen et al., 2009a; Yu and Chen, 2009).

1.3.1.4. Dendrimers

Dendrimers are molecules typically in the range of 2 to 10 nm consisting of an inner core molecule surrounded by a series of branches. The core molecules and branches can be synthesised from many different materials and the terminal groups of the branches chemically modified to produce biologically compatible, hydrophilic, hydrophobic or charged surfaces. The synthesis of dendrimers offers precise control over the chemistry, molecular weight and surface characteristics and therefore offers interesting prospects within the pharmaceutical field in targeted drug delivery, controlled drug release,

creation of new vaccines and modification of DNA expression (Sakthivel and Florence, 2003).

1.3.1.5. Nanoporous Materials

Nanoporous materials are materials with uniformed and ordered pore structures of between 1 and 100 nm. They can be made from a wide range of different compounds such as polymers, ceramics, metal oxides and metals and typically have very large surface area to volume ratios and porosities (volume ratio of pore space to total volume of material) greater than 0.4. Their surfaces can be chemically modified for high adsorption capacity or selectivity and thus they are being increasingly investigated for potential use in hydrogen storage, gas separation, sensing and catalysis (Lu and Zhao, 2004).

1.3.2. NANOINTERMEDIATES

Nanointermediates are materials into which raw nanoparticles are incorporated or materials synthesized with nanoscale features to be used in, or applied to, a final nano-enabled product. Examples include fabrics that have been impregnated with silver nanoparticles for their antimicrobial activity (Perelshtein et al., 2008), paints and coatings with anti-scratch or photocatalytic properties which will then be applied to glass, plastics or construction materials (Allen et al., 2005; Chen et al., 2009b; Geng et al., 2008; Guarino et al., 2008), or electrical components synthesised using nanomaterials which will then be included in larger electrical devices.

Nanocomposites are multiphase solids where one of the phases has a dimension of less than 100 nm (Ajayan et al., 2003), many of which can be classified as nanointermediates. The nanomaterials added (often carbon nanotubes) produce materials with altered mechanical, electrical, optical and catalytic properties such as rubber with increased elasticity and strength (Bandyopadhyaya et al., 2004), and plastics that can conduct and electrical current (Poblete et al., 2009).

1.3.3. NANO-ENABLED PRODUCTS

There are now over 800 'nano-enabled' consumer products already available to the public according to the Project for Emerging Nanotechnologies consumer products inventory (2009), including the 2004 Chevrolet Impala made by General Motors which has body side moulding made from a nanocomposite material, antibacterial kitchen and tableware with a nano-silver coating made by Nano Care Technology Ltd. and Diorskin Forever - Extreme Wear Flawless Makeup by Dior®. The full diversity of nanotechnology however, has yet to be realised, as many prospective products and uses for nanomaterials are still in the research and development stages. The diversity of industries pursuing the development of potential products is highlighted below, however it should be noted that this is by no means a complete list (Table 3).

1.3.4. NANOTOOLS

The progression of the nanotechnology industry has necessitated the development of technical instruments able to produce, manipulate, visualise and model matter at the nanoscale. As previously mentioned, the invention of the scanning tunnelling microscope and the atomic force microscope have been instrumental to the field, allowing for atomic level examination and manipulation of surfaces and have been developed and adapted to create other techniques such as dip pen nanolithography in which nanoscale constructs such as conductive traces can be 'written' onto surfaces (Wang et al., 2008b).

Control over the precise structure, nature and size of the nanomaterials in many applications is paramount, therefore techniques and equipment such as molecular modelling software, nanoparticle production and coating techniques needed to achieve these aims are continually being developed and refined (Mohanraj and Chen, 2006; Rao et al., 2004).

Table 3. Diversity of sectors and applications for nano-enabled products

Sectors	Use	References
Biological, Biomedical and Pharmaceutical	Novel pharmaceuticals • *HIV treatment* • *Virus inhibition* • *Treatment of neuro-degenerative diseases* • *Treatment of age related disorders*	(Elechiguerra et al., 2005) (Sun et al., 2008) (Bosi et al., 2003; Nakamura and Isobe, 2003) (Rzigalinski, 2005)
	Gene therapy	(Tan et al., 2007)
	Drug delivery	(Choi et al., 2007; Klumpp et al., 2006)
	Cancer treatment • *Photodynamic therapy* • *Tumour targeting* • *Countering multi-drug resistence*	(Guo et al., 2008; Hirsch et al., 2003) (Ivanković et al., 2006; Ji et al., 2006; Kong et al., 2008) (Guo et al., 2008; Song et al., 2006)
	Sterilization • *Fabrics/clothing* • *Wound dressings/bandages* • *Surface disinfection* • *Antiseptics* • *Antibacterial*	(Jung et al., 2007; Perelshtein et al., 2008) (Chen and Schluesener, 2008) (Kuhn et al., 2003) (Hetrick et al., 2009) (Chen et al., 2008; Choi et al., 2008; Jayesh et al., 2008)
	Prosthetics	(Webster et al., 2004)
	Imaging • *MRI Contrast* • *X-ray Contrast* • *Cellular imaging*	(Babes et al., 1999; Hacliipanayis et al., 2008; Shapiro et al., 2004) (Bosi et al., 2003) (Qu et al., 2008)
	Nerve re-growth	(Ellis-Behnke et al., 2006)
	Biological tools • *Cellular Probes* • *In vitro and in vivo cellular imaging* • *Biomolecule detection* • *Oligonucleotide synthesis*	(Helmke and Minerick, 2006) (Delehanty et al., 2009; Mukherjee et al., 2009) (Chan and Nie, 1998; Marin and Merkoci, 2009) (Zhao et al., 2009)
	Cosmetics and sunscreens	(Anselmann, 2001; Brezová et al., 2005; Lademann et al., 2000)

Sectors	Use	References
Energy Production	Energy Cell • *Solar* • *Fuel*	(Kamat, 2007; Lee et al., 2009b; Quintana et al., 2007; Yu and Chen, 2009) (Du and Wang, 2009; Ekstrom et al., 2007; Mohapatra et al., 2007)
	Hydrogen storage	(Collins and Avouris, 2000; Geim and Novoselov, 2007; Leonard et al., 2009; Rao et al., 2009)
	Fuel additives	(Jung et al., 2005)
Environmental	Remediation • *Groundwater* • *Wastewater* • *Soil* • *Seawater*	(Barcelona, 2005; Nikolaidis et al., 2003; Ponder et al., 2000; Zhang, 2003) (Liu et al., 2006; Logar and Venčeslav Kaučič, 2006) (Hamerski et al., 1999) (Ziolli and Jardim, 2001)
Materials, Paints and Coatings	Composite Materials • *Light weight, strong e.g. sports equipment* • *Increased elasticity and strength e.g. rubber* • *Increased hardness e.g. drill bits* Paints and Coatings • *Water-repellent* • *Photocatalytic* • *Anti-scratch/mar*	(Esawi and Farag, 2007) (Bandyopadhyaya et al., 2004) (Zhang et al., 2006) (Xue et al., 2009) (Allen et al., 2005; Geng et al., 2008; Guarino et al., 2008) (Chen et al., 2009b)
Electronics, Computing and Data Storage	Components • *Chemiresistors* • *Transistors* • *Heat sinks* • *Circuits/wires*	(Chow et al., 2009) (Weitz et al., 2009) (Kordas et al., 2007) (Lee et al., 2008b; Wang et al., 2008a; Wang et al., 2008b)
	Sensors	(Buso et al., 2008; Guerrini et al., 2009; Valentini et al., 2007)
	Memory/Data Storage	(Snoeck et al., 2008; Vettiger et al., 2002; Yang et al., 2007)
	Displays	(Kang et al., 2008; Walters et al., 2005; Yotani et al., 2009)
General Industrial Processes (Catalysts)	• *Industrial synthesis* • *Degradation*	(Du and Wang, 2009; Kim et al., 2009; Pong et al., 2007; Sharghi et al., 2009; Singh et al., 2009) (Bertelli and Selli, 2006; Dabrowski et al., 2002; Kumar et al., 2008; Liu and Zhao, 2009)

1.4 ENVIRONMENTAL IMPACT OF NANOTECHNOLOGY

1.4.1. ENVIRONMENTAL AND HUMAN HEALTH CONCERNS

With worldwide investment in the nanotechnology industry in the billions of dollars and projected sale of nano-enabled products tipped soon to be in the trillions of dollars, there is no questioning the economic importance of the industry. There is considerable concern, however, from both government bodies and scientists that nanotechnology may have a negative impact on human health and the environment but there is a lack of knowledge in these areas (Colvin, 2003; Dowling et al., 2004; Klaine et al., 2008; Oberdörster et al., 2005; Owen and Handy, 2007; Williams et al., 2005).

The diversity of potential applications also means that nanotechnology is likely to yield considerable benefits to society in terms of general consumer products, healthcare and the environment. These groups and the nanotechnology industries themselves therefore will be anxious to avoid a repeat of the scenario concerning genetically modified (GM) foods where products were released for consumer use before potential health and environmental hazards were adequately assessed, leading to much negative press, a public backlash and blocking of licensing of GM products in some European countries (Brumfiel, 2003; Schmidt, 2009).

Nanoparticles from natural sources such as volcanic eruptions and forest fires have in fact been present in the atmosphere for millions of years (Buzea et al., 2007). Previous anthropogenic sources of nanoparticles include fumes from cooking fires (Buzea et al., 2007) and more recently exhaust from diesel vehicles (Qian et al., 2000), but with ENMs inevitably entering into the environment due to direct application (eg groundwater remediation), general use (eg cosmetics), accidental release or degradation of nano-enabled products, the load on the environment is likely to increase dramatically in the near future.

Despite this however, there is little known about the behaviour of ENMs in the environment, their interactions with biotic and abiotic components or the potential toxic effects they may cause to living organisms.

In addition to this, there are currently no standardised measures or methods of measurement to assess the actual load of ENMs in the environment and consequently levels of ENMs within the various environmental compartments are at present unknown or estimates based on modelling approaches. The next section of text describes what is currently known about the levels and fate of nanoparticles in the atmospheric, soil and aquatic compartments with particular attention to the behaviour and interactions of nanoparticles in the aqueous compartments of soils and surface waters as the physicochemical principles governing their behaviour in these compartments is similar (Lead and Wilkinson, 2006).

1.4.2. ENGINEERED NANOMATERIALS IN THE ATMOSPHERE

Due in part to the mounting evidence to suggest a link between respiratory health and exposure to nanoparticulate matter present in the atmosphere in the 1990's (Peters et al., 1997; Sioutas et al., 2005), there has been considerable study of the atmospheric levels and composition of what are termed ultrafine particles (UFPs, any particles of <0.1 μm in diameter)(Sioutas et al., 2005).

In urban areas, motor vehicles and diesel engines emitting primary combustion products have been found to be the major sources of UPPs, contributing up to 36% of the particle numbers in the atmosphere (Shi et al., 2001). Because of their small size, UFPs do not tend to settle out and they therefore have a long half-life in the atmosphere and can be transported considerable distances. UFP concentrations in proximity to busy roads have been measured at up to $2x10^5$ particles cm^{-3} and their formation, size and atmospheric concentrations have been found to be influenced by the relative distance from roads as well as meteorological factors such as season, temperature and humidity levels, as well as levels of ozone present in the atmosphere (Sioutas et al., 2005). It has also been

shown that adsorption of air pollutants including organic compounds such as polycyclic aromatic hydrocarbons, oxidant gases and transition metals to the surface of UFPs can occur, facilitating co-transport of these compounds (Oberdörster, 2001). Other studies have investigated the concentrations and chemical properties of UFPs in the indoor environment and the likelihood of transport of UFPs between indoor and outdoor environments and have shown that significant levels of particles found indoors are of outdoor origin, indicating that movement of UFPs into buildings occurs (Jones et al., 2000; Thatcher and Layton, 1995).

These findings have clear implications in the area of nanotechnology, especially concerning the safety of workers in factories producing ENMs and the atmospheric transport of ENMs out of factory environments, although few studies tackling the issue of load and transport of ENMs, as opposed to UFPs, in the atmosphere have been carried out.

Two recent studies used modelling approaches to estimate the likely load of various ENMs in the air, soil and aquatic compartments of the environment (Boxall et al., 2008; Mueller and Nowack, 2008). The model by Boxall and colleagues used relative importance of exposure routes to all environmental compartments as well as proportion of available consumer products containing ENMs and concentrations of ENMs in those products as inputs to estimate the likely environmental concentrations of ENMs in the United Kingdom. Their predicted exposure concentrations for a person applying sunscreen containing TiO_2 ENMs outdoors was 7 mg m^{-3} whereas the estimate of atmospheric levels of CeO_2 ENMs as a result of emissions from diesel vehicles was $6x10^{-7}$ mg m^{-3}. The model by Mueller and Nowack used inputs including global production volume, allocation of production volume into product categories, release of particles from products and flow coefficients of ENMs between the environmental compartments to estimate likely loads of silver, TiO_2 and carbon nanotubes in the air, soil and aquatic compartments in Switzerland. Their predicted atmospheric concentrations were $1.7x10^{-3}$, $1.5x10^{-3}$ and $1.5x10^{-3}$ µg m^{-3} for silver ENMs, TiO_2 ENMs and carbon nanotubes, respectively. It is difficult to compare the values for TiO_2 ENM concentration given by the

two models, as the estimation from the Mueller and Nowack model is calculated based on total airborne concentration of TiO_2 and the figure reported by Boxall and colleagues is representative of the airborne exposure of a person after direct application of a sunscreen product containing TiO_2 ENMs.

Exhaust from diesel engines, where cerium oxide nanoparticles were added to fuel to increase combustion efficiency, has been shown to contain low levels of cerium oxide particles, indicating that this will be a likely source of ENMs to the atmosphere in the near future (Costantini et al., 2001). A study by Maynard et al. (2004) found that typical handling of single-walled carbon nanotubes in a laboratory environment resulted in average deposits on workers' gloves of between 0.2 mg and 6 mg per hand and airborne concentrations of up to 53 μg m^{-3}. Another study found that handling of silver and alumina nanoparticles inside fume hoods in a laboratory setting could result in a significant release (>13,000 particles cm^{-3}) of airborne nanoparticles into the laboratory environment (Tsai et al., 2009).

1.4.3. ENGINEERED NANOMATERIALS IN SOILS

According to the modelling approach proposed by Mueller and Nowack (2008), the main direct route of entry of silver, TiO_2 and carbon nanotube ENMs to the soil is *via* run-off through use of paints and cleaning agents and sprays, however the release and subsequent deposition of airborne ENMs to the soil was also a major consideration. Predicted soil concentrations of 0.02, 0.4 and 0.01 μg kg^{-1} of soil for silver ENMs, TiO_2 ENMs and carbon nanotubes, respectively, were calculated. Routes of entry for other ENM types may also include run-off from road surfaces, accidental release from factories, degradation of the products and subsequent leaching of nanoparticles to the soil through landfill. The model proposed by Boxall and colleagues (2008) predicted that the main routes of exposure of ENMs to soils would be through the application of soil and groundwater remediation technologies and application of plant protection products, through the excretion of veterinary medicines, through deposition from the air and

through the application of sewage sludge to agricultural land as fertilizer. Their estimates of exposure concentrations for silver, TiO_2 and fullerenes in soil are 0.43, 1030 and 13.1 μg kg^{-1} and are markedly higher than those proposed by the Mueller and Nowak model, and the estimation for levels of latex and ZnO ENMs in the soil are extremely high (4307 and 3194 μg kg^{-1} respectively). It is difficult to ascertain at this stage which model presents values that are closer to actual environmental levels, as little work has been done in this area to date, and although the Boxall model suggests that their calculated figures are possible overestimations (given that they assumed 10% market penetration of nano-enabled products) the routes of exposure considered are more comprehensive

Entry of nanoparticles into the soil environment has potential implications for soil dwelling organisms such as nematodes (Wang et al., 2009), terrestrial isopods (Jemec et al., 2008), earthworms (Scott-Fordsmand et al., 2008) and soil bacterial communities (Johansen et al., 2008) where reproduction and changes in community structure as a result of exposure to ENMs have been demonstrated. Plant growth and agriculture in affected soils are also likely to be affected as various nanoparticle types have been found to inhibit seed germination (Lin and Xing, 2007) root elongation (Cañas et al., 2008; Lin and Xing, 2007), cause general toxicity (Lee et al., 2008a) and accumulate and translocate within plant tissues (Lee et al., 2008a; Zhu et al., 2008a).

1.4.3.1. Groundwater Remediation

For some ENMs an important route of entry into the environment is *via* direct application for environmental remediation purposes, a technology that has already progressed to full-scale commercial use (Tratnyek and Johnson, 2006; Johnson et al., 2009). Zero-valent iron nanoparticles are highly redox active and are thus being employed to clean up soils contaminated with heavy metal ions, polychlorinated hydrocarbons, pesticides and radionuclides (Zhang, 2003). Application of the nanoparticles is in the form of slurries with high concentration of iron nanoparticles (in the order of grams per litre; Zhang, 2003) which are injected into the soil forming a colloidal reactive barrier

(Giasuddin et al., 2007) or solid permeable reactive membranes coated with zero-valent iron placed into the soil, perpendicular to the flow of groundwater.

Whilst this technology is potentially very beneficial to the environment, there are also potential hazards. The effectiveness of zero-valent iron as a reducing agent for many toxic chemicals also means it has the ability to cause oxidative stress in cells (Phenrat et al., 2009) meaning residual zero-valent iron particles after treatment of soils may themselves become a soil pollutant. Similar problems may arise with the use of static permeable membranes where leaching of up to 70% of iron to the soil means that subsequent iron removal treatments are required if the groundwater is a source of drinking water (Nikolaidis et al., 2003). The generation of toxic treatment by-products, is also a consideration, as the likelihood of this occurring increases with the complexity of the contaminant mixture (Barcelona, 2005). The presence of humic substances which are ubiquitous in soils have also been found to reduce the effectiveness of reduction of pollutions due to competitive binding (Giasuddin et al., 2007).

1.4.3.2. Behaviour and Transport of Engineered Nanomaterials in Soil

The behaviour of naturally occurring nanoparticulate matter in soils known as colloids, has been studied for many years. Colloids are usually defined as material with one dimension between 1 nm and 1 μm (Lead and Wilkinson, 2006) and in natural aquatic systems, they constitute a complex aquatic mixture including viruses and bacteria, natural organic matter (NOM) such as humic and fulvic acids (HA and FA), 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). ENMs entering the soil will thus become components of these colloids and their subsequent behaviour and transport through the soil will depend both on interactions with other colloidal components and with the soil material itself.

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 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 stabilized in suspension. Reducing or eliminating the charge causes the colloids to agglomerate and settle out of suspension or form interconnected matrices (Cao, 2004).

Colloids therefore have a propensity to adsorb to soil particulate matter and to aggregate to particles >1 μm in size (Klaine et al., 2008), which reduces the likelihood of transport (Baalousha et al., 2008). Alteration of the characteristics of the aqueous media however, such as ionic concentration and composition, pH and concentration of NOM have been found to change the degree of aggregation and stabilization (Baalousha et al., 2008). The chemical and physical nature of soil and the ENMs themselves are also important factors in determining the stability and subsequent transport of ENMs in the soil.

A few studies have recently begun to investigate the importance of these factors with regard to the behaviour and environmental impact of ENMs in the soil and the aquatic compartments. Increases in NaCl concentration were found to have minimal impact on fullerene (C60) aggregate size, whereas increasing concentrations of calcium chloride were associated with a seven-fold increase in aggregate diameter (Wang et al., 2008e). The formation of aggregates of iron oxide nanoparticles and aggregate size were also found to be dependent on concentration of humic acid and pH, with maximum aggregate formation at pH 8.5 and at pH 4-5 in the presence of humic acid (Baalousha et al., 2008). Furthermore, the same study reported increasingly larger aggregates formed as pH increased from 2 to 6 and with increasing humic acid concentrations.

However a separate study using TiO_2 nanoparticles, found that although increased aggregation was associated with an increase in ionic strength at any pH, upon the addition and subsequent sorption of fulvic acid to the particles, less aggregation was

observed and nanoparticle dispersions were often found to be stable for environmentally relevant conditions of fulvic acid, pH, and ionic strength (Domingos et al., 2009). This suggests that TiO_2 dispersion in the natural environment might occur.

A recent study found that TiO_2 could remain suspended in soil suspensions for up to 10 days and the amount of TiO_2 remaining suspended after 24 hours was positively correlated with the amount of dissolved organic carbon (DOC) and the clay content of the soil, but negatively correlated with ionic strength, pH and zeta potential (Fang et al., 2009). In the same study, it was reported that in soils containing greater proportions of larger soil particles (20-2,000 µm) and lower ionic strength solutions (<1 mM), 18.8-83% of TiO_2 passed through soil columns but was retained in soils with high clay content and salinity and estimated transport distances for TiO_2 in some soils was between 41.3 and 370 cm.

Another study found that at low ionic strength (3.05 mM), C60 particles were readily transported through 40- to 50-mesh quartz sand, however at higher ionic strengths (30.05 mM) and in finer Ottawa sand (100–140 mesh), greater than 95% of the introduced C60 particles were retained in the column regardless of the electrolyte species. Adjustment of the pH to 10 and 12 allowed 50% recovery of the C60 particles demonstrating that C60 transport and retention in water-saturated sand is strongly dependent on the presence of electrolytes but also on soil/sediment type (Wang et al., 2008e).

Lecoanet et al. (2004) found that fullerenes functionalized to aid dispersion in water had high mobility in porous media and speculated that naturally occurring polyelectrolytes such HAs might enhance nanoparticle transport in a similar way by sorbing to surfaces of particles and reducing attachment efficiencies through steric stabilization and increased hydrophilicity of the particle surface.

Use of ENMs for groundwater remediation requires the stabilization and movement of ENMs through the soil and recent studies have found that addition of the amine hexadecyl-trimethylammonium bromide and mixtures of polyacrylate with poly(4-

styrenesulfonate) and bentonite clay significantly enhanced nano-iron transport through soils (Hydutsky et al., 2007; Seaman and Bertsch, 2000).

1.4.3.3. Co-transport of Pollutants

The interaction of nanoparticles with organic matter such as humic and fulvic acids in soils is now being extensively studied in order to improve the understanding of how these interactions might affect both the stability and mobility of nanoparticles in soils, but also their ability to bind and act as co-transporters of other soil 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 ZnO) is pH dependent and 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 metal oxide nanoparticles with applied coatings of HAs showed enhanced sorption for organic chemicals such as pyrene and phenanthrene (Wang et al., 2008d; Yang and Xing, 2009). Similarly, suspensions of C60 that were stable over a wide range of pH and salinities showed enhanced adsorption of polycyclic aromatic hydrocarbons in the presence of HA suggesting that release of C60 into aqueous environments might affect PAH fate and exposure (Hu et al., 2008).

1.4.4. ENGINEERED NANOMATERIALS IN THE AQUATIC ENVIRONMENT

Colloids in natural surface waters, like the colloids in soils are a complex mixture of inorganic and organic components such as ions, metal and non-metal oxides and NOM including humic and fulvic acids, cellular debris, polysaccharides and amino acids (Lead

and Wilkinson, 2006). The behaviour of nanoparticles in surface waters will therefore be dominated by the same physicochemical principles as those that determine the behaviour of natural colloids in soil. As in soils, there is tendency for nanoparticles to aggregate, leading to sedimentation and decreased transport within the water column and suggests that sediment-dwelling and benthic organisms will be more prone to exposure than pelagic species. However, as previously mentioned, the chemistry and physical characteristics of the ENMs themselves are a key element in determining fate and behaviour and certain conditions such as presence of humic and fulvic acids, pH and specific cation concentrations may favour the stabilization of ENMs in the water column giving them the potential for uptake by aquatic organisms and transport within water systems, although general models for predicting this behaviour have yet to be developed (Baalousha et al., 2008).

The aquatic environment is a sink for pollutants from both the atmosphere, soil and from those entering the water system directly, which suggests that the aquatic environment is particularly vulnerable to contamination from ENMs.

The model proposed by Mueller and Nowack (2008) did not take into account any transport of nanoparticles from the soil to the water compartment, however behaviour and transport of ENMs in soil has important implications for the aquatic environment since it is known that surface water bodies import water from groundwater reservoirs (Schaller and Fan, 2009) and that pollutants present in groundwater can be transported into surface waters (Caruso and Dawson, 2009).

Although methods for detecting and characterizing ENMs in natural waters are being developed (Dubascoux et al., 2008; Hassellöv and Stolpe, 2007) they are still in their infancy and therefore at present, as for the air and soil, information regarding the levels of ENMs in the aquatic environment is scarce. The model by Mueller and Nowack (2008) gave estimated concentrations of silver and TiO_2 nanoparticles and carbon nanotubes in

water of 0.3 μg L^{-1}, 0.7 μg L^{-1}, and 0.0005 μg L^{-1} respectively, however a much higher value of 16 μg L^{-1} in a scenario of high emission of TiO_2 was calculated.

The only direct route of entry proposed by the model is through the washing-off of TiO_2-contatining cosmetic applications into water systems with most ENM entry predicted to pass *via* wastewater treatment works (WWTWs) to surface waters as a result of application of cosmetics and coatings, runoff from paints and spray and abrasion and subsequent run-off of ENM-containing metals and plastics. The washing of fabrics impregnated with silver nanoparticles as an antimicrobial agent may be another important source. Boxall and colleagues (2008) estimated concentrations of silver, TiO_2 and fullerenes in the aquatic environment to be 0.01, 24.5 and 0.31 μg L^{-1} respectively. Estimated ZnO and CeO_2 concentrations in the aquatic environment are 76 and <0.0001 μg L^{-1}. As with the predicted ENM soil concentrations, their model considered more possibilities for direct entry of ENMs to aquatic systems than the model by Mueller and Nowack, including application of ENMs to waters systems for bioremediation, spray drift from agricultural chemicals and deposition of airborne ENMs as well as run-off from contaminated soils and WWTW emissions. The estimated levels in the aquatic environmental are more similar than those generated by the two models for the soil compartment, although again, as few studies have measured actual environmental concentrations in aquatic systems it is difficult to ascertain which model has provided the more realistic estimates.

A study by Benn and Westerhoff (2008) found that socks containing up to 1360 μg silver per gram of material leached as much as 650 μg of silver in 500 mL of distilled water. Clearly, wastewater and effluents from factories producing raw nanoparticles and nano-enabled products are also an important source for contamination into the aquatic environment.

A study by Limbach et al. (2008) used a model wastewater treatment plant to investigate the efficiency of removal of cerium oxide nanoparticles. The majority of nanoparticles were found to adhere to the clearing sludge and were retained within the

plant, however up to 6 % wt of the particles were found in the exit stream. It was further demonstrated that nanoparticle surface charge influenced the levels of cerium oxide found in the effluent, 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 wastewater. This suggests that passage of wastewater through present WWTWs cannot guarantee the removal of ENMs from the discharged wastewater.

There is of course the potential for nanoparticles to enter the aquatic environment through runoff as evidenced in a study by Kaegi et al. (2008). The study, which to date is the only published measurement of ENMs entering the aquatic environment, investigated whether TiO_2 nanoparticles detached from both new and aged façade paints by natural weather conditions resulted in runoff and subsequent discharge into surface waters. Concentrations of titanium from nanoparticles in the runoff collected directly from beneath newly painted façades were approximately 550 mg L^{-1} and concentrations collected from an aged facade were approximately 300 mg L^{-1}. Samples of 'urban' runoff which had a catchment area of a network of roads, pavements and buildings in the vicinity of the painted façades, collected directly before discharge into a stream also had concentrations of titanium from nanoparticles of approximately 300 mg L^{-1}. This suggests that weathering of paints containing ENMs could be responsible for significant discharges of ENMs into the aquatic environment.

Thus from the introductory text above it would appear that development of the nanotechnology industry poses considerable exposure risks to all environmental compartments and there is therefore pressure for assessment of this risk to keep up with innovation and development of new ENMs and nano-enabled products.

This will mean further development of techniques for detection, identification and characterization of ENMs in the environment; development of more comprehensive models to predict environmental exposure and transport between environmental

compartments and models to predict their behaviour and environmental fate and this will require a multi-disciplinary approach. Improving our understanding of these aspects is vital to understanding their risk to human health and potential ecotoxicology.

1.5. NANOTOXICOLOGY

The rapid development of the nanotechnology industry has been fuelled by the unusual properties that materials possess at the nanoscale, however it is also these properties that are in part fuelling concern regarding their potential toxicity and ecotoxicology.

The altered quantum behaviours and enhanced reactivity that make ENMs useful in so many applications may also mean their effects within the environment and their interactions with living organisms are exaggerated or unexpected. In addition, their small size means they may be able to bypass barriers that prohibit the entry of other xenobiotics and enter the cells of living organisms, passing through membranes and junctions between cells. This coupled with their enhanced reactivity may mean ENMs have the potential to induce adverse cellular effects and harm to living organisms. To date however, there are relatively few studies investigating the effects of exposure to ENMs on living organisms, especially those living in aquatic environments.

Since the establishment of a link between mesothelioma and asbestos exposure in the 1960's and 70's (Elmes et al., 1965; Fowler et al., 1964), much research has been conducted 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, showed that ultrafine particles, now synonymous with nanoparticles, are capable of inducing adverse effects in the lungs (Donaldson et al., 1990; Driscoll et al., 1995; Driscoll et al., 1991; Kusaka et al., 1990; Lam et al., 1985) and this has set a precedent for toxicity studies using ENMs. As a consequence, until recently the majority of nanotoxicology studies conducted have been inhalation-based studies in terrestrial vertebrates, with comparatively less attention being paid to alternative routes of exposure and exposure of organisms living in other environments. Now, however, more

studies are emerging that explore other areas such as potential routes of uptake in both terrestrial and aquatic organisms, translocation and fate in the body, interaction of ENMs with cells representative of 'portal of entry' tissues and how the characteristics of the ENMs and the surrounding exposure medium affect uptake and effect.

The next section introduces what is currently known about the toxicology of the most prominent ENM types, including an examination of different routes of exposure in terrestrial vertebrate models, an overview of the published *in vitro* studies and studies that examine the toxicity of ENMs in aquatic organisms.

1.5.1. EXPOSURE OF TERESTRIAL ORGANISMS TO NANOMATERIALS

1.5.1.1. Carbon-Based Nanomaterials

Due to their prominence within industry, a large number of inhalation studies have examined the effects on the lungs of carbon-based nanoparticles. Exposure of mice and rats to ultrafine carbon black particles was often characterized by an inflammatory response which in some cases varied in strength according to the total surface area of particles applied (Shwe et al., 2006; Stoeger et al., 2006; Yamamoto et al., 2006), whereas inflammation accompanied by granulomas in lung tissue were characteristic of exposures to mice and rats with single-walled carbon nanotubes (SWCNTs) (Lam et al., 2004; Warheit et al., 2004). In contrast, Baker et al. (2008) found that exposure to C60 showed minimal toxic effects in rats exposed *via* inhalation.

Studies investigating the fate of inhaled carbon nanoparticles have shown a propensity for accumulation in the liver *via* the blood and possibly the gastrointestinal tract in rats (Oberdörster et al., 2002) and also translocation to the olfactory bulb, cerebrum and cerebellum where the proposed pathway was *via* the olfactory nerve (Oberdörster et al., 2004). A study in humans however, found that there was no significant translocation of inhaled carbon nanoparticles into the circulatory system, indicating that the rodent model may not necessarily be applicable to other mammals (Wiebert et al., 2006).

Other studies detailing the fate and effects of carbon-based nanoparticles administered *via* other routes such as *via* intravenous or intraperitoneal injection and *via* the diet have

also been conducted. Studies in mice have shown that the liver and bones are target organs for both intravenously injected fullerols (Li et al., 2002) and metallofullerols (Cagle et al., 1999) with fullerols also accumulating in the kidney and spleen. In both studies slow clearance *via* the urine and faeces from all tissues occurred except for the bones.

Oral administration of fullerene and single-walled carbon nanotubes (SWCNTs) to rats was associated with oxidative DNA damage in the form of elevated levels of pre-mutagenic 8-oxo-7,8- dihydro-2´-deoxyguanosine in the liver and lungs (Folkmann et al., 2009). A similar study showed that water-soluble fullerene administered orally in rats was excreted rapidly. However on intravenous injection, fullerene was retained in the body (mostly in the liver, lungs, spleen and blood) for up to 160 hours and was also found to cross the blood brain barrier although no acute toxicity was observed (Yamago et al., 1995). A dose of 25 mg kg^{-1} of a different water-soluble fullerene intravenously injected to rats however was found to cause shortness of breath, convulsions and death within 5 minutes of dosing (Rajagopalan et al., 1996).

After intraperitoneal injection of C60 to pregnant mice, C60 was distributed to the yolk sac and embryos resulting in deformities in the embryos and death with the generation of reactive oxygen species (ROS) being the proposed mechanism of toxicity (Tsuchiya et al., 1996). Intranigral infusion of carboxyfullerene to rat brains however, was found to prevent oxidative stress induced by iron citrate (Lin et al., 1999).

Studies in which multi-walled carbon nanotubes (MWCNTs) have been injected into the abdominal cavity of mice have shown that as in inhalation studies, these compounds induce asbestos-like pathogenicity including inflammation, presence of granulomas and induction of mesothelioma (Poland et al., 2008; Takagi et al., 2008).

These studies indicate that administration route as well as differences in both the chemical structure of carbon-based nanoparticles and surface functionalization may have a profound impact on their effects *in vivo*.

Common responses of cells to exposure to fullerene include cytotoxicity related to generation of ROS, lipid peroxidation and apoptosis (Isakovic et al., 2006; Sayes et al.,

2004; Sayes et al., 2005). Several studies have indicated that non-functionalized fullerenes are more toxic to cells than hydroxylated or otherwise functionalized fullerenes, despite their poor solubility (Isakovic et al., 2006; Sayes et al., 2004). The toxicity observed from pristine C60 was commonly found to be as a result of generation of ROS and subsequent lipid peroxidation whereas functionalized fullerenes were found to cause cell death by inducing apoptosis (Isakovic et al., 2006; Sayes et al., 2004; Sayes et al., 2005). Water-soluble fullerenes have also been found to cross cell membranes *in vitro* and localise preferentially in the mitochondria (Foley et al., 2002).

In vitro studies examining the effects of carbon nanotubes on cells have shown that both SWCNTs and MWCNTs can have a dose-dependent inhibitory effect on cell proliferation and growth (Cui et al., 2005; Magrez et al., 2006; Murr et al., 2005; Raja et al., 2007; Zhang et al., 2007a) and induce apoptosis (Cui et al., 2005). In contrast to the results seen in exposures to fullerenes, increases in toxicity have been associated with functionalization of the nanotube surface as a result of acid treatment (Magrez et al., 2006).

Studies investigating the interactions of carbon nanotubes (CNTs) with cell membranes have shown that CNTs were able to cross cells membranes in rat macrophages, and whereas CNTs caused no acute toxicity, trace metal contaminants induced intracellular ROS generation (Pulskamp et al., 2007). In contrast, highly purified MWCNTs injured the plasma membrane of mouse macrophages through interaction with proteins on the membrane surface (Hirano et al., 2008). The degree and kind of agglomeration of carbon nanotubes has also been shown to affect their toxicity *in vitro* with suspended CNT-bundles found to be less cytotoxic than rope-like agglomerates of CNTs which in turn were found to be more cytotoxic than asbestos (Wick et al., 2007).

These studies demonstrate that many aspects of the behaviour and toxicity of carbon-based nanoparticles in the bodies and cells of terrestrial organisms, are highly dependent on their basic chemical structure (e.g. single or multi-walled CNT), the degree to which their surfaces are functionalized, the nature of their agglomeration and the route of exposure to the organism.

1.5.1.2. Titanium Dioxide Nanoparticles

Titanium dioxide nanoparticles have also been the focus of many toxicity studies as, like carbon-based nanoparticles, they are also already widely used within industry.

A common response in mice administered with TiO_2 nanoparticles *via* inhalation and intratrachaeal instillation, as with the carbon-based nanoparticles, was induction of an inflammatory response (Grassian et al., 2007; Park et al., 2009). Increasing levels of macrophages and macrophage inflammatory protein-2 in lung lavage fluid as well as membrane damage and lipid peroxidation in the alveolar macrophages and peripheral red blood cells have been observed in rats exposed to TiO_2 nanoparticles *via* intratracheal instillation (Afaq et al., 1998; Dick et al., 2003). In contrast to this however, a study by Rehn et al. (2003) where rats were intrachaeally instilled with two types of TiO_2 nanoparticle (untreated and silanized surface) and found no evidence of inflammation. Differing levels of inflammatory response have also been observed between rats, mice and hamsters during sub-chronic inhalation exposures by Bermudez et al. (2004) with rats being apparently the more sensitive.

The size of the TiO_2 nanoparticle used in the exposures has been implicated in determining the level of toxic effect in some inhalation studies, however results have so far been conflicting in their findings. Larger sized TiO_2 nanoparticles have been found to induce higher lactate dehydrogenase (LDH) activity in mice (Grassian et al., 2007) however nano-sized TiO_2 particles were found to induce more inflammation and toxicity than bulk TiO_2 in rats (Renwick et al., 2004) and another study found that the level of inflammatory response was not dependent on size or surface area of the particles but was related to the surface chemistry of the particle (Warheit et al., 2006).

Other particle characteristics, such as chemical nature, crystal structure and surface chemistry, have also been suggested as factors determining the toxicity observed in inhalation studies with TiO_2 nanoparticles (Renwick et al., 2004; Warheit et al., 2007a).

Studies investigating the fate of inhaled or instilled TiO_2 nanoparticles have found that inhaled particles can be transported to the luminal side of airways and alveoli, to all

major tissue compartments in the lung and to the capillaries (Geiser et al., 2005). Transport to the mediastinal lymph nodes in rats (van Ravenzwaay et al., 2009) and the liver and spleen has also been reported but with no associated tissue response (Lee et al., 1985). Both anatase and rutile TiO_2 particles exposed to mice intranasally were found to enter the brain through the olfactory bulb and deposit in the hippocampus region where lipid peroxidation was observed. This effect was more pronounced for anatase TiO_2 than for rutile TiO_2 and the authors suggested this was due to differences in the crystal structure (Wang et al., 2008c). A study investigating the transport of nanoparticulate (21 nm) and bulk (120 nm) TiO_2 particles in a rat tracheal explants system found that both nano- and bulk particles were transported to the epithelium and subepithelial space, but that there was no preferential transport of particles (in terms of particle number) related to size (Churg et al., 1998).

A number of studies have demonstrated that major target organs for intravenously injected TiO_2 nanoparticles include the liver, kidney spleen, lungs and skin (Olmedo et al., 2008; Sugibayashi et al., 2008). Systemic migration of particles was found to occur regardless of administration route (Olmedo et al., 2008) and several studies demonstrated increasing macrophage activity and inflammatory responses in the target tissues (Olmedo et al., 2008; van Ravenzwaay et al., 2009). In contrast to this, one study found no evidence of inflammation or organ toxicity despite limited clearance of TiO_2 from the liver at 28 days post-injection (Fabian et al., 2008) although a further study found that slow clearance of (approximately 30%) TiO_2 from the liver did occur 1 month after injection in mice (Sugibayashi et al., 2008).

Oral administration of TiO_2 nanoparticles to mice resulted in a similar biodistribution pattern with particle transported to the liver, spleen, kidneys and lungs, with evidence of hepatic injury and nephrotoxicity and increased serum LDH and α-hydroxybutyrate dehydrogenase levels indicating myocardial damage (Wang et al., 2007).

Induction of ROS, oxidative stress and apoptosis are common observations in a wide variety of cell types exposed to TiO_2 nanoparticles. Oxidative stress was seen in canine

alveolar macrophages (Beck-Speier et al., 2001) and human bronchial epithelial cells (Gurr et al., 2005; Park et al., 2008) and the generation of ROS was observed in rat neurons (Long et al., 2007) and human lung epithelial cells (Singh et al., 2004). Decreasing cell area, cell proliferation, mobility and ability to contract collagen in human dermal fibroblasts exposed to TiO_2 nanoparticles were found as a result of the generation of ROS (Pan et al., 2009). In addition, cell apoptosis and accompanying necrosis were observed in a number of studies in human lung epithelial cells (Park et al., 2008), human monoblastoid cells (Vamanu et al., 2008) and Syrian hamster embryo fibroblasts (Rahman et al., 2002). In contrast, a number of studies have shown limited cytotoxicity or mitochondrial damage associated with TiO_2 exposure in mouse neuroblastoma cells and rat liver cells (Hussain et al., 2005; Jeng and Swanson, 2006; Lu et al., 2009).

Uptake into cells has been demonstrated by Komatsu et al. (2008) where TiO_2 nanoparticles taken up by mouse Leydig cells affected viability, cell proliferation and gene expression, potentially affecting reproduction. A study examining uptake mechanisms of TiO_2 nanoparticles in human dermal fibroblasts showed that both individual TiO_2 nanoparticles and TiO_2 particle clusters were able to penetrate the cell membranes and whereas particle clusters entered the cells by endocytosis, single particles were able to penetrate the membrane independent of this mechanism. Once inside the cells, particles were sequestered in vesicles, which filled up with increasing incubation time and eventually ruptured (Pan et al., 2009).

Some investigation into possible genotoxic effects has resulted in varying findings. A study in which TiO_2 nanoparticles were exposed to Chinese hamster ovary cells demonstrated that exposure resulted in the induction of sister chromatid exchanges and micronuclei (Lu et al., 1998), however, another study in rat liver epithelial cells found no evidence of cytotoxicity and a decrease in micronuclei formation (Linnainmaa et al., 1997). Similarly, a further study showed there was no cytotoxicity or mitochondrial damage as a result of TiO_2 exposure to human alveolar basal epithelial cells, but DNA damage was demonstrated which was more pronounced in exposure to micro-sized particles than nanoparticles (Karlsson et al., 2009).

Due to their known photoactivity and their usage within sunscreen applications, a number of studies have investigated the effects of ultraviolet (UV) irradiation of cells in the presence of TiO_2 nanoparticles. These studies have shown that upon irradiation with both UVA and UVB, TiO_2 can induce the generation of ROS and oxidative stress (Ashikaga et al., 2000; Park et al., 2007; Vileno et al., 2007) where DNA damage (Ashikaga et al., 2000; Dunford et al., 1997; Hidaka et al., 1997; Wamer et al., 1997), apoptosis (Park et al., 2007) and decreased cell stiffness (Vileno et al., 2007) were observed. Nakagawa et al. (1997) however, found no genotoxic or cytotoxic effects in the absence of UV.

Serpone et al., (2006), however, found that TiO_2 nanoparticles that had been photo-inactivated by thermal surface modification, suppressed damage caused to DNA plasmids and human keratinocytes by UVB/UVA simulated solar radiation.

To summarise, studies investigating the effects of TiO_2 nanoparticles in terrestrial organisms have therefore shown that inhaled TiO_2 nanoparticles are able to be transported from the lungs to other parts of the body, commonly inducing an inflammatory response. They have also been shown to be able to penetrate cell membranes and induce general toxicity responses such as oxidative stress and apoptosis as well as genotoxic effects *in vitro* and their effects appear to be dependent on physicochemical characteristics such as size and crystal structure and presence of UV radiation.

1.5.1.3. Silver Nanoparticles

Due to their known antimicrobial properties, concern over the exposure of silver nanoparticles to workers in manufacturing plants *via* inhalation and dermal contact (Choi et al., 2008; Fernandez et al., 2008) has recently prompted a number of inhalation studies in rat models.

An increase in the size and number of goblet cells in the lungs of rats after repeated acute exposures over a month-long period, but no obvious toxicity was observed (Hyun et al., 2008). In contrast, two further studies (Sung et al., 2009; Sung et al., 2008)

demonstrated that lung tidal volume and minute volume decreased significantly, and that lung tissue showed evidence of thickened alveolar walls and small granulomatous lesions after exposure to silver nanoparticles. A dose dependent increase in bile-duct hyperplasia was also observed in the liver, indicating that both the lungs and liver are target organs for silver nanoparticles.

The effects of silver nanoparticle exposure *via* intravenous injection and *via* the diet have also been investigated in exposures to rats and mice. Dose-dependent changes in alkaline phosphatase and cholesterol were observed in rats exposed orally to silver nanoparticles indicating that some liver damage occurred, however, accumulation of silver in other tissues was also found, notably the kidneys where a gender-related difference in accumulation was seen with a two-fold increase in female kidneys compared to male kidneys (Kim et al., 2008). Examination of the expression of a suite of genes related to oxidative stress in different regions of the mouse brain after intravenous injection of silver nanoparticles showed differences in expression in different regions of the brain and suggested that silver nanoparticles may induce neurotoxicity through the generation of free radical-induced oxidative stress (Rahman et al., 2009).

Cytotoxicity has been observed in a number of *in vitro* studies with silver nanoparticles, with commonly observed responses including LDH leakage (Braydich-Stolle et al., 2005), decreased mitochondrial function (Braydich-Stolle et al., 2005; Hussain et al., 2005), depletion of glutathione (GSH) (Arora et al., 2008; Hussain et al., 2005) and apoptosis (Arora et al., 2008; Braydich-Stolle et al., 2005; Hsin et al., 2008). Furthermore, the generation of ROS has also been demonstrated (Hsin et al., 2008) and it was shown to be size-dependent with smaller sized silver particles generating the most ROS (Carlson et al., 2008). Interestingly, a number of studies by the same research group have indicated that effects observed *in vitro* may be dependent on the cell type used. Oxidative stress was observed in both human sarcoma and epithelial carcinoma cells as a result of exposure to silver nanoparticles (Arora et al., 2008), however, although entry of silver nanoparticles into primary mouse fibroblasts and liver cells occurred, no

accompanying oxidative stress was observed (Arora et al., 2009). This suggests that caution must be taken when extrapolating results from *in vitro* nanoparticle toxicity assays between cell types and species.

These studies demonstrate that the liver, lungs and lungs are target organs for silver nanoparticles exposed to terrestrial animals *via* a variety of exposure routes and they have been shown to induce adverse effects at the cellular level such as apoptosis and generation of ROS.

1.5.1.4. Other Types of Nanomaterial

Inhalation and intratrachaeal instillation studies with other ENM types have given further evidence of ability of nanoparticles to cause inflammation in the lung. Zinc oxide particles have been shown to cause an increase in neutrophils and increased activity of alkaline phosphatase, acid phosphatase and LDH in the lavage fluid of guinea pigs (Conner et al., 1988) and latex nanoparticles induced both innate and adaptive immunity-dominant lung inflammation in mice and exacerbated inflammation caused by an allergen (Inoue et al., 2009). The inflammatory response of latex nanoparticles in rats was also found to be size-dependent, with smaller particles showing a greater response than larger particles and the response was also found to be directly proportional to the total surface area of instilled particles (Brown et al., 2001).

Instilled iron oxide (Fe_2O_3) nanoparticles were found to invoke increases in both inflammatory and immune cells in rat lungs as well as increasing microvascular permeability and causing disturbances in blood coagulation parameters (Zhu et al., 2008b).

Studies examining the translocation potential of inhaled gold nanoparticles have found that although the lungs are the main target organ, they are also translocated to the blood, oesophagus, kidney, spleen and heart (Takenaka et al., 2006; Yu et al., 2007). Furthermore, uptake of gold particles into alveolar macrophages and type I epithelial cells after inhalation was demonstrated and in both cell types, nanoparticles were

enclosed in vesicles in the cells' cytoplasm, showing the particles were processed by endocytotic pathways (Takenaka et al., 2006).

Other translocation studies have found that a variety of nanoparticles such as iron oxide (Wang et al., 2005), manganese phosphate (Dorman et al., 2002) and manganese oxide (Elder, 2006), can be transported to the olfactory bulb of the brain via the olfactory neuronal pathway after inhalation. Further to this, Elder, (2006) also found increases in protein levels and inflammatory marker tumour necrosis factor-α in the olfactory bulb.

The fate and effects of a variety of other nanoparticle types administered via the oral and intravenous route have also been investigated. The target organs for copper nanoparticles administered to mice *via* oral gavage were found to be the kidney, liver and spleen, where toxicity was observed in all these organs. Interestingly, male mice displayed more severe toxic symptoms compared with female mice. This was supported by a study from Lei et al. (2008), who also found that orally administered nano-sized copper particles induced hepatotoxicity and nephrotoxicity in rats. In addition, these studies demonstrated the differences in toxicity of particles of different sizes, as it was shown that micro-sized copper particles, in comparison to nanoparticles, were found to be virtually non-toxic to mice (Chen et al., 2005).

The liver was also a target organ for orally administered ZnO nano- and microparticles in mice, however the micro-sized particles in this exposure caused the more severe liver damage (Wang et al., 2006).

Intravenous injection of biocompatible magnetic nanoparticles resulted in their distribution to the liver, kidneys, spleen, heart, testes and uterus and brain in mice, indicating that the nanoparticles were able to penetrate the blood/brain barrier, however no toxicity in any organ was observed (Kim et al., 2006). Intravenous and intraperitoneal injection of gold nanoparticles in mice resulted in particles being taken up by endocytosis into Kupffer cells in the liver and to a lesser extent by macrophages in the spleen, lymph nodes and small intestine, but not into the kidneys or brain (Sadauskas et al., 2007).

A large number of other nanoparticle types have also been examined for their effects *in vitro*. Cerium oxide nanoparticles in particular have been studied due to their increasing use within catalytic applications, however the results have been extremely variable.

The major factor influencing uptake of cerium oxide nanoparticles in human lung fibroblasts was found to be size, with larger cerium oxide nanoparticles more readily taken up by than smaller nanoparticles with nanoparticle number density and total particle surface area of minor importance with regards to uptake (Limbach et al., 2005).

A few studies have described oxidative stress (Eom and Choi, 2009) and associated DNA lesions and chromosome damage (Auffan et al., 2009) as a result of cerium oxide nanoparticle exposure. Decreased cell proliferation and mitochondrial activity in human mesothelioma and rodent fibroblasts (Brunner et al., 2006) and increased catalase activity in cultured lung slices (Fall et al., 2007) have also been reported.

A number of reviews and studies however, have described the protective capabilities that cerium oxide nanoparticles have on cells. The potential for cerium oxide nanoparticles to increase cell longevity and perhaps increase lifespan as a result of their ability to protect against free radical-mediated toxicity have been discussed (Rzigalinski, 2005; Strawn et al., 2006) and a number of studies have demonstrated that cerium oxide nanoparticles give protection to mouse and rat neurons against oxidative injury (Das et al., 2007; Schubert et al., 2006).

Quantum dots have also been the focus of a number of *in vitro* studies due to their increasing use for biomedical imaging purposes. Dose-dependent decreases in cell viability and eventual cell death in monkey kidney cells and human primary hepatocytes (Shiohara et al., 2004) as well as decreased mitochondrial activity and autophagy in porcine kidney cells (Stern et al., 2008) have been described, with the metal content of the quantum dots an important factor in determining toxicity.

Surface coating and other physicochemical properties of the quantum dots such as size, charge, and oxidative and photolytic stability have also been implicated as factors in

determining the toxic effects of quantum dots *in vitro* (Clift et al., 2008; Hardman, 2006).

The toxicity of a number of other metal and metal oxide nanoparticle types has also been investigated *in vitro*. Zinc oxide nanoparticles were found to induce neural stem cell apoptosis (Deng et al., 2009), LDH leakage in mouse neuroblastoma cells (Jeng and Swanson, 2006) and have shown evidence of genotoxic potential in human epidermal cells (Sharma et al., 2009). The toxicity of vanadium oxide nanoparticles and bulk particles (V_2O_3 and V_2O_5) to human lung cells was found to be dependent on the oxidation state of the nanoparticle with the higher toxicity of V_2O_3 attributed to the higher catalytic activity on the surface of the particle although size-dependent toxicity was also observed (Wörle-Knirsch et al., 2007). Size-dependent toxic effects have been observed *in vitro* for both copper oxide and gold particles with damage to mitochondria in human alveolar basal epithelial cells more pronounced with smaller sized particles (Karlsson et al., 1991) and the mechanism of cell death (necrosis or apoptosis) in exposure to gold particles dependent on the size of nanoparticle administered (Pan et al., 2007).

Uptake and translocation studies with other particle types *in vitro* have shown that various nanoparticle types have the ability to bypass barriers and enter into cells. Polystyrene nanoparticles for example, were taken up by diffusion and adhesion mechanisms and shown to remain free within the cytosol of pulmonary macrophages (Geiser et al., 2005) and surface-functionalized gold nanoparticles were able to penetrate the nucleus in human cervical cancer cells (Gu et al., 2009). Wax nanoparticles have also been shown to both cross the blood-barrier in rat brain perfusions and alter the integrity and permeability of the barrier depending on their surface charges (Koziara et al., 2003; Lockman et al., 2004).

1.5.5. EXPOSURE OF AQUATIC ORGANISMS TO NANOMATERIALS

Most studies on aquatic organisms to date have been centred around work on daphnid species such as *Daphnia magna* and *Ceriodaphnia dubia* and model fish species such as *Danio rerio* (zebrafish) and *Oncorhynchus mykiss* (rainbow trout).

1.5.5.1. Effects of Nanomaterials in Aquatic Invertebrates

Daphnid organisms are crustacean filter feeders and thus are capable of interacting with a large proportion of their surrounding environment. As a result they can be more susceptible to xenobiotics than some other aquatic organisms. Daphnids are therefore routinely used as bioindicators for pollutants in aquatic systems and in aquatic toxicity testing.

Exposure of daphnids and other aquatic invertebrates to carbon-based nanomaterials has so far shown that they can be associated with a number of detrimental effects, which often were linked to the chemical nature of the nanoparticles being tested and preparation method of the nanoparticles.

Fullerenes prepared by either sonication in the exposure medium or filtered in tetrahydrofuran (THF) and subsequent evaporation of the THF were found to cause significant mortality in *Daphnia magna* (Lovern and Klaper, 2006). Filtered fullerenes in THF however, were markedly more toxic causing 100% mortality at 800 ppb whereas for sonicated fullerenes, only 65% mortality was observed at the highest dose of 9 ppm. Total elimination of THF from the exposure medium, however, was not demonstrated so it is possible some of the toxicity observed in the filtered fullerene may be attributable to THF (Lovern and Klaper, 2006). In another study by the same research group in which *D. Magna* were exposed to fullerene dispersed using THF and a water soluble fullerene C60HxC70Hx, fullerene was found to cause a change in heart rate and both fullerene and C60HxC70Hx suspensions caused increased hopping frequency and appendage movement, behaviours associated with increased risk of predation and reproductive decline. In this case the elimination of THF from the exposure media was demonstrated

suggesting the effects seen were due to the fullerene compounds and not THF (Lovern et al., 2007).

Fullerene suspensions prepared by stirring were also found to cause delay in moulting and reduced offspring production at concentrations of 2.5 and 5 ppm after a 21 day exposure. Concentrations high enough to cause 50% mortality could not be achieved using this preparation method as fullerene precipitated out of solution (Oberdörster et al., 2006).

In exposures of the estuarine meiobenthic copepod *Amphiascus tenuiremis* to three different fractions of prepared SWCNTs, exposure of 'as-prepared' SWCNTs caused increased mortality, reduced fertilization rates and reduced moulting success. Exposure to a fraction of short length fluorescent nanocarbon byproducts resulted in increased life-cycle mortality, however the purified SWCNTs had no effect on mortality, development or reproduction (Templeton et al., 2006).

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 of TiO_2 in the exposure medium and the physicochemical characteristics of the particles themselves.

Sonicated TiO_2 nanoparticles caused a mortality of no more than 9% at concentrations up to 500 ppm in *Daphnia magna*, however TiO_2 filtered in THF had an LC50 of 5.5 ppm and caused 100% mortality at 10 ppm (Lovern and Klaper, 2006).

Another study exposed *Daphnia magna* to two types of TiO_2 nanoparticle. Type 1 was 25 nm and was composed from mostly anatase TiO_2 crystals, and type 2 was 100 nm and 100% anatase. Both particle types were found to cause immobilization, although type 1 was markedly more potent at the same concentrations, and illumination of the exposure vessels at 250 W increased immobilization to 73% for type 1 and 30% for type 2 (Hund-Rinke and Simon, 2006).

In contrast, similar studies in *Daphnia magna* have shown exposure to TiO_2 nanoparticles causes limited adverse effects. Exposure of *Daphnia magna* to 30 nm TiO_2 particles at 2 ppm caused no change in behaviours such as hopping frequency, feeding appendage and

post-abdominal curling movements or heart rate (Lovern et al., 2007), and exposure of 7 nm and 20 nm TiO_2 particles at 1 mg mL^{-1} to *D. magna* had no effect on reproduction or mortality and no effect on mortality and growth in the larvae of the aquatic midge *Chironomus riparius* (Lee et al., 2009a). In the same study however, exposure to cerium oxide nanoparticles of 15 nm and 30 nm were found to cause DNA strand breaks in both *D. magna* and *C. riparius* and associated mortality in *C. riparius*.

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 as high as 110 ppb, however, transfer of quantum dots to *C. dubia* from dosed algae was demonstrated, suggesting that transfer of quantum dots between trophic levels could occur (Bouldin et al., 2008).

1.5.5.2. Effects of Nanomaterials in Fish

Fish are excellent sentinels of environmental health as they are sensitive to a wide range of xenobiotic chemicals. Their position in the aquatic food chain means assessment of the populations and health of fish can give an indication of the health of other lower trophic levels. Understanding the effects of ENMs on fish is therefore an important aspect when considering the effects of ENMs on the aquatic environment as a whole.

Potential routes of uptake for ENMs in fish include absorption *via* the gill epithelia, *via* the gut epithelia as a result of dietary exposure and drinking or *via* the skin (Handy et al., 2008). The few studies that have been conducted to date have mostly been concerned with recognising nanoparticle types that cause overt toxic effects, identification of target organs and biodistribution of nanoparticles in the bodies of fish, with little evidence in the literature so far regarding comparison of responses between nano-sized particles and their bulk counterparts or how the characteristics of the exposure media affect uptake or toxicity. As mentioned above, the behaviour of ENMs in aquatic media is likely to be affected by a large number of different physicochemical factors, which in turn are likely to influence their likelihood of uptake into aquatic organisms. This means that conducting exposures of aquatic organisms to ENMs that

deal with these factors comprehensively is more complex than conducting equivalent inhalation or oral exposures in terrestrial animals.

1.5.5.2. i *Carbon-Based Nanomaterials*

One of the first *in vivo* exposure studies of fish to ENMs to emerge was an exposure of fullerenes to juvenile largemouth bass *Micropterus salmoides* (Oberdörster, 2004). The finding that oxidative stress was detected in the brain alerted the scientific community to the potential hazards associated with release of ENMs into the aquatic environment. Like the study by Lovern and Klaper (2006) on *D. magna* however, the fullerene used in the exposure was prepared by dispersing fullerenes in THF, followed by the addition of water and subsequent evaporation of the THF. The concentration of residual THF in the exposure media was not quantified. Henry et al. (2007) conducted a similar study using larval zebrafish and found changes in gene expression and reduced survival in zebrafish exposed to fullerenes dispersed using THF, but also in water to which THF had been added and evaporated off. Analysis of the THF-fullerene media and THF-water by gas chromatography-mass spectrometry did not detect THF but detected an oxidation product γ-butyrolactone which has an LC50 of 47 ppm for zebrafish, which suggested that the effects observed in fish exposed to fullerene prepared in this way may be attributable to this degradation product.

A subsequent study by Oberdörster et al. (2006) exposed fathead minnow (*Pimephales promelas*) and Japanese medaka (*Oryzias latipes*) to fullerene prepared by a long-term stirring method and found that the mRNA and protein-expression levels of cytochrome P450 isozymes CYP1A, CYP2K1 and CYP2M1, which are traditional biomarkers for lipophilic xenobiotic exposure, were unchanged.

A study by Shinohara et al. (2009) exposed European carp *Cyprinus carpio in vivo* and carp brain homogenates *in vitro* to a fullerene suspension of 4.5 mg L^{-1} prepared without the use of solvents for 48 hours. Significant lipid peroxidation was detected in the brain homogenates, but none in the brains of the exposed fish and it was found that no fullerene had reached the brain tissues as a result of the exposure.

An exposure of zebrafish embryos to fullerene and fullerol showed that fullerol exhibited no inherent toxicity, however exposure to fullerene caused delayed embryonic and larval development, decreased survival and larval hatch rates and pericardial oedema (Zhu et al., 2007). Addition of the antioxidant GSH was found to reduce toxicity, suggesting that toxicity was mediated by generation of free radicals or other oxidative stress mechanisms. The preparation method for fullerene in this study, however, employed the use of benzene, acetone and THF, which suggests the results of this study should be viewed with caution.

A subsequent study by Zhu et al. (2008d) employed the stirring method for fullerene preparation and exposed the goldfish *Carassius auratus* to the resultant fullerene aggregate suspension for 32 days. Significant induction of antioxidant enzymes superoxide dismutase and catalase were seen in the gills and liver along with a depletion of GSH levels. Lipid peroxidation was decreased in the gills and brain but increased in the liver suggesting that the liver is the main target organ for fullerene. In this study, fullerene was also found to have an inhibitory effect on fish growth.

As a result of the issues surrounding the preparation of stable suspensions of fullerene, the present literature concerning *in vivo* exposure of fish species to fullerene does not yet provide clear information regarding the effects, as so many studies have employed the use of solvents to aid dispersion, however a recent study employing more comprehensive *in vitro* work, suggests that oxidative stress is a likely effect in some biological compartments, if not the brain (Zhu et al., 2008d).

A number of studies have assessed the effects of carbon nanotubes in zebrafish embryos. Embryos treated with SWCNTs and double-walled carbon nanotubes (DWCNTs) caused hatching delay at concentrations over 120 mg L^{-1} and 240 mg L^{-1} respectively however 99% of embryos hatched by 72 hour post fertilization (hpf). Embryonic development was not found to be affected and the authors suggested that as chorion pores are nano-scaled, they may provide a protective barrier against penetration by the micro-scaled CNT aggregates. Hatching delay was therefore attributed to trace levels of

residual cobalt and nickel catalysts in the CNTs. Carbon black nanoparticles were found to have no effect on hatching at similar concentrations (Cheng et al., 2007).

Embryos treated with MWCNTs exhibited dose dependent increases in mortality and decreased hatching as well as bradycardia, slowed blood flow and apoptosis. Mortality of 100% was reached at a concentration of 200 μg mL^{-1} and at concentrations of 60 μg mL^{-1} and above, embryos showed deformation of the notochord and increased mucus production in the intrachorion region (Asharani et al., 2008a). The authors did not provide details of trace metal concentrations in the nanotube preparations, however, the concentrations where hatching delay was observed are similar to that seen in the study by Cheng et al. (2007).

A further study by Cheng et al. (2009) found fluorescent-labeled MWCNTs introduced to zebrafish embryos at the 1-cell stage by microinjection were distributed to all blastoderm cells and excluded from the yolk cell and when introduced to the circulation system were removed by the body after 96 hours. Accumulation of white blood cells in the trunk region of embryos was noted in early stages of the exposure as well as lysosome-like vesicles in the blastoderm cells. The injected larvae developed normally and displayed no reduction in egg production, however the survival of the second generation larvae was 50% lower by than that of control fish at 14 days post hatch.

In a 10 day semi-static exposure of juvenile rainbow trout *Oncorhynchus mykiss* to SWCNTs, a dose-dependent rise in ventilation rate was observed. Accumulation of SWCNT aggregates associated with gill mucus was visible to the naked eye and increased mucus secretion in the gills, oedema, altered mucocytes and hyperplasia as well as an increase in Na^+K^+-ATPase activity in the gills and intestine were also observed. Thiobarbituric acid reactive substances (TBARS) were decreased in the gill, brain and liver and increases in GSH were noted in the gills and livers, suggesting evidence of lipid peroxidation and oxidative stress in these tissues (Smith et al., 2007).

Genotoxicity in erythrocytes and acute toxicity related to mortality and growth were studied in an exposure of the larvae of the amphibian *Xenopus laevis* to DWCNTs. No genotoxicity as a result of exposure was noted but acute toxicity at all concentrations

was exhibited and was related to physical blockage of the gills and digestive tract (Mouchet et al., 2008).

The results of these studies suggest that 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.

1.5.5.2. ii *Titanium Dioxide Nanoparticles*

Exposures of zebrafish embryos to TiO_2 nanoparticles have so far shown them to be relatively non-toxic when compared with effects seen in exposures to carbon-based nanoparticles. No toxicity was observed in zebrafish embryos exposed to 30 nm TiO_2 comprised of 20% rutile and 80% anatase crystals at concentrations of up to 10 mg L^{-1} (Griffitt et al., 2008) and a further study by the same research group found exposure of adult zebrafish to 1000 µg L^{-1} of the same TiO_2 nanopartices caused no change in molecular or histological parameters in zebrafish gills (Griffitt et al., 2009).

Two similar studies found TiO_2 nanoparticles showed no toxicity to zebrafish embryos or larvae and that there were no apparent differences in effect between nano-sized and bulk TiO_2 particles (Zhu et al., 2008c) and the LC50 for TiO_2 nanoparticles was over 500 mg L^{-1} in fathead minnow *Pimephales promelas* (Hall et al., 2009).

In contrast however, a 14 days semi-static exposure of rainbow trout to comparable concentrations of TiO_2 nanoparticles 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 also observed 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).

A subsequent 8-week oral exposure to rainbow trout by the same research group found concentrations of up to 100 mg kg^{-1} in the food had no impact on growth. An increase in TiO_2 content in the liver and spleen was observed in the early stages of the exposure but

no effect was seen on haematological parameters or TBARS suggesting that adverse effects of TiO_2 nanoparticles on rainbow trout are more severe as a result of water-borne exposure than by exposure *via* the diet (Handy et al., 2008).

Very few studies in fish have examined the uptake and partitioning of TiO_2 nanoparticles within the body as result of exposure, probably due in part to the difficulties involved in measuring low levels of TiO_2 and limitations in analytical equipment. A study by (Moger et al., 2008) however used coherent anti-Stokes Raman Scattering (CARS) to examine the gills of rainbow trout exposed to 5000 µg L^{-1} TiO_2 nanoparticles and confirmed the presence of small numbers of particle aggregates within the gill tissue.

In a study using a rainbow trout gonadal cell line (RTG-2 cells), no adverse effects were observed in cells at concentrations of up to 50 µg mL^{-1} TiO_2 particles, however, significantly increased levels of DNA strand breaks were reported when the exposure was illuminated with UVA radiation (3 kJ m^{-2}) (Vevers and Jha 2008).

Two further studies, however, have demonstrated the enhanced accumulation of the heavy metals arsenic and cadmium in the viscera and gills of carp when exposed in the presence of TiO_2 nanoparticles (Sun et al., 2007; Zhang et al., 2007b) and showed significant accumulation of TiO_2 nanoparticles in the gills and gut of carp.

1.5.5.2. iii *Silver Nanoparticles*

To date, the majority of *in vivo* exposures of silver nanoparticles to fish have been in zebrafish models. Effects in embryo exposures have shown similarities between effects seen with carbon-based nanoparticles although the exact mechanisms of toxicity have not yet been elucidated.

Zebrafish embryos exposed to 5-20 nm silver particles capped with starch or bovine serum albumin (BSA) to aid dispersion exhibited a dose-dependent increase in mortality and hatching delay and dose-dependent toxicity which was typified by larvae with deformations of the notochord, slow blood flow, pericardial oedema and cardiac arrhythmia. Distribution of silver nanoparticles to the brain, heart, yolk and blood was

demonstrated by transmission electron microscopy (TEM) and apoptosis was also seen in 50% of the embryos treated at 50 μg mL^{-1} and above (Asharani et al., 2008b).

Another study similarly found decreased hatch rate, notochord abnormalities and weak heart beat in embryos treated with 10-20 nm silver nanoparticles. Catalase activity was found to be increased in exposed embryos and the expression of Sel N1, a gene associated with notochord development and heart disease in zebrafish was significantly lower in exposed fish. The study also confirmed the presence of silver ions (Ag^{+}) in the exposure media to which the authors attributed the detrimental effects seen (Yeo and Kang, 2008).

Unlike the aggregates of carbon nanotubes, that were unable to pass through chorion pores, silver nanoparticles of 5-46 nm have been shown to be transported in and out of chorion pore channels by Brownian diffusion (Lee et al., 2007). The particles were observed inside exposed embryos at all developmental stages and dose-dependent toxicity characterized by developmental deformities and mortality was observed.

A study investigating the effects of silver nanoparticles in zebrafish fry found that although 26 nm silver particles exhibited toxicity, silver ions were found to be over 300 times more toxic to zebrafish fry on a mass basis (Griffitt et al., 2008). This led to a further study where zebrafish were exposed to 26 nm silver nanoparticles at their previously ascertained no observable effect concentration (NOEC) of 1000 μg L^{-1}. Alongside this, fish were also exposed to the equivalent concentration of silver ions that would dissociate from the silver nanoparticles, to try to distinguish effects between silver ions and silver nanoparticles. Whole body burdens of silver were significantly higher in nanoparticle exposed fish than in controls or fish exposed to dissolved silver, however gill filament width increased on exposure to dissolved silver but not silver nanoparticles despite the finding that silver nanoparticles were associated with the gill tissue. Analysis of global gene expression in the gills found differences in response between nanoparticle-exposed fish and fish exposed to soluble silver ions suggesting that the biological effects of exposure to silver nanoparticles do not appear to be driven solely by the release of silver ions (Griffitt et al., 2009).

1.5.5.2. iv *Other Types of Nanomaterial*

A small number of other *in vivo* studies have investigated the effects of other nanoparticle types in fish species.

In a study investigating the biodistribution of fluorescent latex nanoparticles of different sizes in embryonic and adult see-through medaka (*Oryzias latipes*), all particles (39.4–42,000 nm) were found to adsorb to the chorion of eggs and accumulate in oil droplets, however biodistribution of particles to eggs, yolk and gallbladder were found to vary according to particle size. Particles of 474 nm were shown to have the highest bioavailability to eggs. In medaka embryos, particles of 39.4 nm were transported into the yolk and gallbladder during development and in adult fish, they accumulated in the gills an intestine.

Increased salinity in the exposure media caused an increase in particle aggregation and was accompanied by increasing mortality that reached 100% in an exposure media 10 times more concentrated (333 mOsm L^{-1}) than the regular exposure media used. There was no mortality at the same exposure media concentrations in the absence of the nanoparticles. In the adult medaka, uptake of the nanoparticles was demonstrated with the majority accumulating in the gills and intestine, although they were also detected in the brain, testis, liver and blood, indicating that they are able to cross the blood-brain barrier (Kashiwada, 2006).

Copper (along with silver) has been the focus of a number of studies by Griffitt et al. 2007, 2008, 2009). Copper nanoparticles of 80 nm were found to have a 48 hour LC50 value of 1.5 mg L^{-1} in zebrafish (Griffitt et al., 2007). Addition of the particles to the water resulted in rapid aggregation and subsequent settling of the particles with 50-60% of the particles leaving the water column. Although some dissolution of the copper occurred, it was not sufficient to explain the mortality observed. Histological and biochemical analysis revealed the gills to be the primary target organ demonstrated by visible gill damage and dose-dependent decreases in Na^+K^+-ATPase activity. A second exposure of zebrafish to either 100 µg L^{-1} copper nanoparticles, or to the corresponding concentration of copper ions released due to dissolution, revealed 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).

Exposure to a NOEC in terms of lethality of nano-copper (100 µg L^{-1}), was also found to increase metal burdens in gill tissue and caused increases in the mean gill filament width by three to fourfold between 24 and 48 hours exposure. Unlike the results seen with silver however, the amount of metal associated with gill tissue did not differ substantially between fish exposed to soluble copper and nanoparticulate copper. The gene expression profiles obtained also differed substantially both from those seen in the exposure to silver nanoparticles and the controls suggesting that biological effects as a result of exposure to these two nanoparticle types are a result of distinct mechanisms (Griffitt et al., 2009).

In a 72 hour static exposure of zebrafish to aluminium nanoparticles, evidence of ingested nanoparticles was seen in the intestines of the fish and although no lethality was recorded at concentrations up to 500 g L^{-1}, reduced gill ATPase activity was observed indicating compromised gill function (Barber et al., 2005).

In rainbow trout hepatocytes exposed to newly produced and 'aged' (2 years old) cadmium telluride quantum dots, increases in levels of metallothionein and heat shock proteins, increases in labile zinc, and DNA strand breaks at concentrations of 0.1 mg L^{-1} and above were reported with the cytotoxic responses of aged quantum dots found to be due to the liberation of cadmium ions (Gagne et al., 2008). Similar findings were seen in zebrafish embryos exposed to quantum dots, where the surface coating of the quantum dots was found to influence their toxicity and influence their stability in the exposure media. The toxicity observed was characteristic of that observed with exposure to cadmium and cadmium concentration correlated weakly with metallothionein expression. Not all toxicity observed however, could be explained simply by release of cadmium (King-Heiden et al., 2009).

1.5.6 COLLECTIVE ANALYSIS OF THE REPORTED BIOLOGICAL EFFECTS OF ENGINEERED NANOMATERIALS

The field of nanotoxicology is still in its infancy and therefore the lack of standardized testing makes gleaning meaningful information from the literature presently available difficult. There is an almost unlimited variety of nanomaterials available. Many nanomaterial types are manufactured in different sizes, and can be modified by the addition of a variety of coatings or functional groups which make them more suited to a specific purpose. This, coupled with the numerous methods used in preparing nanoparticles for biological exposure experiments, such as the use of solvents or biologically compatible capping agents that aid dispersion, means drawing firm conclusions on biological effects, relative potencies of different nanomaterials etc., from the present evidence should be approached with caution.

Inhalation studies have shown ENMs of a range of different types have the potential to induce inflammatory responses and membrane damage. Inflammatory responses as a result of introduced stimuli are characterised by an increased blood supply to the affected area and the migration of neutrophils and macrophages out of the capillaries to the affected tissue. Both macrophages and neutrophils are phagocytes, and produce ROS after engulfing foreign bodies to aid in their breakdown. They may also release ROS to the surrounding tissue where they can react with a variety of cellular components including lipid membranes, proteins and nucleic acids, leading to oxidative stress (Iwama and Nakanishi, 1996; Rand, 1995).

Many ENM types have also been shown to be translocated after inhalation to other organs, notably the liver, blood and brain, where the olfactory nerve has served as a transport channel. Carbon nanotubes have been likened to asbestos in their ability to cause granulomas within lung tissue suggesting the physical shape of an ENM may influence its toxic effects (Warheit et al., 2004).

ENMs administered orally or *via* injection to rodent models have found that the liver, bones, kidney and spleen are often target organs for these particles. Penetration of the blood-brain barrier has also been demonstrated and toxicity and oxidative stress in a

number of tissues has been observed, most notably in the lungs and kidneys. Carbon nanotubes again were found to have an asbestos-like pathogenicity causing granulomas in the mesothelial lining of the abdominal cavity (Poland et al., 2008; Takagi et al., 2008).

Some ENMs have been found to be able to penetrate intact skin, although their toxicity in the skin or ability to be transported after penetration has not yet been comprehensively examined (Larese et al., 2009; Tokunaga et al., 2006).

In vitro studies have shown that exposure to many different ENM types can induce a various deleterious effects. Generation of ROS and free radicals have been observed and implicated in the cause of oxidative stress, lipid peroxidation, decreased mitochondrial activity and apoptosis in a wide variety of different cell types. Particle uptake by diffusion into cells has been demonstrated for a range of ENM types suggesting observed toxicity may be both as a result of interaction of the ENMs with cell membranes and interactions within the cytosol. In the case of photoactive nanoparticle types such as TiO_2, the presence of UV radiation can induce the generation of ROS and oxidative stress in cells which has direct implications for the safety of sunscreen applications, particularly since some TiO_2 nanoparticles have been shown to penetrate lower skin layers.

Exposure studies of aquatic organisms to date have demonstrated that ENMs are capable of inducing a wide range of biological effects. Both carbon-based and metallic nanoparticles have been shown to exhibit toxicity in embryonic fish models characterized by developmental abnormalities and mortality whereas TiO_2 nanoparticles have shown limited toxicity to both embryonic and adult fish. In a number of studies the dissolution of metal ions from nanoparticles into the exposure media, or the presence of metal impurities have been implicated as important factors in the toxicity of ENMs in aquatic exposure systems.

Drawing commonalities between aquatic exposure studies at this stage, however, is complicated by the lack of standardisation in exposure methods and nanoparticle preparations.

The above examples of nanotoxicity studies clearly show that a wide variety of parameters influence the fate and effects of nanoparticles in cells and living organisms. Particle type, size, crystal structure, the presence and type of coating and the use of dispersants all need to be investigated for their potential differing effects. In some cases the species and gender of the experimental organism may also make a difference to the effect. How the characteristics of ENMs and environmental parameters affect uptake and toxicity will be discussed in more detail in the next section.

1.6. PARAMETERS GOVERNING THE TOXICITY OF NANOMATERIALS

One of the key requirements for the development of standardized tests for examining the toxicity of ENMs is the identification of an appropriate dose metric. The use of parts or mass per unit volume or molar concentrations are used in exposures to conventional xenobiotic compounds however may not be appropriate in the case of biological exposures to ENMs. 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.

Since then *in vitro* and *in vivo* exposures to ENMs have shown that a wide range of particle parameters have been implicated in influencing toxicity and various reviews and studies have emerged discussing the problems associated with using mass concentration as a dose metric for comparing toxicity responses from different ENM types. The importance of incorporating measurements of particle uptake into cells when examining effects (Lison et al., 2008; Teeguarden et al., 2007) and various other dose metrics

which may better explain the responses seen in exposure to nanoparticles (Wittmaack, 2007) have also been discussed.

1.6.1. SIZE AND SURFACE AREA

Materials manufactured at the nano-scale, have properties that differ from their 'bulk' sized equivalents, often due to their large surface area to volume ratio. Size and therefore surface area, have thus been implicated as factors influencing the toxicity of nanoparticles.

Although in one study larger intracheally instilled TiO_2 particles were found to be more toxic in mice (Grassian et al., 2007), inhalation studies in other rodents and *in vitro* studies have in general indicated that smaller nanoparticulates generate greater inflammatory responses than larger particles (Brown et al., 2001; Inoue et al., 2009; Singh et al., 2004). Though 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 though smaller sized nanoparticles cause a greater inflammatory response than larger sized particles on a mass basis, the level of inflammatory response is dependent on the total surface area of particles instilled (Brown et al., 2001; Oberdörster, 2000; Stoeger et al., 2006; Yamamoto et al., 2006).

Toxicity studies *in vitro* have also shown that the size of a particle, and therefore its surface area, play a role in mediating response. Many studies have shown smaller sized particles to be more toxic than larger sized particles (Karlsson et al., 2009; Renwick et al., 2004; Tamura et al., 2004) and size-dependent generation of ROS (Cagle et al., 1999; Choi and Hu, 2008) has also been demonstrated. One study showed that the type of toxicity observed was also dependent on the size of nanoparticle applied, with 1.4nm particles causing cell death by necrosis and 1.2nm particles causing programmed cell death by apoptosis in human cell lines (Pan et al., 2007).

Investigations into the transport of particles *in vitro* or *in vivo* have, however, shown mixed results. Smaller-sized iridium nanoparticles were found to be more readily transported from the lung epithelium to extrapulmonary organs than were larger nanoparticles (Kreyling et al., 2002) however another study showed there was no preferential transport of TiO_2 particles in a tracheal explants system related to size (Churg et al., 1998). Larger cerium oxide nanoparticles were found to be more readily taken up in human lung fibroblasts compared with smaller nanoparticles and it was suggested that this was due to the increased propensity of smaller nanoparticles at high concentrations to aggregate in the exposure media (Limbach et al., 2005).
Many studies acknowledge that size and surface area may be important factors in determining biological effect but suggest that other factors may also be important.

An investigation into the antibacterial properties of nano- and bulk-sized TiO_2 particles in suspension found there was an inverse relationship between size and activity but also noted that antibacterial activity was dependent on the ability of the nanoparticles to generate free radicals (Verran et al., 2007). A non-linear size-dependency of adverse effects of polystyrene nanoparticles was observed in human blood and the charge on the particle surface was also found to play a role in determining toxicity (Mayer et al., 2009) and in another study although a size-dependent inflammatory response was observed in alveolar basal epithelial cells exposed to TiO_2 particles, in the absence of cells there was no difference in levels of ROS generation between particles of different sizes suggesting that interaction of the nanoparticles with the cells is an important factor (Singh et al., 2004).

1.6.2. SURFACE CHEMISTRY

Several studies by Warheit et al. have indicated that aspects of the surface chemistry of nanoparticles, such as their catalytic activity, ability to generate reactive species and general cytotoxicity are the dominant factors in determining toxicity and that neither size nor surface area may be accurate dosemetrics (Warheit et al., 2007b). Rat lungs instilled

with pigment grade bulk TiO_2 or nanoscale rods and nanoparticles 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 inflammation and cytotoxicity whereas exposure to micro-sized TiO_2 and two types of nanosized rutile TiO_2 particles produced only transient inflammation. This suggested differing crystal structure and/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 intratracheal exposures of rats to mined and synthetic quartz particles of varying sizes produced effects that were similar qualitatively, but differed in their potency in a way that was not consistent with particle size, but correlated with surface activity such as haemolytic potential (Warheit et al., 2007a).

1.6.3. FUNCTIONAL GROUPS

The addition of functional groups to the surface of nanoparticles, particularly carbon-based nanoparticles has also been shown to alter toxicity *in vitro*.

Increasing derivatization of the surface of fullerenes has been shown to be associated with a decrease in toxic effect in two human cell lines, with pristine fullerene being around three orders of magnitude more toxic than a polyhydroxylated fullerene (Sayes et al., 2004). Further to this, different mechanisms of toxicity were observed between functionalised and non functionalised fullerenes with generation of ROS by pristine fullerenes causing cell death and cell death by apoptosis observed on exposure to functionalized fullerene (Isakovic et al., 2006).

In contrast, functionalization of the surface of carbon nanotubes however was found to increase their cytotoxicity (Magrez et al., 2006).

1.6.4. COATINGS

The addition of surface coatings to many ENMs to help aid their dispersion in aquatic media or make them more biologically compatible, or the embedding of ENMs into inert matrices to improve their function is common, but this also has implications for their biological effects.

In an exposure of iron-doped silica and pure iron oxide nanoparticles to human lung epithelial cells the pure iron oxide particles that contained 20-100 times more iron than the iron-doped silica produced a weaker induction of ROS and therefore were less cytotoxic. The authors suggested that this was due to the presence of 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 nanosized zerovalent 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 were most toxic to murine macrophages, however carboxylated quantum dots were readily taken up by cells but had little impact on cell viability, and amine-poly(ethylene glycol) (PEG) quantum dots showed much slower uptake compared with other coating types (Clift et al., 2008).

Coating type has also been demonstrated to influence cytotoxicity in gold nanoparticles. Triphenylphosphine-stabilized gold particles were shown to exhibit size-dependent toxicity in a range of human cell lines (Pan et al., 2007) however PEG-stabilized 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).

1.6.5. CHARGE AND AGGREGATION

Modification of ENMs by the addition of functional groups or coating is likely to alter the charge on the nanoparticle. The stability and aggregation behaviour of ENMs within aquatic media is determined by both the physicochemical properties of the media, and the charge on the surface of the ENMs. The degree of aggregation of nanoparticles has been shown in some cases to affect toxicity *in vitro*.

One study demonstrated that in human mesothelioma cells exposed to SWCNTs dispersed in non-cytotoxic media or rope-like agglomerated SWCNTs using asbestos as a reference, the dispersed SWCNT bundles were found to be less cytotoxic than asbestos but the rope-like agglomerates produced a higher toxic response than asbestos at the mass concentrations (Wick et al., 2007).

Transport and uptake of cerium oxide nanoparticles in human lung fibroblasts were studied as a function of particle size, agglomeration and diffusion in another study. Smaller nanoparticles had low diffusion coefficients and were found to aggregate more quickly and form aggregates containing a greater number of particles whereas larger particles with low number densities slowly agglomerated and were found to penetrate into the cells much more efficiently before sedimentation occurred. The uptake of cerium oxide nanoparticles into cells was therefore found to correlate best with relative particle size as a result of the aggregation behaviour (Limbach et al., 2005).

Charge in itself has also been implicated as an influencing factor in ENMs transport and toxicity. Emulsifying wax nanoparticles with different surface charges were applied to an *in situ* rat brain perfusion to investigate the integrity of the blood-brain barrier. Nanoparticles with neutral charge and low concentration of anionic nanoparticles 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).

1.6.6. PARTICLE CHEMISTRY AND SOLUBILITY

Other factors relating to the physical chemistry of the nanoparticles have been found to determine 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 both the surface and shape of the particle and its composition and degree of solubility (Brunner et al., 2006) and work by Gagne et al. (2008), Griffitt et al. (2009) and Griffitt et al. (2007) has shown that the toxicity of both copper and silver nanoparticles to zebrafish can be attributed in part to the 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 nanoparticles 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.

1.6.7. 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 and found that carbon nanotube aggregates had a very similar cytotoxicity index to asbestos. Soto et al. (2005) suggested that the similarity in toxicity may be due to the physical similarities between the two particle types. 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).

1.6.8. PHOTOCHEMISTRY

The presence of ultraviolet light may also influence the toxicity of some nanoparticles. A number of oxide nanoparticles including TiO_2, and ZnO exhibit photocatalytic activity when exposed to light and as a result can generate reactive oxygen species in aquatic media.

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 to those kept in the dark (Adams et al., 2006) and damage to cell structure characterized 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).

1.6.9. 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 compounds such as THF to aid the dispersion of hydrophobic carbon-based nanoparticles (Henry et al., 2007) and sonication of fullerenes (Oberdörster et al., 2006) has also been found to influence their toxicity.

1.6.10. 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) and the presence or absence of proteins on the surface of SWCNTs and silica nanoparticles has been found to affect their toxicity (Barrett et al., 1999; Dutta et al., 2007).

Albumin-coated SWCNTs were found to inhibit the induction of 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 qualitatively different however, to 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; Sun et al., 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.

1.6.11. ENVIRONMENTAL PARAMETERS

In addition to characteristics of the particles in their raw state, the characteristics of the exposure media will subsequently have an effect on the behaviour and interaction of nanoparticles with cells and organisms. As aforementioned the pH, and composition and concentration of ions in the media and presence of NOM will be likely to affect the charge on a particle and therefore its aggregation behaviour and likelihood of interaction with cells and 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 media and the behaviour of a particular particle in that

exposure media must all be considered in a nanoparticle exposure scenario 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 therefore 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).

1.7. AIMS AND OBJECTIVES OF THIS PHD

The aquatic environment is particularly at risk from accumulation of a vast array of anthropogenic compounds and the continuing expansion of the nanotechnology industry will undoubtedly see the release of nanomaterials into the aquatic environment. Development of a knowledge base on the effects that ENMs might have on aquatic organisms is therefore urgently needed in order to be able to assess the associated risks. In order to do this, a multi-disciplinary approach must be adopted to fully characterize the behaviour of ENMs in aquatic media. Their propensity to aggregate, their longevity in the water column, and interaction with natural organic matter in the environment must all be better understood in order to predict the likelihood of uptake by aquatic organisms.

Likewise, the likely routes of exposure, mechanisms of uptake and fate and behaviour of ENMs in the bodies of aquatic organisms must be elucidated in order for the potential impact of ENMs in the aquatic environment to be understood.

The main aim of these PhD studies was therefore to investigate the general ecotoxicology of a range of commercially available ENMs that are already in widespread use within industry and in consumer products, using fish as a vertebrate model. The work intended to establish the uptake, biodistribuition and biological effects of a range of metal and metal oxide nanoparticles in fish. Both rainbow trout (*Oncorhynchus mykiss*), and to lesser extent zebrafish (*Danio rerio*), were used as study species as they are easy to keep under laboratory conditions and their general physiology, genetics and behaviour

are well studied. Rainbow trout in particular are also sensitive to a variety of toxicants and environmental stressors and are often used as sentinels for indicating the health of an aquatic system and therefore served as a good model organism for investigating the ecotoxicology of many ENMs.

The key objectives of the work undertaken were:

Objective I

To gain a better understanding of the potential routes of exposure for selected metal and metal oxide ENMs in fish

Objective II

To identify target organs for selected metal and metal oxide ENMs in fish

Objective III

To examine characteristic effects of selected metal and metal oxide ENMs in target organs in order to provide groundwork for the development of relevant and effective *in vitro* screening assays

Objective IV

To establish any differences in effect between exposure to nano-sized metal and metal oxide particles and larger (bulk) particles

Titanium dioxide and silver were chosen as the main study compounds, principally because both compounds have widespread industrial applications in their bulk form and their nanoparticulate forms are already in widespread use in consumer products and in industry. The toxicity of cerium oxide, zinc oxide and copper nanoparticles were also investigated in some *in vitro* studies using trout hepatocytes.

In this work nanoparticles were dispersed in the exposure media or dosing stocks by means of sonication in order to eliminate the possibility of toxic effects caused by the use of chemical dispersants.

At the start of this study there was virtually nothing known about the levels and behaviour of ENMs in the aquatic environment, therefore it was difficult to be able to conduct an exposure experiment that is in its truest sense environmentally relevant. The dosing regimes used were therefore estimations based on regimens used in previous nanotoxicology studies in the literature, data from modelling studies, and best guesses.

The studies that were conducted to address these objectives are outlined below and are presented in the form of scientific papers that have been accepted or submitted for publication in peer-reviewed scientific journals.

Study 1.

In the first study zebrafish (*Danio rerio*) were exposed to ZnO and CeO_2 nanoparticles *via* the water column, and rainbow trout (*Oncorhynchus mykiss*) were exposed to TiO_2 nanoparticles and bulk particles *via* the water column and *via* the diet to test the null hypothesis: fish exposed to metal oxide nanoparticles *via* the water column or the diet, do not accumulate metal oxides in their tissues as a result of exposure. The aim of these experiments was to elucidate the bioavailability and uptake of nanoparticles into the bodies of fish, identify target organs and to determine the relative importance of these routes of exposure.

Study 2.

In the second study, rainbow trout were exposed to TiO_2 nanoparticles *via* intravenous injection in order to test the null hypothesis: TiO_2 nanoparticles introduced intravenously to rainbow trout are excreted rapidly and are not retained within the body. The aim of these experiments was to investigate the target organs and inherent toxicological effects of TiO_2 nanoparticles if they are taken up into the blood.

Study 3.

Rainbow trout were exposed to low and high concentrations of silver nanoparticles, bulk silver particles and silver ions *via* the water column in order to test the null hypothesis: neither nanoparticulate, bulk or ionic silver show differences in uptake in rainbow trout exposed *via* the water column and observed toxic effects do not differ between particle type or ion. The aim of these experiments was to establish any differences in uptake, target organs or inherent toxicity of silver in the fish depending on the size of the particle.

Study 4.

With a view to developing a high through-put screening assay suitable for assessing the uptake and toxicity of a wide variety of ENM types, primary trout hepatocytes cultures were established and exposed to a variety of commercially available nanoparticles and their bulk equivalents and responses were assessed using a suite of well known toxicity assays: lactate dehydrogenase (LDH) release, measurement of thiobarbituric acid reactive susbstances (TBARS) and glutathione-S-transferase (GST). In these experiments the following null hypothesis was tested: primary trout hepatocytes are not a good model for assessing toxicity of ENMs so are not suitable for use in the development of high through-put screening assays for ENM toxicity.

CHAPTER 2

Bioavailability of nanoscale metal oxides, TiO_2, CeO_2, and ZnO to fish

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Bioavailability of nanoscale metal oxides, TiO_2, CeO_2, and ZnO to fish

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UPTAKE AND EFFECTS OF NANOPARTICLES IN FISH

Abstract

Nanoparticles (NPs) are reported to be a potential environmental health hazard. For organisms living in the aquatic environment there is much uncertainty on exposure because of a fundamental lack of understanding and data regarding the fate, behaviour 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, ZnO, CeO_2, and TiO_2 from the water and diet to determine their potential ecotoxicological impacts on fish as a function of concentration.

Tissue concentrations were quantified and particle characterization was performed with TEM, DLS, filtration and ultrafiltration, ICP-MS/OES, EEM fluorescence spectroscopy, ESEM, and EDX. Definitive uptake from the water column and localization of TiO2 NPs in gills are demonstrated for the first time using CARS microscopy. Using ICP-MS as a detection method, zinc concentrations (0.33 ± 0.03, brains; 1.14 ± 0.09 mg/g, skin) in zebrafish (and titanium (0.24 ± 0.04, brains; to 0.88 ± 0.27 mg/g, liver) and in trout did not differ in exposed fish, compared with controls. Significant uptake of cerium (1.35 ± 0.58 mg/g) in zebrafish liver *via* water exposure and ionic titanium (0.75 ± 0.066 mg/g) in trout gut *via* dietary exposure was observed. Aggregate formation was shown to limit considerably the bioavailability of unmodified metal oxide NPs in fish.

UPTAKE AND EFFECTS OF NANOPARTICLES 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 CeO2 is used as a fuel additive to enhance combustion efficiency [2, 3]. These compounds are wide band-gap semi-conductors that produce excited electron–hole pairs when irradiated with light and as such are also showing great potential for use in solar driven energy production [4-6], as catalysts in various industrial applications [2, 7-9] and as groundwater and soil remediation agents [2, 10, 11]. Due to their diverse applications, human and environmental exposures are likely to increase substantially in the near to mid-term 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 [12-14]. In mammalian models, routes of exposure examined include inhalation [15-20], oral administration (TiO_2 NPs) [21] and adsorption *via* the skin (microfine ZnO and TiO_2)

[22]. Where toxicity has been demonstrated as a result of inhalation or *in vitro* exposures, a common finding has been the incidence of an inflammatory response [15, 18, 23-30]. In addition, several studies have indicated the capacity of TiO_2 and other metal oxides to induce oxidative stress in various cell types [21, 27, 29-35]. Long-term toxicity has also been indicated through *in vitro* studies with the induction of DNA damage [32, 36-38] and apoptosis [39]. 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 [40, 41]. 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 [12, 42, 43]. A few studies however have 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 behaviour and mode of preparation of TiO_2 NPs to resultant toxicity; there was an LC50 of 6 mg L-1 for exposure via the water to filtered TiO_2 whereas, the mortality rate for sonicated TiO_2 did not differ from controls [44]. In the gills of fish (rainbow trout, *Oncorhynchus mykiss*) exposure to TiO_2 NPs have been reported to cause respiratory toxicity, as demonstrated by decreased Na+/K+-ATPase activity, induction of oedema and thickening of the lamellae and increased levels of glutathione [45].

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 (<10mg/L) in zebrafish [46]. 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.

Therefore, the purpose of this study was to determine the chemical fate of the metal oxide NPs, zinc oxide, cerium oxide, and especially titanium dioxide, in the aquatic environment and to fully characterize the NPs to provide an exposure assessment and determine bioavailability to fish following exposure *via* 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 using zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*) exposing them to various sonicated metal oxide NPs *via* the water column under semi-static conditions, for between 24h and 14 days, or *via* an oral dose by incorporation into feed pellets over a 21 day period (see Supporting Information for details on exposure regimes; see Fig. S. I. 1). 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 (ICPMS) or optical emission spectroscopy (ICP-OES).

Nanochemicals and Exposures

NPs were characterized for particle size, particle number and mass concentration, particle shape, aggregate size and zeta potential using transmission electron microscopy (TEM), ICP-MS, a dynamic light scattering particle-sizer (Malvern Instruments zetasizer), and Coherent anti-stokes Raman Scattering (CARS) multi-photon microscopy. Stock suspensions (10 µL) of NPs were diluted to 250 µg/L, 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 < 100nm), and CeO_2 (>99.9%, nominal size < 25nm), and TiO_2 (>99.9%, nominal size < 100nm) powders (both SigmaAldrich, UK) were produced by suspending 2.5 g/L of powder in ultrapure water and sonicating for 30 minutes 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. Control tanks included bulk zinc, cerium, or titanium oxides, as well as ionic titanium (titanium metal standard solution, catalogue no. J/8330/05, Fisher Scientific U.K.) to determine if size and form of particle suspension had an effect on uptake in fish (Fig. S. I. 1). The contribution of soluble ions to the exposures in this experiment is not known, however, Zn is the most important to consider as it is the most soluble. Franklin et al. [53] 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 in water from the NPs used in this study is <10 µg L^{-1} (Lead et al., unpublished data).

Water and Tissue Samples.

To determine NP exposure levels in the tank, water samples (3mls) were digested in concentrated acid (3ml HCl for ZnO, 4 ml HNO_3 – for CeO_2 and TiO_2) boiled in a Gerhardt Kjeldatherm digester before being reconstituted in 10 ml of nitric acid (10% for ICP-OES, 2% for ICP-MS, ICP standard Ti, VWR). CeO2 and ZnO exposed water and fish tissue sample analysis was carried out on a Vista-MPX CCD Simultaneous ICP-OES. Zinc (ICP Multi-element Standard IV, Merck) and cerium (ICP standard Ce, VWR) standards were used. Analysis of TiO2 and quality control of exposed water and fish tissue samples were carried out on a Thermo Elemental PlasmaQuad PQ2 + STE at the NERC ICP facility at the Kingston University in Kingston-upon-Thames, UK. 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 non-invasive, label-free, three-dimensional imaging of biological structures at depths of up to several hundred microns with sub-cellular 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 cellular level [47]. CARS microscopy was performed using a custom built imaging system (further details of the CARS set-up can be found in Supporting Information and ref. 47). Rainbow trout gill tissue was excised, gently rinsed in ice-cold trout ringers solution, and fixed in an ice-cold solution of 3% glutaraldehyde/2.5% paraformaldehyde. 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 (250 mg L^{-1}) (Fig. 1) indicated that the zinc oxide were rod shaped with a low aspect ratio, while the CeO_2 was irregular but roughly symmetrical and the TiO_2 was 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. CeO_2 NPs had an average size of 10.2 ± 0.78 nm and TiO_2 NPs had an average size of 34.2 ± 1.73 nm. Bulk TiO_2 particles had an average hydrodynamic diameter of 801 nm.

Analysis of water samples from tanks dosed with NPs by ICP showed decreasing concentrations of all metal oxides in experimental tanks over time, both in the presence or absence of fish (see Fig. S.I. 6) The detection limit for Zn, Ce and Ti was 10 ppb. 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 Fig. 2a, the hydrodynamic diameter of TiO2 measured by DLS surprisingly was in good agreement with the TEM results [48], with small aggregates present of about 25 nm.

These measured z average diameters did not vary with NP concentration in the ultrapure MilliQ water (MQ). However, z average diameters increased in reverse osmosis water (RO, low but detectable salt concentrations—details in Supporting Information) that was used in the trout exposures, and synthetic (SY, high added salt concentrations--details in Supporting Information) water that was used in zebrafish exposures. The differences in water conditions reflect the different water supplies used in the two aquaria. This tendency to aggregate can be explained by the reduction in the zeta potential and charge screening by the cations present in the RO and, especially, the SY waters (Fig. 2b). Hydrodynamic diameters of particles from TiO_2 exposure tanks (Fig. 2a) and ESEM images with EDX elemental analysis on filtered samples (Fig. 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 (Fig 2c).

Analysis of tissues from rainbow trout exposed to TiO_2 NPs *via* the water showed no significant uptake at any of the exposure concentrations (Table 1). There was an increase in gill tissue concentrations in positive control fish, exposed to elemental 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 NPs. Analysis of the tissues of zebrafish exposed to ZnO NPs 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 μg L^{-1} or 5000 μg L^{-1}; Fig. S.I. 7). 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 μg L^{-1} CeO_2 compared to controls and an apparent but not significant uptake in fish exposed to 5000 μg L^{-1} CeO_2 (Fig. S.I. 8). 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 associated *in vivo* and this may account for the higher Ce levels seen in the 500 μg L^{-1} CeO_2 exposure compared with the 5000 μg L^{-1} CeO_2 dose exposure. There was no

significant uptake into any of the other tissues analyzed. CARS imaging of rainbow trout gill tissue clearly showed large aggregates of TiO2 (up to 3µm) on the surface of the gill epithelium following 24h to 96h exposures (Figs. 4 & 5). NPs were detected in several samples of gill tissue on the surfaces of the primary or secondary lamellae. One sample analyzed showed the presence (Fig. 5) of several NPs in the marginal channel in the outer tip of the secondary lamellae, following a 14-day exposure.

Discussion

The purpose of this extensive series of exposure studies was to determine if 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 of the metal oxide NPs, zinc oxide, cerium oxide, and titanium dioxide in the aquatic environment was determined through a comprehensive evaluation of uptake in fish with full characterization of the NPs under a wide variety of exposure conditions.

Our results show little CeO_2 uptake and no measureable uptake of TiO_2 or ZnO in fish tissues, as determined by ICP-MS/ICP-OES, following acute and short-term exposures in the water column across all treatment groups, up to a nominal exposure concentration of 5,000 µg L^{-1}, or following a 21-day feeding exposure up to 300 mg g^{-1} of TiO_2 NPs to food content, by weight. ESEM/EDX elemental analysis of filtered water samples (450 nm, 100 nm, and ultra-filtered at 1kdD) coupled with CARS imaging of gill tissue, however, clearly show that limited uptake can occur directly from the water column and across the epithelial membrane in the gill. However, it is clearly the case that under these conditions, NP behavior such as aggregation and association with biological material, results in reduced bioavailability of metal oxides and therefore limits the uptake of these compounds into fish.

It is important to understand 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 potential of any compound, it is crucial to understand the concentration and form of NPs that aquatic organisms such as fish are/will be exposed. This in turn will influence the route of exposure and likely target organs, should any uptake occur. Such information will also help us to better target 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 [49-51].

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 [52]. Our adopted approach, without the use of a solvent or prior functionalization provides information regarding uptake of metal oxide nanoparticles in their raw form, and may therefore represent 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.

The physico-chemical factors that govern the fate and behavior of metal oxide NPs in the aquatic environment are important determinants of the potential ecotoxicity of these compounds to fish and other aquatic organisms. Our knowledge to date in this area, however, is limited both in natural and experimental systems, making predicting the potential for ecotoxicological effects difficult. 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 or coagulate 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 [46] although, currently no studies have investigated the effect of NOM on NP bioavailability to fish. An exception to this may be ZnO, which is highly soluble and produces significant amounts of free Zn^{2+} cations, up to 16 mg L^{-1} at equilibrium [53]. In this study, although the concentration of Zn^{2+} cations were not measured, no significant uptake of zinc in fish tissues was observed for concentrations in the water spanning 500 µg L^{-1} to 5000 µg L^{-1} and using ICP-MS (or ICP-OES) as a quantification technique (see Fig. S. I. 8). 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 by ICP-MS (or OES) being masked by high background levels of Zn in fish, seen to be between 0.3 and 1.1 mg g^{-1} dry weight (see Fig. S.I.7).

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 (Fig. 3, see also Supporting Information; Figs. S.I. 2, 4, & 5). 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 came 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 behaviour 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 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 [54], implies that NPs are unlikely to enter the fish *via* the gills at toxic concentrations under relevant environmental conditions. The tissue concentration data determined using ICP showed no strong indication of uptake of any of the three metal oxide NPs or TiO_2 bulk particles into any tissues from either a water-borne exposure in rainbow trout and zebrafish or an oral exposure in rainbow trout. Although low levels of Ce were measured in the livers of zebrafish exposed to 500 μg L^{-1} CeO_2 this was likely due to contamination from the gut during sampling. CARS imagery demonstrated that when coming into contact with fish gills nanoaggregates are likely to adhere to mucus on the gill surface and remain bound, possibly in the mucus layer, for short periods - likely from 12h to 40h as has been shown for mucal clearance of bacteria from rainbow trout gill [54]. 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.

Although bioavailability is limited, small amounts of metal oxide NP uptake in fish may occur (i.e. below the limits of most conventional methods of detection). 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.

Although individual NPs are too small to be resolved by CARS microscopy, the signal obtained is sufficient to provide the location of NP aggregates 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 [41]. 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 of the gill tissue (Fig. 5). At this time, we are not able to specify whether the signal represents internal co-localisation 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 [56] and isolated Kupffer cells [58], NPs have also been shown to be taken up by a murine macrophage line [59]. In order to build a greater understanding on 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.

Taken together our results indicate that unmodified, manufactured metal oxide NPs, in the absence of NOM, are likely have low bioavailability in high-cation environments.

This would indicate that for many non-benthic fish, metal oxide NPs are unlikely to be a major ecotoxicological hazard. This needs to be considered against the context of a general lack of knowledge of the fate, behaviour 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 manuscript, are included. This material is available free of charge via the Internet at http://pubs.acs.org.

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Figure Legends

Figure 1. TEM micrographs of nanoparticle (250 mg L^{-1}) suspensions a) zinc oxide, b) cerium oxide c) titanium dioxide. Scale bars represent 200 nm.

Figure 2. Hydrodynamic diameter and zeta potential of nanoscale titanium dioxide under different water conditions and exposure regimes. Panel a) Particle size vs. concentration and water type: MQ: MilliQ ultrapure water, RO: Reverse osmosis treated city water, SY: synthetic water (containing high ion concentrations, details in Supporting Information). Panel b) Zeta potential under different water conditions. Panel c) Z-average data from dynamic light scattering (DLS) analysis of the fish tank waters according to experimental condition; just prior to adding fish; 24 hours after fish were added; 5 days and 9 days after fish were added: Where A is control, rainbow trout added no nanoparticles (NPs), B is Control, no fish, 5000 µg L^{-1} TiO_2 NPs; C is Control, no fish 5000 µg L^{-1} Bulk TiO_2; D is Fish added 500 µg L-1 TiO_2 NPs; E-fish added 5000 mg L^{-1} TiO_2 NPs; and F is fish added 5000 µg L^{-1} bulk TiO_2. Data represent means ± S.E.

Figure 3. Environmental scanning electron micrographs (ESEM) of fish exposure water and the corresponding EDX spectrum analysis (white square) at day 9 for, a) rainbow trout, no particles, b) rainbow trout with 5000 µg L^{-1} bulk TiO_2, c) rainbow trout with 5000 µg L^{-1} TiO_2

NPs, d) rainbow trout with 500 µg L^{-1} TiO_2 NPs. Images were analysed at 4.5 Torr, 10kV, 80% humidity at 4°C.

Table 1. Concentration of zinc and cerium in zebrafish tissues and titanium in rainbow trout (mg g^{-1} dry weight) exposed *via* tank water or diet to various concentrations and preparations of zinc oxide, cerium oxide, titanium dioxide and ionic titanium. Values represent means ± S.E.

* indicates a value significantly different from controls; n.d. = not detected; n=16.

Figure 4. CARS image of TiO2 nanoparticles on a section of the primary lamellae (large panel) and 3-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 indicates TiO2 nanoparticles.

Figure 5. CARS images of gill tissue of rainbow trout, *Oncorhynchus mykiss*, following a waterborne exposure to TiO2 nanoparticles (NPs). The cellular structure of the primary (PL) and secondary (SL) gill lamellae, comprised of pillar cells (PC) and pavement cells (PV), was obtained by epi-detection of the CH2 vibration (shown in green). The red blood cells are effectively separated from the lamellae cells by forwards detection of the CH2 vibration (shown in blue)47. Panel (A) shows gill tissue following a 28 day exposure. A nanoparticle aggregate can be seen occupying the space between the pillar cells. Panel (B) shows the same NP aggregate under a three times increase in magnification. Panel (C) shows a projection of a 300 x 100 µm 3D data set of gill tissue following a 14 day exposure. A cluster of NPs can been seen in the region of the marginal channel (MC). Panel (D) shows a multi-planar view of the same exposure. The two adjacent sub-panels specifically locate the NPs inside the tissue near the surface of the marginal channel (MC).

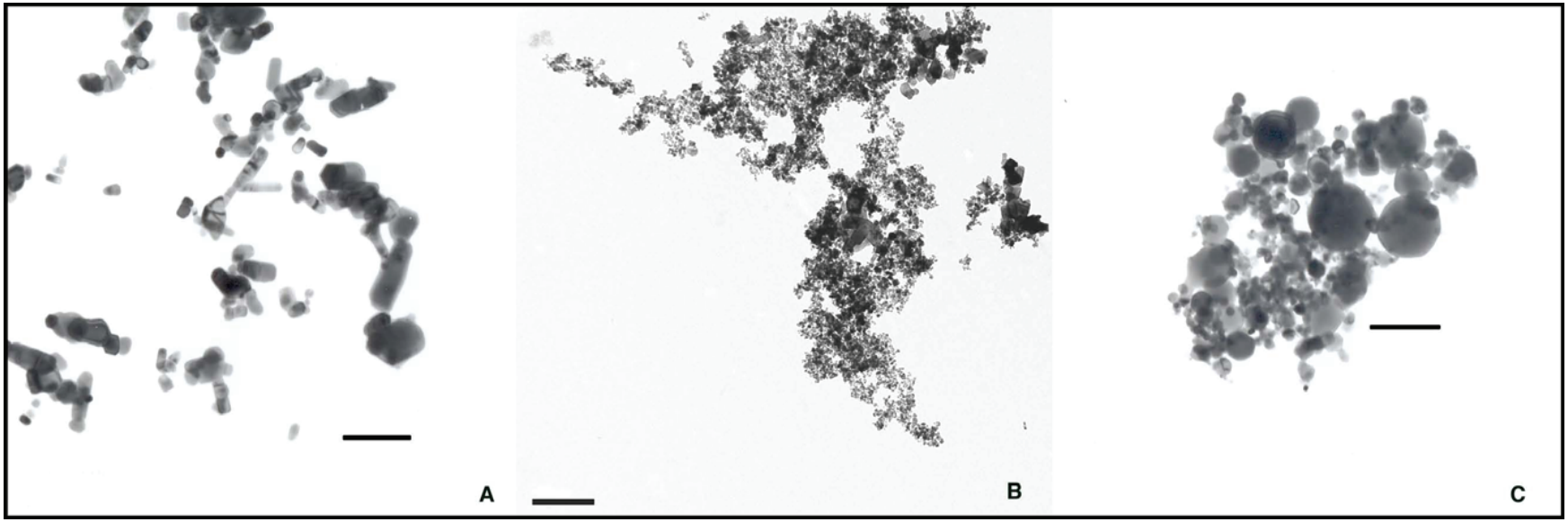

Figure 1

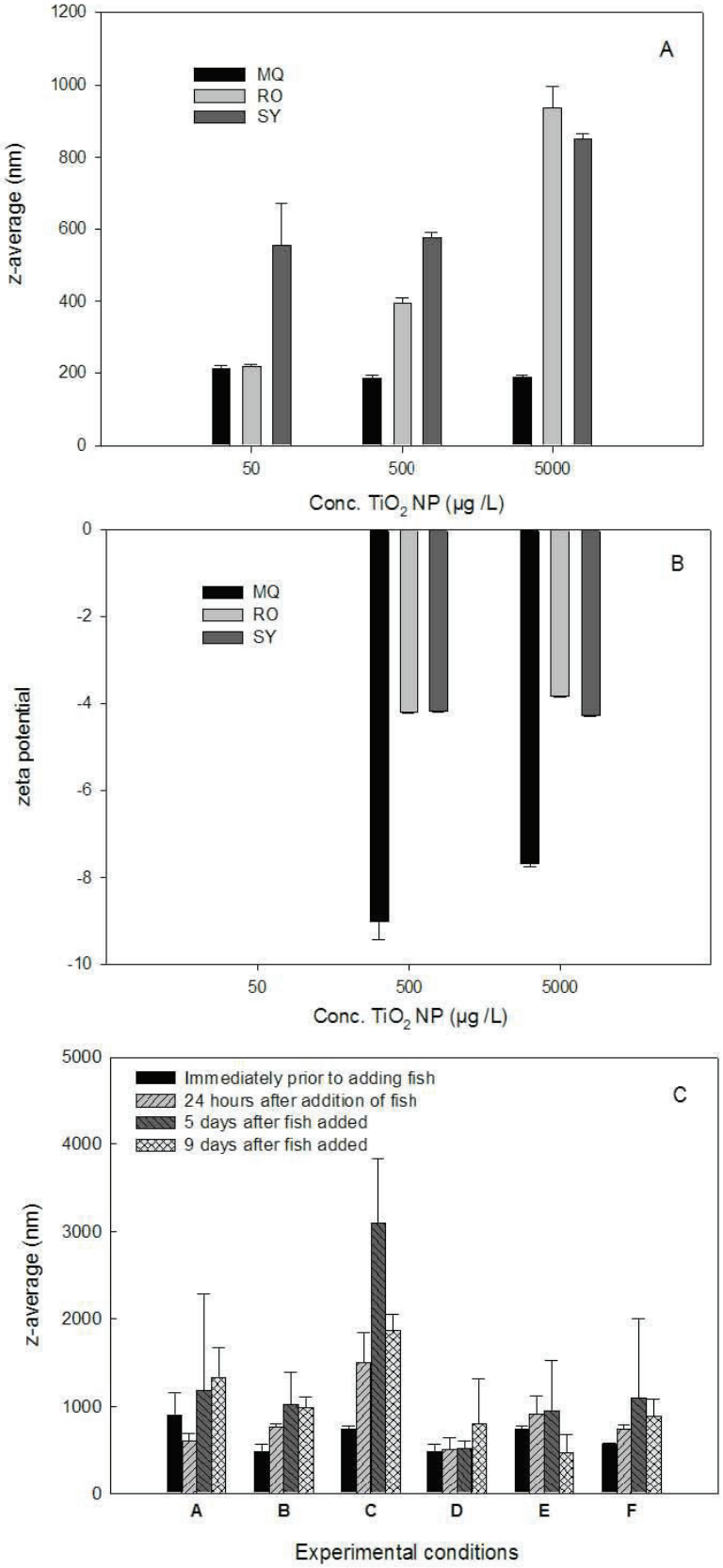
A
MQ
RO
SY
z-average (nm)
1200
1000
800
600
400
200
0
50
500
5000
Conc. TiO2 NP (µg /L)
B
MQ
RO
SY
zeta potential
0
-2
-4
-6
-8
-10
50
500
5000
Conc. TiO2 NP (µg /L)
C
Immediately prior to adding fish
24 hours after addition of fish
5 days after fish added
9 days after fish added
z-average (nm)
5000
4000
3000
2000
1000
0
A
B
C
D
E
F
Experimental conditions

Figure 2

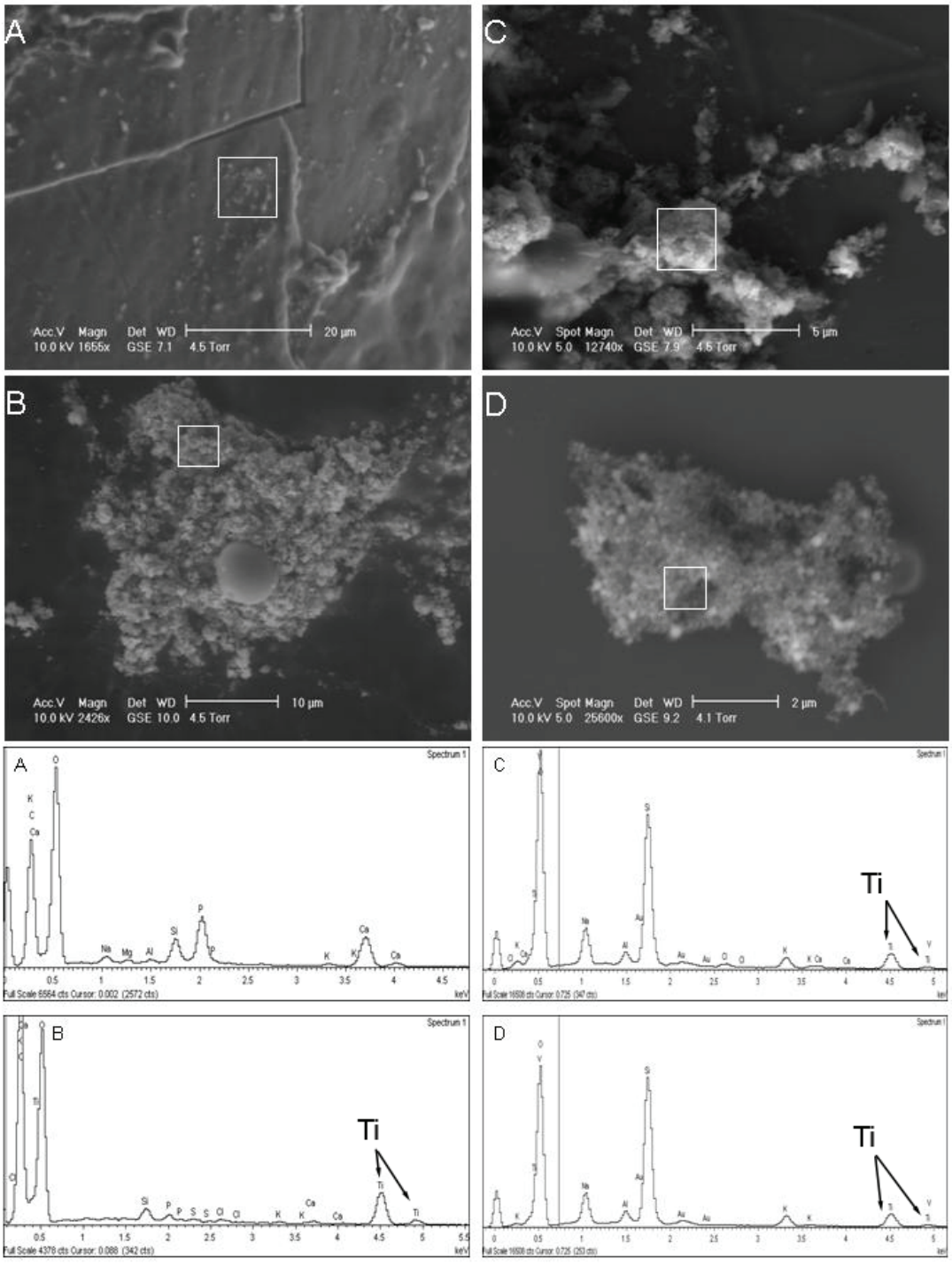
A
Acc.V Magn Det WD
10.0 kV 1655x GSE 7.1 4.5 Torr
20 µm
C
Acc.V Spot Magn Det WD
10.0 kV 5.0 12740x GSE 7.9 4.6 Torr
5 µm
B
Acc.V Magn Det WD
10.0 kV 2426x GSE 10.0 4.5 Torr
10 µm
D
Acc.V Spot Magn Det WD
10.0 kV 5.0 25600x GSE 9.2 4.1 Torr
2 µm
Spectrum 1
A
O
K
C
Ca
Na
Mg
Al
Si
P
K
Ca
Ca
Full Scale 6564 cts Cursor: 0.002 (2572 cts)
keV
Spectrum 1
C
Ti
Si
Ti
Na
Al
Au
Au
Au
O
O
K
K Ca
Ca
Ti
V
Ti
Full Scale 16936 cts Cursor: 0.725 (347 cts)
keV
Spectrum 1
B
Ti
Ti
Cl
Si
P
P
S
S
Cl
Cl
K
K
Ca
Ca
Ti
Ti
Full Scale 4378 cts Cursor: 0.088 (342 cts)
keV
Spectrum 1
D
Ti
O
V
Si
Ti
Na
Al
Au
Au
Au
K
K
Ti
V
Ti
Full Scale 16936 cts Cursor: 0.725 (253 cts)
keV

Figure 3

Water Exposure

	Tissue	Control	500µg/L nano	5000µg/L nano	5000µg/L bulk	5000µg/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	n.d.	n.d.	n.d.	-	-
	Liver	0.03 ± 0.03	1.35 ± 0.58*	1.01 ± 0.59	-	-
	Brain	n.d.	n.d.	n.d.	-	-
	Skin	n.d.	n.d.	n.d.	-	-
Titanium Dioxide						
	Gill	n.d.	n.d.	n.d.	0.01 ± 0.01	0.32 ± 0.06*
	Liver	n.d.	n.d.	0.88 ± 0.27	n.d.	0.03 ± 0.02
	Brain	0.24 ± 0.04	0.20 ± 0.01	0.19 ± 0.04	n.d.	n.d.
	Skin	n.d.	n.d.	n.d.	n.d.	n.d.
	Blood					
	Gut	n.d.	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	n.d.	0.02 ± 0.01	0.15 ± 0.04
	Liver	n.d.	n.d.	n.d.
	Brain	n.d.	n.d.	n.d.
	Skin	n.d.	n.d.	n.d.
	Blood	n.d.	n.d.	n.d.
	Gut	0.11 ± 0.01	0.36 ± 0.03*	1.49 ±0.14*

Table 1.

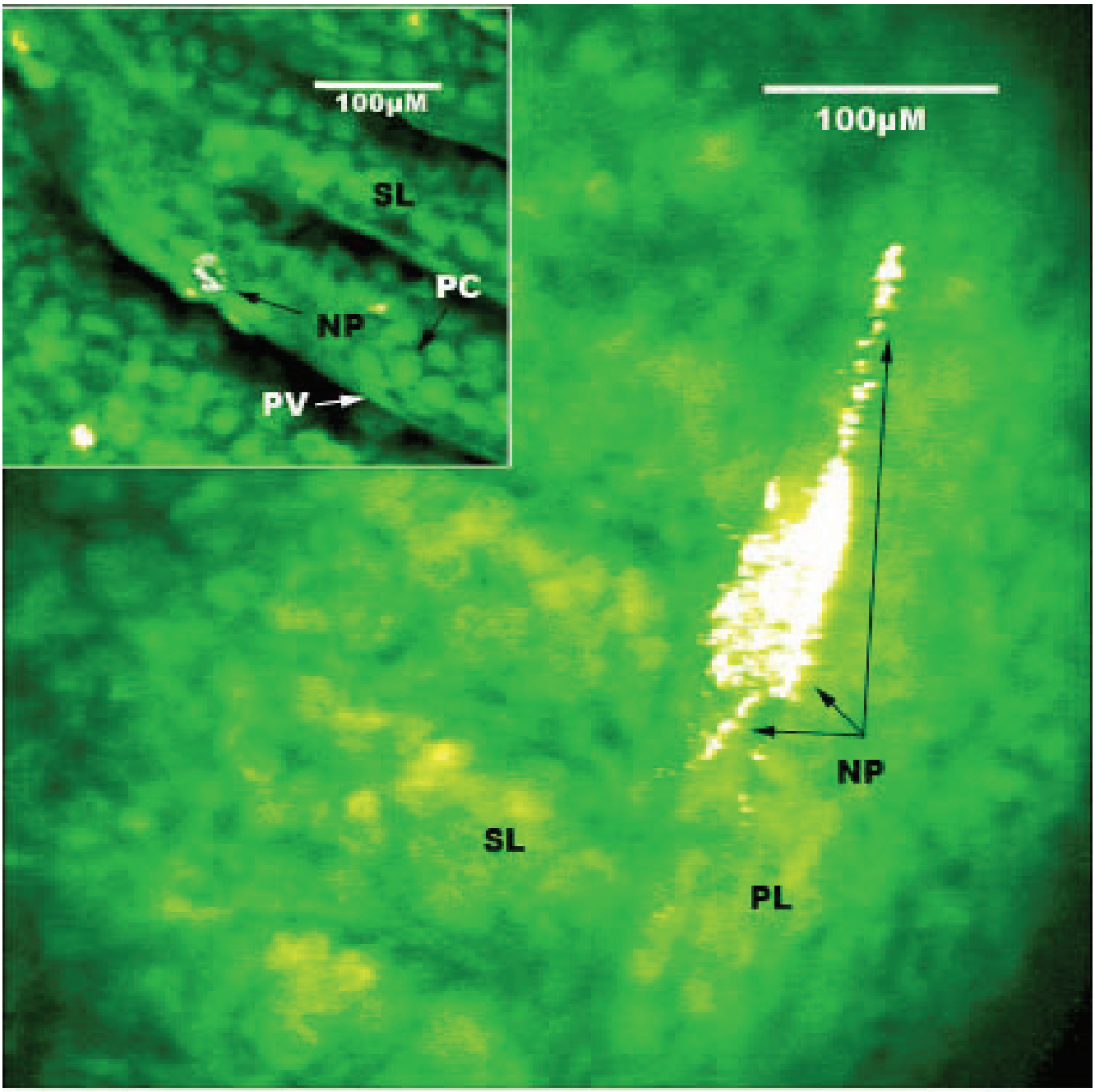

Figure 4

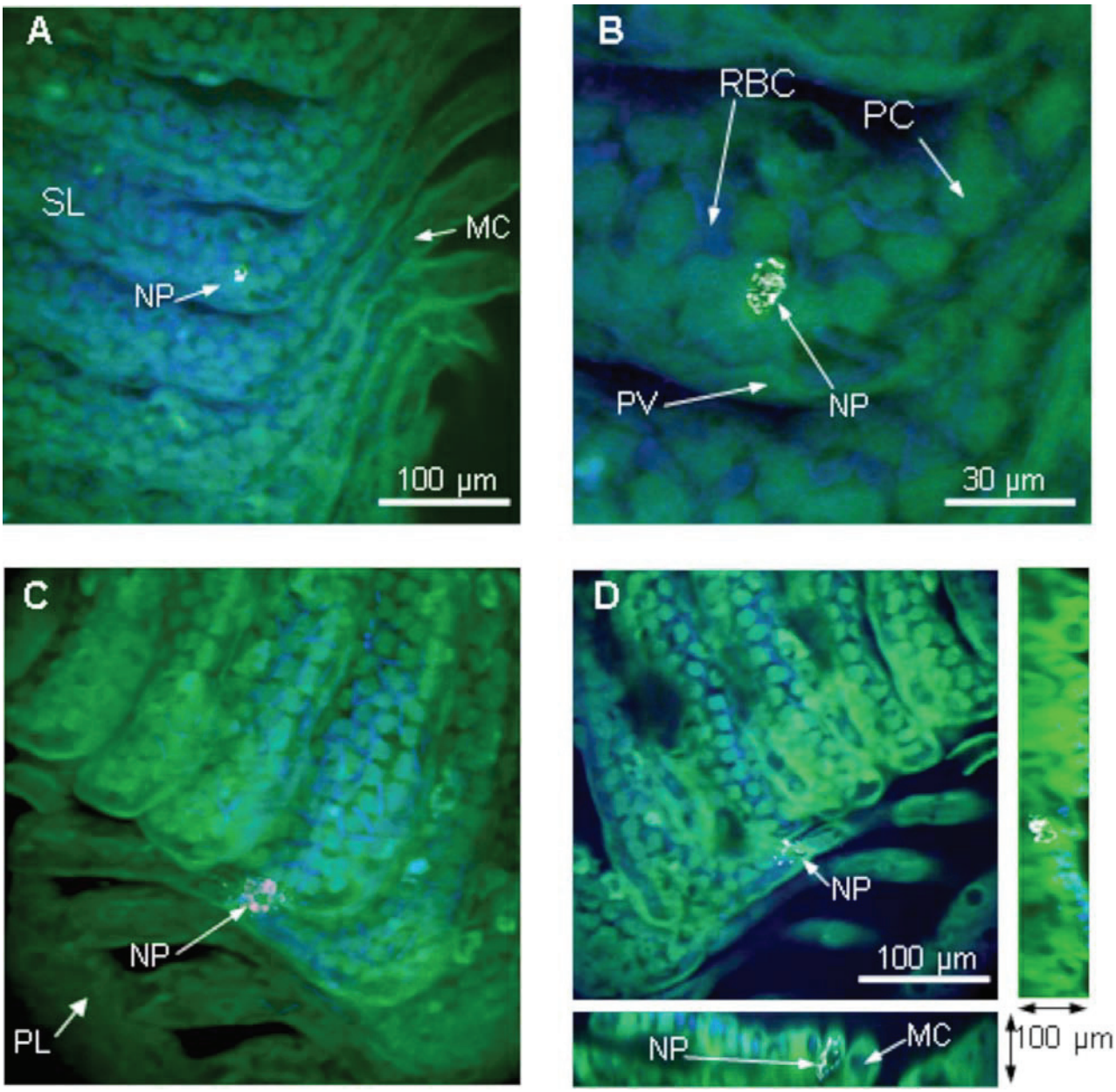

Figure 5

SUPPORTING INFORMATION

Bioavailability of nanoscale metal oxides, TiO_2, CeO_2, and ZnO to fish

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1.0 Objective

The purpose of this study was to provide a comprehensive evaluation of the fate of metal oxide nanoparticles, such as zinc oxide, cerium oxide, and titanium dioxide, in the aquatic environment using fish as a model organism.

2.0 Exposures

2.1 Experimental animals and water conditions

Zebrafish (*Danio rerio*, n = 200) were obtained from Exeter Aquatics (Devon, UK). Fish were kept in 60 L tanks supplied with mains tap water filtered by reverse osmosis (RO) and reconstituted with Analar grade mineral salts to standardized synthetic freshwater (SY): Na^+ = 19.50 mg/L (34.16 µS), K^+ = 1.94 mg/L (1.33 µS), Mg^{2+} = 6.98 mg/L (6.04 µS), Ca^{2+} = 27.62 mg/L (15.84 µS). Prior to exposure, fish were maintained *via* a flow-through system and fed to satiation twice daily, each morning on freshly hatched *Artemia nauplii* and each afternoon on TetraMin® dry tropical flake food (Tetra Werke, Melle, Germany). Water temperature was maintained between 28 and 29°C. Fish were starved for 1 day prior to the experiments.

Rainbow trout (*Oncorhynchus mykiss*, n=80), ~200g (~25cm) were obtained from Houghton Springs Fish Farm (Dorset, UK). Prior to exposure, fish were maintained in 500 L tanks supplied *via* a flow-through system with reconstituted RO water: Na^+ = 8.27 mg/L (14.07 µS), K^+ 2.07 mg/L (1.48 µS), Mg^{2+} = 4.38 mg/L (3.82 µS), Ca^{2+} = 24.50 mg/L (14.13 µS) and were fed a maintenance ration of food (Emerald Fingerling 30, Skretting, UK) 1% body weight. Water temperature was maintained between 9 and 11°C. Fish were deprived of food for 3 days prior to the experiments. A summary of all exposure regimes can be found in Fig S.I. 1.

2.2.1 Zebrafish exposed via water to zinc oxide or cerium dioxide

In two separate experiments (zinc oxide and cerium oxide), three 50 L tanks were filled with RO water. Two tanks were dosed with 10 mL and 100 mL zinc oxide/cerium oxide NP stock suspension (2.5 g/L) to give final concentrations of 500 µg/L and 5000 µg/L

zinc oxide/cerium oxide in the tanks. The third tank was left as a control. After dosing, the water in each tank was agitated with a glass rod and a 10 mL water sample taken *via* glass pipette from the middle of the tank for analysis of nanoparticle concentration. Zebrafish (n=20 for zinc oxide; n=25 for cerium dioxide) were added to tanks and exposed to nanoparticles for 7 days. Airstones in the tanks provided continual movement of the water column throughout the exposure and water samples were taken from the middle of the exposure tanks daily. Fish were deprived of food for the duration of the experiment and were observed daily for 10 minutes to examine their behaviour. At the end of the exposure fish were euthanized with benzocaine according to UK Home Office guidelines. The brain, gills, skin and liver were removed and frozen in liquid nitrogen, stored overnight at -80°C, and freezedried for 24 hours before weighing.

2.2.2 TiO_2 - Oncorhynchus mykiss via water column

Five pairs of 60 L tanks were filled with RO water. Two pairs were dosed with 12 ml and two pairs dosed with 120ml of the TiO_2 nanopowder stock suspension (2.5 g/L) to give final concentration of nanoparticles in the tanks of 500 µg/L TiO_2 and 5000 µg/L TiO_2, respectively. A further pair of un-dosed tanks were used as control tanks. The tanks were left for 24 hours with air stones providing continual movement of the water column then half drained and re-dosed to allow for nanoparticles adhering to the glass, which may affect the actual tank concentration. Eight rainbow trout were then added to each of the control, the 500 µg/L TiO_2, and the 5000 µg/L TiO_2 tanks.

Two 1ml water samples were taken from each of the ten tanks one hour before addition of the fish, immediately after the fish were added, at 1, 2, 4, 12 and 24 hours and every 24 hours subsequently for 8 days (total exposure time = 9 days). Water changes of 50% were carried out on days 2, 4 and 6 and the tanks filled and re-dosed accordingly. Fish were euthanized at the end of the exposure and blood, brain, gills, skin, liver, gut and gall bladder were dissected as in previous exposures and stored at - 20°C until further analysis.

2.2.4 TiO_2 - Oncorhynchus mykiss via oral exposure

Three pairs of 80 L tanks were supplied with flow-through RO water. Eight trout were then added to each of the tanks and allowed to acclimatize for one week before the start of the dosing. Three batches of pelleted feed containing varying amounts of TiO_2 nanoparticles were used for the feeding experiments. The feed base was made by grinding 500g of the standard feed (Emerald Fingerling 30, Skretting, UK) in a blender and passing through a 1mm sieve. Low and high dose feed batches were made by thoroughly mixing 50 mg and 500 mg TiO_2 nanoparticles respectively with 50 g wheat flour. The control batch contained only 50 g wheat flour. The flour mixture was then added to the feed base and mixed thoroughly before addition of 0.5-1 L water and mixing to form a smooth paste. The paste was then passed through a piping bag (Ø 3mm) and dried over night in a drying cabinet at 45ºC. The dried food was then broken up in to 10mm pieces. Fish in paired tanks were fed 16 g of the control, low or high dose food daily based on 1% maintenance for 21 days *via* an automatic feeder.

Water samples of 1ml were taken daily from the top of each tank to ascertain the level of leaching from the food and faeces into the water. Fish were euthanized at the end of the exposure and blood, brain, gills, skin, liver, gut and gall bladder were dissected as in previous exposures and stored at -20°C until further analysis.

2.3 CARS Microscopy

2.3.1 Light source

An Optical Parametric Oscillator (Levante Emerald, APE Berlin) pumped with a frequency doubled Nd:Vandium picosecond oscillator (High-Q Laser Production GmbH) was used. The pump laser generates a 6 ps, 76 MHz pulse train of 532 nm laser light with adjustable output power up to 10 W. The Optical Parametric Oscillator (OPO) uses non-critically phased matched optical parametric generation to produce signal and idler beams which exit the laser cavity collinearly with a perfect temporal overlap. The OPO provides continuous tuning over a wide range of wavelengths; from 670 nm to 980 nm for the signal, which is used as the pump, and between 1130 nm and 1450 nm for the

idler output, used for the Stokes beam. The maximum combined output power of the signal and idler is approximately 2 W.

2.3.2 CARS microscope

Imaging was performed using a modified commercial inverted microscope and confocal laser scanner (IX71 and FV300, Olympus UK) which is ideal for conversion to multiphoton microscopy (Majewska *et al*., 2000; Wang *et al*., 2005). To maximize the NIR throughput the standard galvanometer scanning mirrors were replaced with silver galvanometric mirrors and the tube lens was replaced with a MgF2 coated lens. A 60X, 1.2 NA water immersion objective (UPlanS Apo, Olympus UK) was used to focus the laser excitation into the sample. The scanning confocal dichroic was replaced by a silver mirror with high reflectivity throughout the visible and NIR (21010, Chroma Technologies). Due to the directional nature of the CARS generation, simultaneous forwards- and epi- detection is desirable (Cheng & Xie, 2004). The redundant internal detectors were replaced by external, non-descanned, detection, which has the advantage of increased efficiency for deep tissue imaging.

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 using 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. The anti-Stoke signal was isolated at each photodetector by a single band-pass filter cantered at 750 nm (HQ750/210, Chroma Technologies). Three-dimensional data was acquired by taking stacks of 2-dimensional images in the x-y plane each separated by an increment in the z-direction, which was achieved by alteration of the objective focus.

3.0 Results and Analysis

3.1 *Analysis of aquarium water*

3.1.1 *Size & zeta potential of TiO_2 nanoparticles in experimental aquaria*

From Fig. 2c in the main text of the manuscript, it can be seen that in the tanks where there were no fish, the particle size of the aggregated TiO_2 increased with time.

In tanks that contained fish, the largest mean z-value was 1100 nm ± 900 nm. A possible reason for this was a mechanism which aggregated the nanoparticles (i.e. fish mucus) and caused them to settle out of solution. With the higher concentration (5000 µg/L TiO_2) the particle sizes increased more before sedimenting to the bottom of the aquarium. The net result was that by T=day 9, only smaller particles were left suspended within the water column. Fig. 2a in the main text also shows that increased concentration had an effect on particle size. This is likely due to an increased rate of particle collision due to Brownian motion and thus an increased chance of aggregation. This process is also controlled by charge (e.g. less charge, more aggregation). Fig. S.I. 2 shows the electrophoretic mobilities for unfiltered aquarium water samples. Although no size-dependent or treatment dependent effects could be ascertained from the zeta potentials, it appears that the lowest charge occurs in tanks with the highest particle concentrations (including fish). This also supports the conclusion, that higher concentrations of nanoparticles or the presence of an exudates from the fish was increasing the aggregation of the nanoparticles and sedimentation of larger aggregates to the bottom of the tank. This effect was confirmed by visual observation, as large aggregates of TiO_2 formed shortly after introduction of the fish to the experimental aquaria.

3.2 Fluorescence EEM scanning

3.2.1 *FEEM scanning methods & peaks identified*

Fluorescence EEM (excitation-emission matrix) can be used for identifying the presence of specific organic compounds within environmental samples and scans were analyzed for all exposure tank water samples using a Varian Cary Eclipse fluorescence

spectrophotometer. Analysis was temperature controlled in 4 ml capacity cuvettes with 1 cm pathway at 20oC. Excitation scans were between 200 and 400nm and emission between 280 and 500nm in 2 nm steps. Results were standardized to a mean Raman intensity of 20 units collected at ex 348 nm. DOC analyses were with a Shamadzu 5050 total carbon analyser. Two peaks were identified at Ex/Em 225-230/324-342 and at Ex/Em 280- 285/324-354 both of which can be attributed to the protein, tryptophan (Baker, 2005), possibly related to the presence of faecal matter. Other peaks were present, presumably from the TiO_2 nanoparticles, but were not identified. Raman corrected intensities for peak 1 were 10 – 172.9 and for peak 2, 3 – 72.6 units. One sample, (Fig. S.I. 5) also showed florophores for fulvic and humic-like fluorescence.

3.2.2. FEEM scanning results for experimental aquaria (TiO_2 exposure)

There was a higher fluorescence signal for all the tanks containing the fish compared with tanks with no fish that we attribute to the tryptophan-like protein fluorophore (Fig. S.I. 5). For the tanks containing the bulk material and the nanoparticles there was slightly less (but not significantly so) intensity which may be attributed to one or two factors: 1) the signal was quenched slightly by the TiO_2 particles – this has yet to be investigated fully, 2) the fish did not produce as much tryptophan, 3) The nanoparticles aggregated to the proteins and fell out of solution. We did not find a fluorophore which could exclusively be attributed to fish mucus. The results compare favorably to the dissolved organic carbon DOC results and show a reasonable correlation to the tryptophan intensities (data not shown).

3.4 Fate of nanoparticles in fish

Analysis of the tissues of fish exposed to ZnO nanoparticles showed there was no significant uptake of zinc into any of the tissues examined. ICP-OES cannot distinguish between zinc oxide and any other form of zinc present in the tissues and as endogenous levels of zinc in fish tissue are relatively high, any zinc taken up must be measured against the background level of zinc already present.

There was no significant uptake of cerium in the brains, gills or skin of zebrafish exposed to CeO2 nanoparticles, however there was a significant uptake of cerium to liver tissues of zebrafish exposed to CeO2 nanoparticles. In contrast to zinc, relatively small concentrations of cerium taken up into the tissues can be detected using ICP-OES.

Inter-individual variation in liver concentration was high, a few livers contained large amounts of cerium and some contained none at all.

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Cheng J.X. and Xie X.S., Coherent anti-Stokes Raman Scattering Microscopy: Instrumentation, Theory, and Applications. *J. Phys. Chem.* B **108**, 827-840 (2004).

Majewska A., Yiu G., and Yuste R., A custom-made two-photon microscope and deconvolution system. Pflugers *Arch. Eur. J. Physiol*. **441**, 398-408 (2000).

Wang H. F., Fu Y., Zickmund P., Shi R. Y., and Cheng J. X., "Coherent anti-Stokes Raman Scattering Imaging of axonal myelin in live spinal tissues," *Biophys. J.* **89**, 581-591 (2005).

Figure Legends

Figure S.I. 1. Summary of exposure regimes for all experiments for this study including zebrafish, *Danio rerio,* exposures to ZnO and CeO2 *via* the water column and rainbow trout, *Oncorhynchus mykiss*, exposures to TiO_2 *via* the water column and *via* the diet.

Figure S.I. 2. Electrophoretic mobilities of the water from the exposure tanks; just prior to adding fish; 24 hours after fish were added; 5 days and 9 days: Where A is control, fish added, no nanoparticles (NPs); B is Control, no fish, 500 µg L^{-1} TiO_2 NPs; C is Control, no fish, 5000 µg L^{-1} TiO_2 NPs; D is fish added, 500 µg L^{-1} TiO_2 NPs; E is fish added, 5000 µg L^{-1} TiO_2 NPs; and F is fish added, 5000 µg L^{-1} bulk TiO_2. Bars represent means ± S.E.

Figure S.I. 3. pH of experimental aquaria dosed with TiO_2 (NPs, bulk, or elemental) over the course of a water-borne exposure to rainbow trout, *Oncorhychus mykiss*. Data points indicate mean concentrations taken daily from two replicate tanks at midtank level.

Figure S.I. 4. EEMs from the fish tanks before and after exposure to fish (a) 5000 µg L^{-1} TiO_2 NPs, time = Prior to fish introduction ; (b) 500 µg L^{-1} TiO_2 NPs, time = Prior to fish introduction; (c) 5000 µg L^{-1} TiO2 bulk particles, time = Prior to fish introduction ; (d) Tank water only, no particles; (e) Fish only, day 9; (f) 5000 µg L^{-1} TiO_2 bulk particles, with fish, day 9 (g) 5000 µg L^{-1} TiO2 NPs with fish, day 9 (h) 500 µg L^{-1} TiO_2 NPs, with fish, day 9.

Figure S.I. 5. Concentration of cerium oxide (x) and zinc oxide (□) in tank water dosed at 5,000 µg L^{-1}. Data points indicate mean ± SE of concentrations taken daily from two replicate tanks at mid-tank level

Figure S.I. 6. Zinc concentration in brains, gills, livers and skin of zebrafish, *Danio rerio*, exposed to two concentrations of zinc oxide nanoparticles. Error bars indicate ± S.E. (Numbers of fish sampled: control n=19; 500 µg/L n=11; 5000 µg/L n=17).

Figure S.I. 7. Cerium concentration in livers of zebrafish, *Danio rerio*, exposed to two concentrations of zinc oxide nanoparticles. Error bars indicate ± S.E. (n=25). indicates significant uptake of cerium (Mann-Whitney, $p<0.0001$).

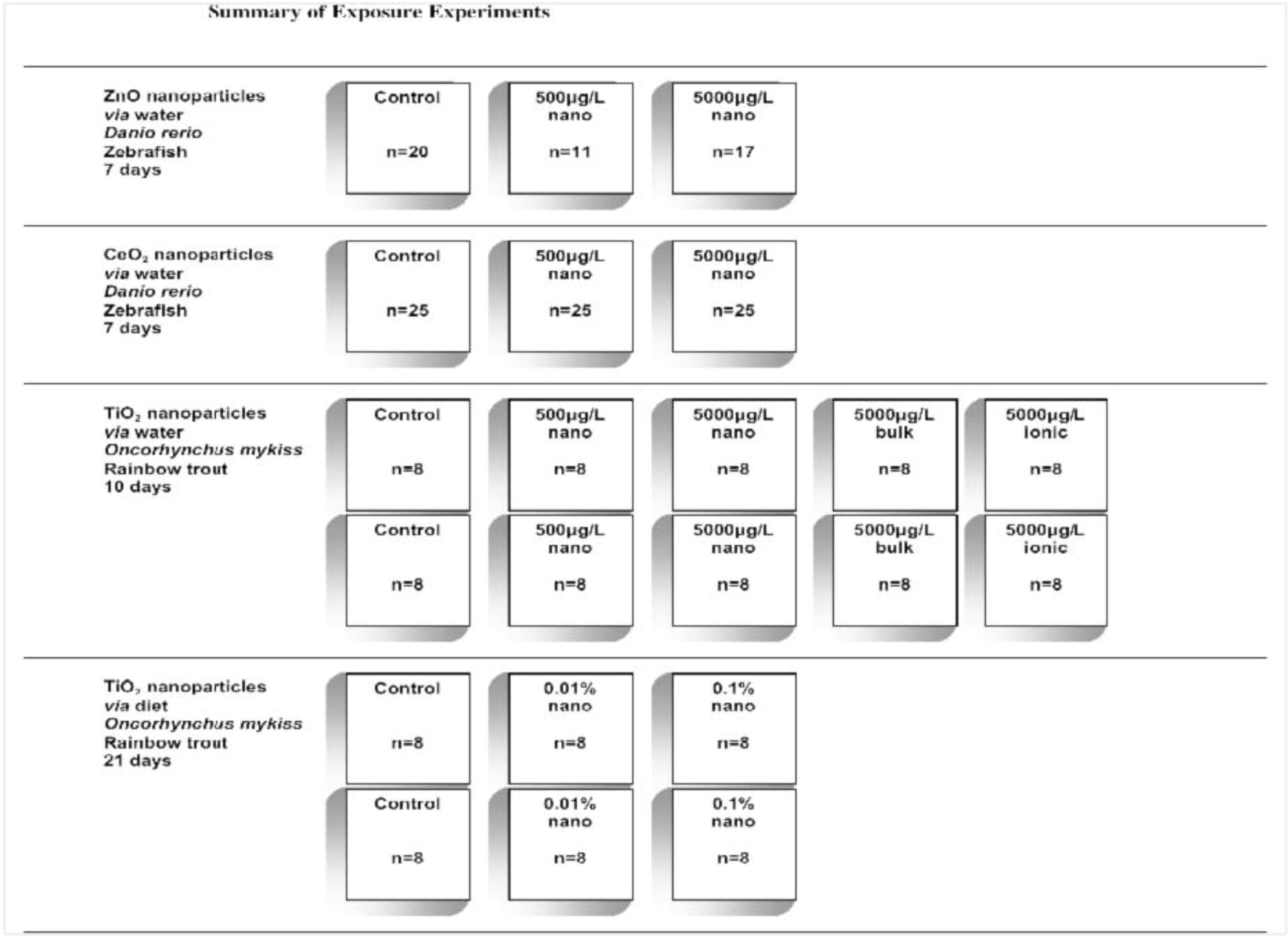
Summary of Exposure Experiments
ZnO nanoparticles
via water
Danio rerio
Zebrafish
7 days
Control
n=20
500µg/L nano
n=11
5000µg/L nano
n=17
CeO2 nanoparticles
via water
Danio rerio
Zebrafish
7 days
Control
n=25
500µg/L nano
n=25
5000µg/L nano
n=25
TiO2 nanoparticles
via water
Oncorhynchus mykiss
Rainbow trout
10 days
Control
n=8
500µg/L nano
n=8
5000µg/L nano
n=8
5000µg/L bulk
n=8
5000µg/L ionic
n=8
Control
n=8
500µg/L nano
n=8
5000µg/L nano
n=8
5000µg/L bulk
n=8
5000µg/L ionic
n=8
TiO2 nanoparticles
via diet
Oncorhynchus mykiss
Rainbow trout
21 days
Control
n=8
0.01% nano
n=8
0.1% nano
n=8
Control
n=8
0.01% nano
n=8
0.1% nano
n=8

Figure S.I. 1.

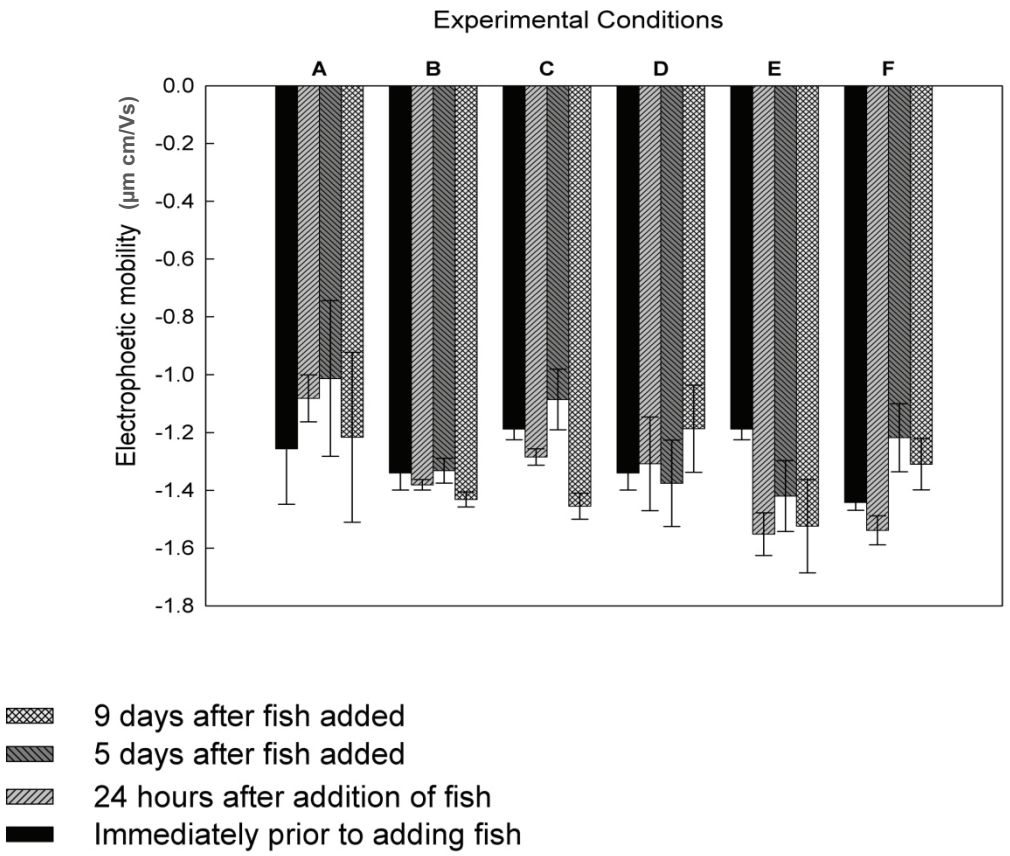
Experimental Conditions
A
B
C
D
E
F
Electrophoetic mobility (µm cm/Vs)
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
-1.6
-1.8
9 days after fish added
5 days after fish added
24 hours after addition of fish
Immediately prior to adding fish

Figure S.I. 2.

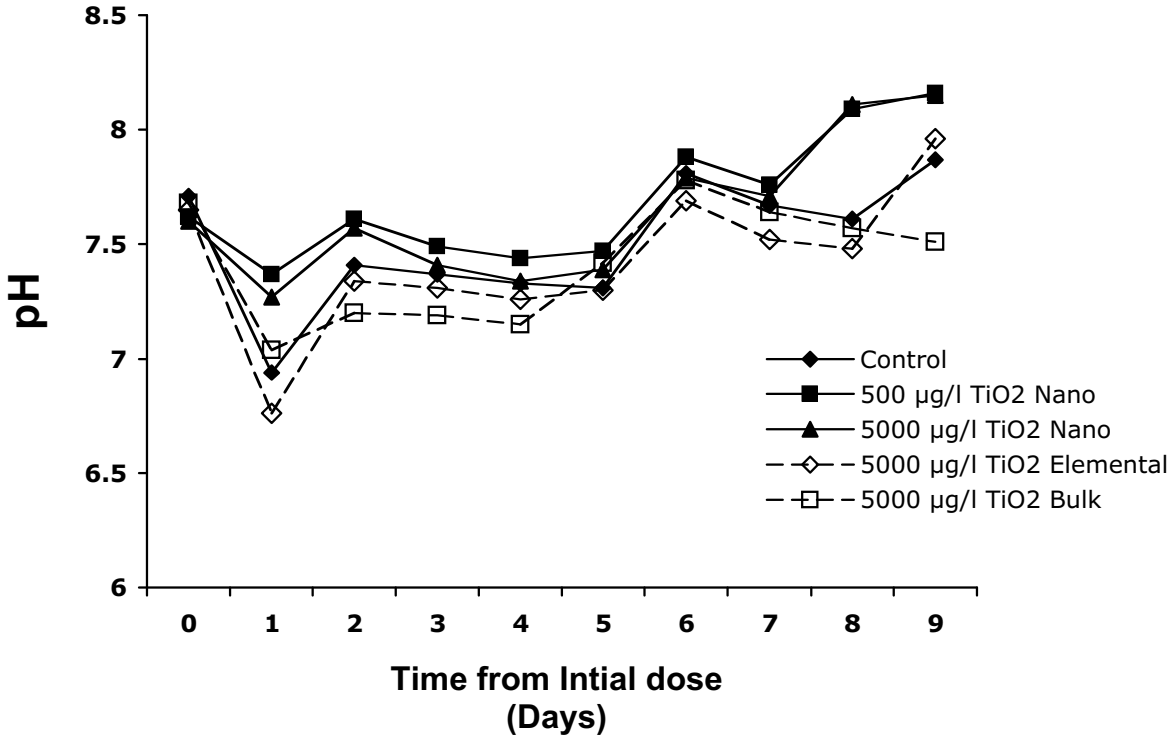
8.5
8
7.5
7
6.5
6
pH
0
1
2
3
4
5
6
7
8
9
Time from Intial dose
(Days)
Control
500 µg/l TiO2 Nano
5000 µg/l TiO2 Nano
5000 µg/l TiO2 Elemental
5000 µg/l TiO2 Bulk

Figure S.I. 3.

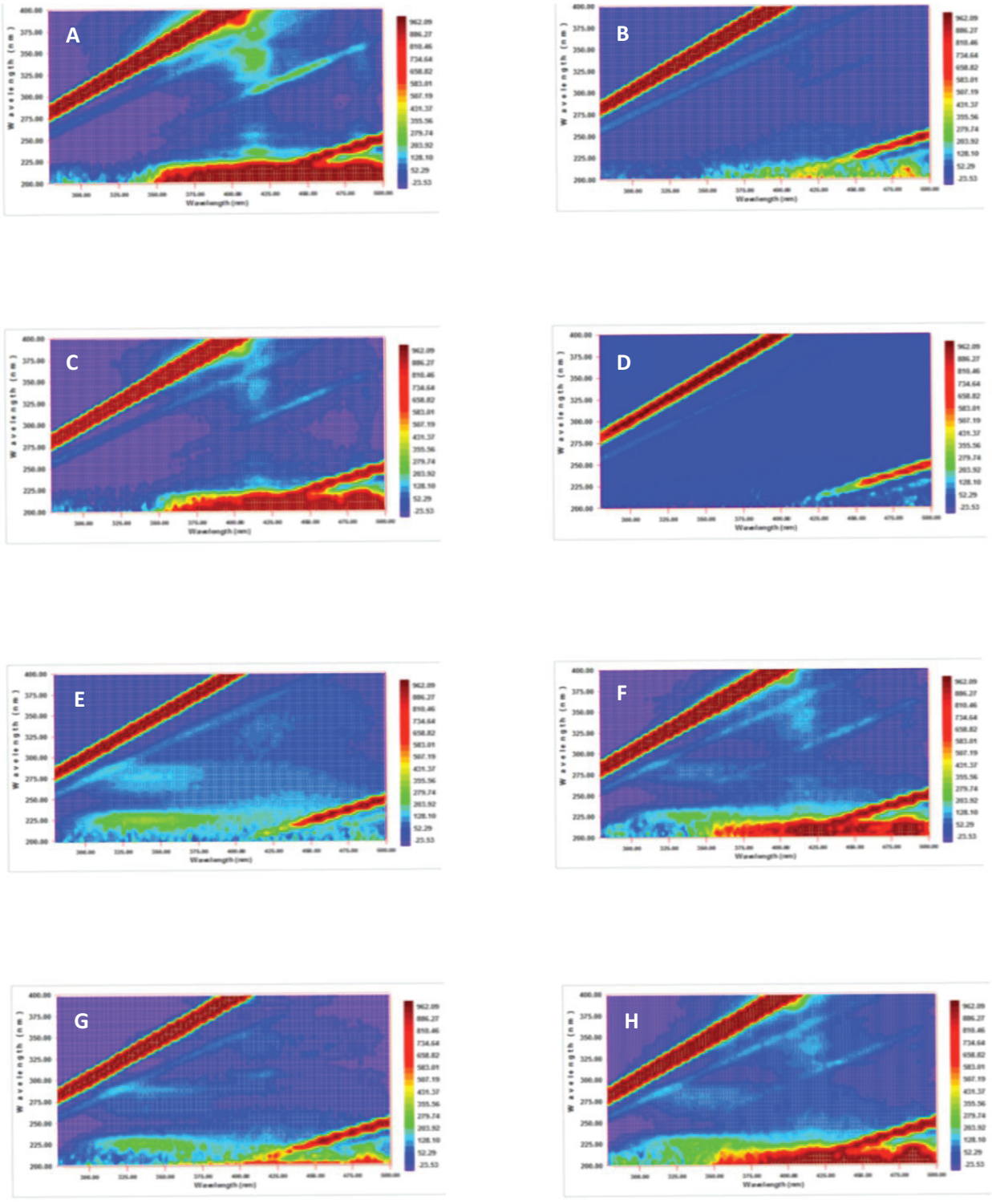

Figure S.I. 4

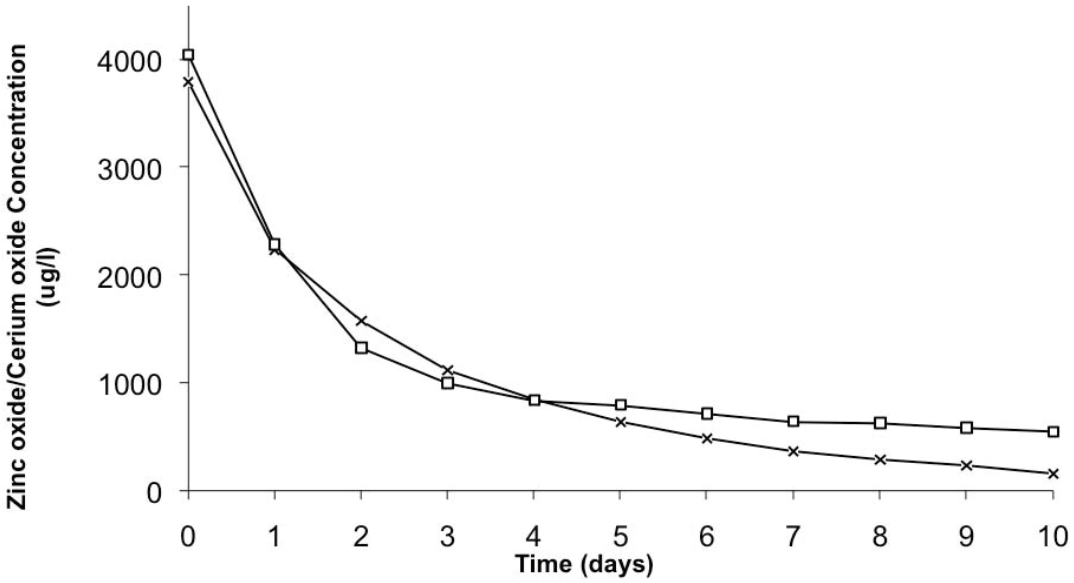
Zinc oxide/Cerium oxide Concentration (ug/l)
4000
3000
2000
1000
0
0
1
2
3
4
5
6
7
8
9
10
Time (days)

Figure S.I. 5.

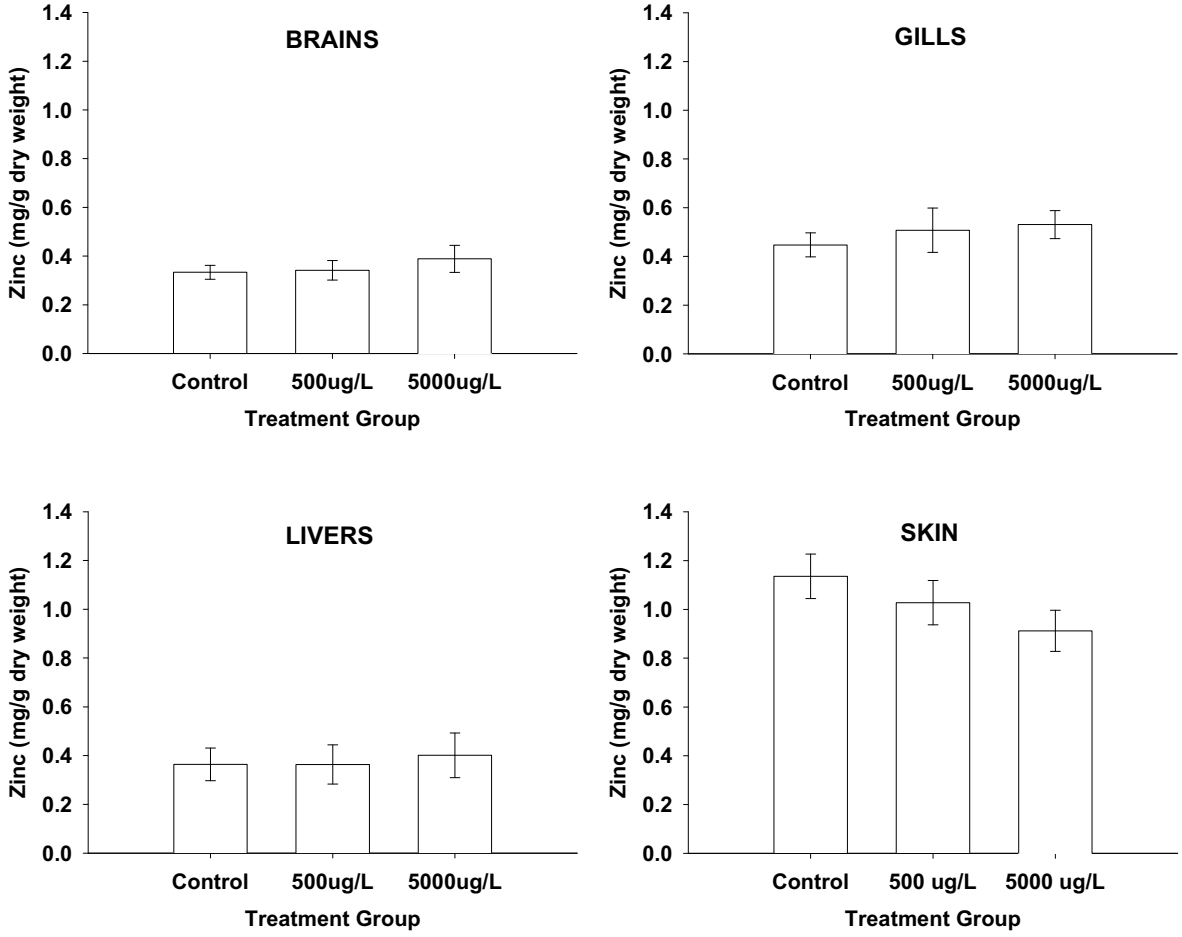
BRAINS
GILLS
LIVERS
SKIN
Zinc (mg/g dry weight)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
500ug/L
5000ug/L
500 ug/L
5000 ug/L
Treatment Group

Figure S.I. 6.

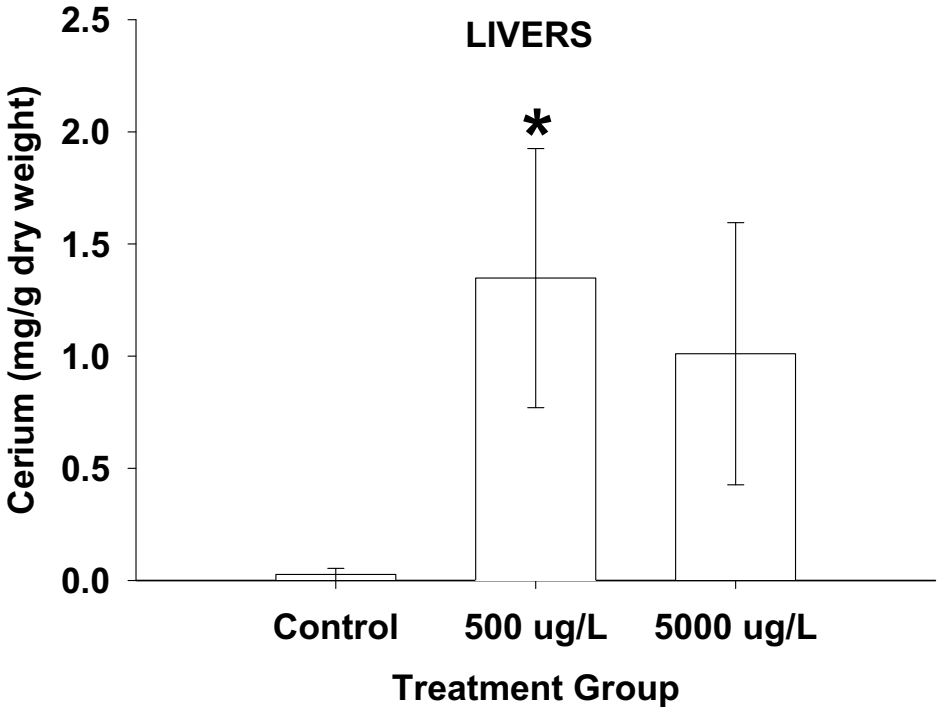
LIVERS
Cerium (mg/g dry weight)
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
500 ug/L
5000 ug/L
Treatment Group

Figure S.I. 7

CHAPTER 3

High doses of intravenously administered titanium dioxide nanoparticles accumulate in the kidneys of rainbow trout but with no observable impairment of renal function.

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High Doses of Intravenously Administered Titanium Dioxide Nanoparticles Accumulate in the Kidneys of Rainbow Trout but with no Observable Impairment of Renal Function

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Our recent work suggests limited uptake of unstabilized metal oxide nanoparticles via water into fish, however, some other studies have indicated such exposures can induce oxidative stress. To investigate tissue distribution and toxicity of titanium dioxide (TiO_2) nanoparticles that may enter into fish, we conducted a series of injection studies. Rainbow trout (*Oncorhynchus mykiss*) were intravenously injected with 100 μg TiO_2 nanoparticles and the content of titanium in blood, brain, gills, liver, and kidney quantified at time points between 6 h and 90 days using inductively coupled plasma optical emission spectroscopy. Injected Ti was concentrated in the kidneys and remained there up to 21 days, however, there was evidence of clearance of TiO_2 at 90 days. Ti accumulation in the liver was 15 times lower than in the kidney with no apparent clearance. Using TEM we showed nanoparticles were localized in tissue vesicles surrounding the kidney tubules. In a second injection study, rainbow trout were injected with 100 μg TiO_2 and plasma samples from individual fish analyzed for total protein and creatinine content at time points between 6 h and 21 days to assess for possible effects on kidney function. No effect of TiO_2 on total plasma protein content or creatinine concentrations were found indicating that neither urine production nor glomerular filtration rate were affected. We conclude that in trout upon a single high dose exposure of TiO_2 nanoparticles via the bloodstream, TiO_2 accumulates in the kidneys but has minimal effect on kidney function.

Key Words: **titanium dioxide; rainbow trout; lipid peroxidation; intravenous injection; nanoparticles; nanotoxicology.**

Production and use of engineered nanoparticles (ENPs) is increasing rapidly with global investment in nanotechnology in the billions of dollars. Large-scale production combined with diverse industrial uses means that the environmental contamination with these nanoparticles is set to become a major issue (Aitken *et al.*, 2006). Little is known, however, about the fate and behavior of ENPs in the aquatic environment. Many are likely to interact with the organic material and natural colloids present in these systems, which in turn will likely affect their bioavailability to aquatic organisms (Handy *et al.*, 2008a). Similar knowledge gaps exist regarding the fate and behavior of nanoparticles entering into the bodies of aquatic organisms and their subsequent biological effects (Colvin, 2003; Dowling *et al.*, 2004; Oberdörster *et al.*, 2005). There are therefore serious concerns about the relative safety of ENPs for both human health and their environmental impact.

Titanium dioxide (TiO_2) is a versatile compound that is already used in nano-form in a variety of consumer products, such as sunscreens and other cosmetics (Wakefield *et al.*, 2005), specialist coatings and paints (Guarino *et al.*, 2008; Kandavelu *et al.*, 2004), and in industrial photocatalytic processes (Guillard *et al.*, 2003; Zhang *et al.*, 2006). Therefore, nanoparticulate TiO_2 is probably already present in the environment, although current levels are unknown. Estimates for levels of TiO_2 in the aquatic environment based on modeling approaches, have suggested concentrations could be as high as 0.7–16 μg/l in water (Mueller and Nowack, 2008).

Many *in vitro* studies have focused on elucidating the effects of nanoparticulate TiO_2. Common findings include general cytotoxicity (Soto *et al.*, 2005) induction of an inflammatory response (Sayes *et al.*, 2006), as well as generation of free radicals (Donaldson *et al.*, 1996), reactive oxygen species and oxidative damage (Long *et al.*, 2007; Sayes *et al.*, 2006). These studies have also shown the ability of TiO_2 to cross cell membranes (Geiser *et al.*, 2005) and to induce micronuclei formation and apoptosis (Rahman *et al.*, 2002). *In vivo* studies on TiO_2 have generally been inhalation studies in mammalian models and findings are characterized by the induction of an inflammatory response (Bermudez *et al.*, 2004; Grassian *et al.*, 2007; Warheit *et al.*, 2007). General cytotoxicity in lung tissue (Warheit *et al.*, 2007) as well as lesions within the lung have also been reported (Bermudez *et al.*, 2004).

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TABLE 1
Size and Charge Characterization Data for TiO_2 Nanoparticles Suspended in Water and Trout Ringer

			Size				Charge			
Dilutant	Concentration of TiO_2 (mg/l)	pH	Z-average (nm)	SD	Polydispersity Index	Range (nm)	Electrophoretic mobility (μm cm/Vs)	SD	Zeta potential (mV)	SD
Trout Ringer	10	7.98	1200	255.7	0.6	400–1100	−8.5	0.7	−0.6	0.1
Trout Ringer	0	7.66					−4.9	1.9	−0.3	0.1
HPLC grade water	10	5.88	227.4	10.8	0.23	100–450	−6.2	1.1	−0.4	0.1
HPLC grade water	0	6.02					−0.2	2.3	−0.0	0.2

Note. HPLC: high performance liquid chromatography.

Few studies have investigated the fate and behavior of TiO_2 nanoparticles within the aquatic environment or their effects on aquatic organisms. In one study on the rainbow trout, however, exposure to TiO_2 nanoparticles via the water, resulted in some physical injury to the gills and a suggestion of lipid peroxidation in the gills, intestine and brain (Federici *et al.*, 2007). Uptake of TiO_2 into these tissues, however, was not demonstrated, making interpretation of these biological effects difficult. Furthermore, no toxicity was observed in an exposure of TiO_2 nanoparticles to zebrafish embryos (Zhu *et al.*, 2008).

Work by Lovern and Klaper (2006) on *Daphnia magna* highlighted the importance of agglomeration of TiO_2 nanoparticles on their toxicity. Filtered TiO_2 with an average diameter of 30 nm caused a 50% mortality at an exposure to 6 ppm and 100% mortality at 10 ppm, whereas nonfiltered TiO_2 with aggregate diameters from 100 to 500 nm showed a maximum mortality of 9% at concentrations up to 500 ppm.

Our previous work, examining the fate and behavior of TiO_2 nanoparticles in rainbow trout (Johnston *et al.*, in press, manuscript submitted), has shown limited uptake of titanium for exposures via either the water or via the diet due to aggregation of nanoparticles in water.

The propensity of nanoparticles to agglomerate in aquatic media may mean that the bioavailability of such nanoparticles to aquatic organisms is low, however, agglomeration is likely to vary with temperature, pH, ionic strength, presence of humics in the aquatic media which may have implications for uptake into aquatic organisms (Handy *et al.*, 2008b).

In the current study, in order to investigate the intrinsic hazard potential of TiO_2 nanoparticles that may enter into fish from the aquatic environment, we conducted a series of studies injecting TiO_2 directly into the bloodstream of rainbow trout. Our results show that nanoparticles are removed from the bloodstream and concentrate in the kidneys, with small amounts also present in the liver. TEM imaging revealed that nanoparticles were encapsulated in vesicles in the kidney and persisted, with limited and very slow clearance. A further study investigating the impacts of high levels of TiO_2 within the kidney tissue, found no effects on normal kidney function, as assessed by the levels of plasma creatinine and total protein.

MATERIALS AND METHODS

Rainbow trout husbandry. Juvenile rainbow trout with total body weight of 75.7 ± 3.25 g (mean ± SE) and fork length of 19.3 ± 0.20 g (mean ± SE) were obtained from Hatchlands Trout Farm, Devon, UK. Prior to exposure, fish were maintained in 500-l tanks supplied via a flow-through system with dechlorinated tap water on a 12-h light/12-h dark cycle and were fed on a maintenance food ration (Emerald Fingerling 30, Skretting, UK), of 1% body weight daily. Water temperatures were maintained between 9°C and 11°C throughout.

Materials. All chemicals were purchased from Sigma-Aldrich, UK, unless otherwise stated. Trout perfusion ringer was made up in ultrapure water (Maxima ultrapure water, Elga, Marlow, UK) with the following ionic strength: 130mM NaCl, 4.2mM KCl, 0.1mM $(NH_4)_2SO_4$, 1mM Na_2HPO_4, 2mM $CaCl_22H_2O$, 1.2mM $MgSO_4{\cdot}2H_2O$, and 5mM $NaHCO_3$. The final pH of the ringer was 7.4.

Nanoparticle characterization. According to the manufacturers specifications (Sigma-Aldrich), TiO_2 nanoparticles were a mixture of rutile and anatase with a primary crystallite size of 23.2 nm, equivalent spherical diameter of 32.4 nm, specific surface area of 46.3 m^2/g and purity of > 99.9% based on trace metal analysis. We characterized the TiO_2 nanopowder (Sigma-Aldrich) as a dry powder by transmission electron microscopy and BET to determine mean particle diameter and specific surface area. The mean particle diameter was 34.2 ± 26.1 nm and the specific surface area was 18.6 ± 1.2 m^2/g. Characterization of the nanopowder in water and in trout ringer was also carried out (Table 1). All measurements are taken after sonication of the suspensions for 30 min.

Injection studies. Preliminary short-term injection studies carried out on rainbow trout revealed that intravenously injected TiO_2 accumulated in the kidney and liver (data not shown). In the first injection study, trout were injected via the caudal peduncle to the caudal vein, with TiO_2 nanoparticles and sampled at time periods between 6 h and 90 days (2160 hours) in order to investigate the kinetics of TiO_2. In the second study, blood samples were taken from individual trout injected with TiO_2 at the same sampling intervals to investigate whether kidney function was compromised as a result of accumulation of TiO_2 in this tissue.

First TiO_2 injection study. To prepare the TiO_2 for injection, 1 l of trout perfusion ringer was supplemented with 100 mg TiO_2 nanopowder and sonicated for 30 min. Trout were anaesthetized with benzocaine until partial loss of equilibrium was achieved and 56 individuals were injected intravenously with 1mL trout perfusion ringer supplemented with the TiO_2. The

mean administered dose was 1.3 mg TiO_2 nanoparticles per kg body weight which is lower but comparable to the dosage used in a previous study (Fabian *et al*., 2008) where rats were injected with a dose of 5 mg/kg TiO_2 nanoparticles. The injected fish were placed in tanks ($n = 8$ fish per treatment) filled with dechlorinated water and maintained on a flow-through system. A further eight trout were injected with 1 ml of trout perfusion ringer (control) and maintained in a separate tank under the flow-through conditions.

Fish injected with TiO_2 were sampled at 6, 12, 24, 96 h, 7, 21, and 90 days. Control fish were sampled at 21 days. Fish were euthanized with an overdose of benzocaine and their fork lengths and body weights measured. Liver and kidneys were dissected out, weighed and flash frozen in liquid nitrogen and stored at −20°C before preparation for inductively coupled plasma optical emission spectroscopy (ICP-OES) analysis. One milliliter of blood was taken from each fish for ICP-OES analysis at each sampling time point. A further 100 µl of blood was collected from each fish and centrifuged at 12,000 × g for 5 min at 4°C and the plasma removed and stored at −20°C for analysis of thiobarbituric reactive substances (TBARS).

Sections of approximately 5 mm^3 were taken from the anterior and posterior kidney of the first two fish from each sampling period and fixed in formalin for 4 h before being washed and stored in 70% industrial methylated spirits (IMS) for histology. Sections from the posterior kidney of approximately 4 mm^3 were taken from two control fish and two TiO_2 injected fish sampled at 21 days and fixed in 2.5% paraformaldehyde and 3% gluteraldehyde in trout ringer for 2 h in preparation for transmission electron microscopy (TEM).

Second TiO_2 injection study. To prepare the TiO_2 for injection, 1 l of trout perfusion ringer (pH 7.4) was supplemented with 100 mg TiO_2 nanopowder and sonicated for 30 min. Sixteen rainbow trout were anaesthetized in benzocaine until partial loss of equilibrium was achieved and a 500 µl of blood sample taken and the plasma collected and stored at −20°C as described above. The trout were allowed to recover for 24 h. Eight individuals were then injected intravenously with 1 ml of trout perfusion ringer and eight individuals with 1 ml of nanopowder supplemented ringer (100 µg TiO_2). Control and injected fish were maintained in two separate tanks supplied with dechlorinated water on a flow-through system.

Blood samples of 500 µl were taken at 6, 12, 24, 96 h, 7 and 21 days postinjection and plasma collected as described above and stored at −20°C prior to analyses for creatinine and protein.

Determination of titanium concentrations in fish tissues. Tissue and blood samples were defrosted and digested with a combination of 4 ml of concentrated HNO_3 (AR grade, Fisher Scientific, Loughborough, UK) and 1 ml of hydrogen peroxide (Laboratory reagent grade, Fisher Scientific). The tissues were left to cold-digest for 24 h before being heated for 24 h on a Gerhardt Kjeldatherm digester unit at 125°C. The temperature was then increased to 190°C to evaporate off the nitric acid and the samples were subsequently redissolved in 10 ml of 10% HNO_3. Two hundred microliters of 10% Triton-X 100 (2% Triton-X 100) was added to the samples which were then analyzed on a Vista-MPX CCD Simultaneous ICP-OES. Calibration standards containing 500 and 1000 µg/l of titanium (Titanium ICP Standard, Merck, Darmstadt, Germany) and quality control standards of 1, 10, 100, 500, and 1000 µg/l were made using TiO_2 nanoparticles suspended in 10% HNO_3 dispersed with 2% Triton-X 100. All samples and quality control standards were sonicated for 30 min prior to measurement. The detection wavelength for titanium was 336.122 nm and the detection limit of the ICP-OES for titanium in the blood plasma was 10 ppb. The digestion efficiency of titanium dioxide in spiked tissue samples was 28.82 ± 1.7% SD. Results are expressed in terms of injected titanium (from titanium dioxide) unless otherwise stated.

Histological analyses. Trout kidney tissue was fixed in 10% neutral buffered formalin for 6 h before transferring the tissue to 70% IMS. The kidney samples were then placed in labeled plastic tissue processing cassettes (VWR, UK) and into a Shandon Hypercenter XP Tissue Processor where the samples were dehydrated in series with IMS (AR grade Fisher Scientific) and embedded in paraffin wax. The samples were then embedded in blocks of paraffin wax and sectioned on a rotary microtome (Leica, Nussloch, Germany). Six microscope slides were prepared for each tissue sample (with three to four sections per slide) and placed on a hotplate at 45°C for 16 h to ensure sufficient adhesion of the sections, which were then stained with hematoxylin and eosin in a Shandon automatic slide stainer (Thermo Scientific, UK). Tissues were examined for histopathological changes using a Zeiss Axioskop 40 light microscope (Welwyn Garden City, UK) with an Olympus DP70 Digital Microscope Camera (Watford, UK) and AnalySIS Image Processing Software (Watford, UK).

Transmission electron microscopy. Kidney tissues were fixed for TEM adopting an in-house method. Briefly, tissue was placed in 2% paraformaldehyde/2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 2 h, washed three times in 0.1M phosphate buffer for 5 min and fixed in 1% osmium tetroxide in phosphate buffer for 1 h. The tissues were then washed in deionized water for 5 × 5 min, before being cut into 1-mm^3 pieces and subsequently suspended in 2% uranyl acetate for 1 hour. The tissues were then dehydrated in an ethanol series: 30, 50, 70, 90, and 100% (×2), in each for 10 min and embedded in TAAB resin. The tissues were blocked in shallow planchets and placed in a 60°C oven for 20 h. Tissues were sectioned on an ultramicrotome (Ultracut, Reichert) and sections of the tissue were examined for structural alterations and subcellular localization of TiO_2 using a Joel TEM 1400 transmission electron microscope (Welwyn Garden City, UK).

Measurement of lipid peroxidation. Measurement of 1,1,3,3-tetramethoxypropane (malonaldehyde; MDA) using the TBARS assay is widely used as an indicator of lipid peroxidation. The protocol adopted was similar to that described previously (Conner *et al*., 2006). Briefly, eight standards of 1,1,3,3-tetramethoxypropane (MDA) at concentrations ranging from 0.625 to 100µM were made up for the TBARS assay using dilutions of a 500µM stock solution of MDA in 2% ethanol. 100 µl of plasma and standards were mixed with 500 µl of 0.4% thiobarbituric acid (40 mg in 10 ml) in 10% acetic acid, pH 5.0 in 1.5-ml microcentrifuge tubes. The tubes were heated to 90°C for 1 h and then cooled under cold tap water to room temperature. Six hundred microliters of butanol was added and the mixture vigorously mixed for 5 s on a vortexer at full speed. The mixture was then centrifuged at 3100 × g for 10 min and the 150 µl of butanol phase removed and placed in duplicate in a flat-bottomed, 96-well microtiter plate and the absorbance measured at 532 nm on a Molecular Devices SpectraMax 340pc microtiter plate reader (Wokingham, UK). A standard curve was prepared and MDA concentrations were determined accordingly.

Measurement of plasma creatinine levels. A series of eight standards of creatinine at concentrations ranging from 0.15 mg/l to 20 mg/l were made up in ultrapure water (Maxima ultrapure water, Elga) from a 100 mg/l stock. Fifty microliters of each standard and plasma sample were added to a standard flat-bottomed 96-well microtiter plate in duplicate. Ultrapure water was used as a control. One hundred microliters of alkaline picrate solution (1:5 vol/vol 1M NaOH/0.13% picric acid) was added and the solution, which was then incubated for 30 min at room temperature. Absorbance was read at 490 nm in a Molecular Devices SpectraMax 340pc microplate reader and plasma creatinine concentrations determined against a standard curve

Determination of total plasma protein using Biuret protein assay. Biuret reagent was prepared by dissolving 2.25 g sodium potassium tartrate, 0.75 g copper sulfate pentahydrate, and 1.25 g potassium iodide in order in 100 ml of 0.2M NaOH. The volume was then brought up to 250 ml with distilled water.

Protein standards of 0, 0.77, 1.55, 3.1, 6.2, 12.5, 25, and 50 mg/ml were prepared using bovine serum albumin dissolved in ultrapure water (Maxima ultrapure water, Elga). One hundred microliters of plasma or standards were vigorously mixed with 900 µl of Biuret reagent and left to stand for 20 min. One hundred fifty microliters of each sample was then

transferred to a 96-well microtiter plate and the absorbance measured at 550 nm on a Molecular Devices SpectraMax 340pc microplate reader. Protein concentrations of the plasma samples were determined against the prepared standard curve.

Statistical analyses. The data are expressed as mean values ± SE and were analyzed using SPSS version 16.0 (SPSS Inc., Chicago, IL), with α set at 0.05. All data were checked for conformity with the assumptions of normality (homogeneity of variance and normality of error). If these assumptions were not met, data were transformed to meet these assumptions. If the data was still non-normal, a suitable nonparametric test was used.

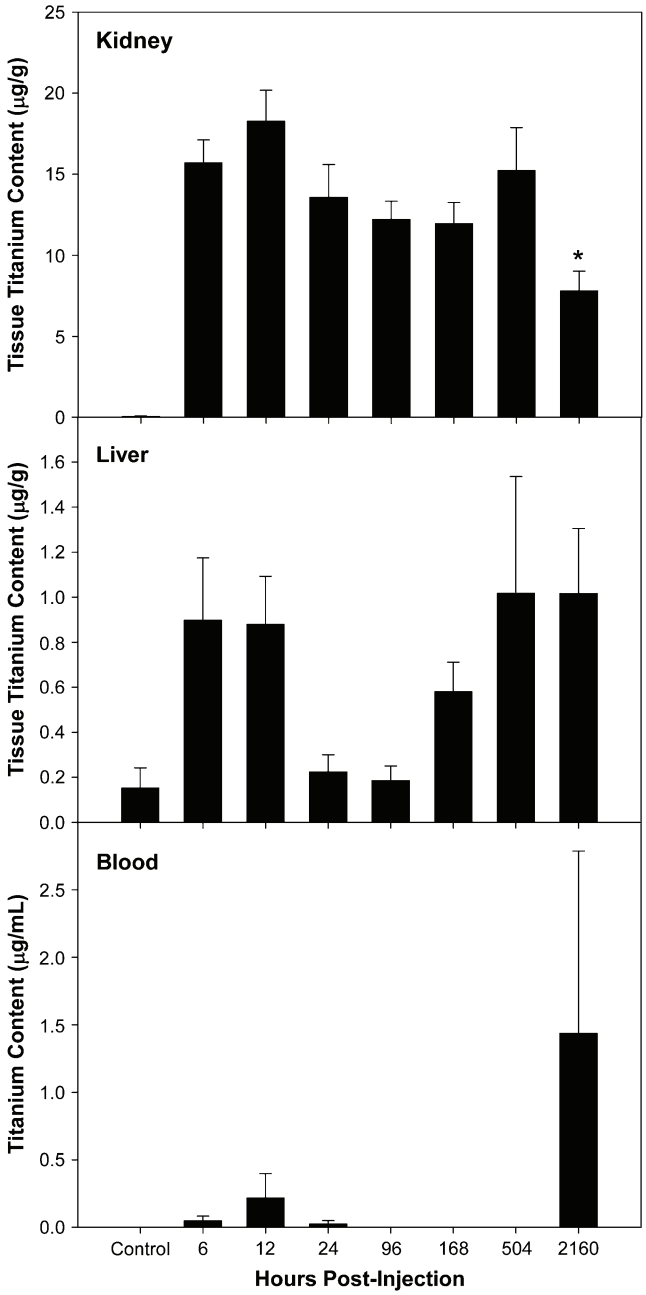

FIG. 1. Titanium levels in the kidney, liver, and blood of trout at sampling time points from 6 h to 90 days after intravenous injection of titanium dioxide nanoparticles (34.2 nm). Data are means ± SE. $n = 8$. *Significantly different from 6, 12, and 504 h (Tukey's $p = 0.003$).

RESULTS

First TiO_2 Injection Study

Trace metal analysis of the trout tissues by ICP-OES showed that 10–19% of injected Ti accumulated in the trout kidneys, equivalent to a TiO_2 concentration in the kidneys of 22.81 µg/g tissue ± 1.23 µg/g (mean ± SE) (Fig. 1). Concentrations of titanium in the kidneys did not change significantly from the first sampling after injection (6 h) to the sampling point at 21 days (ANOVA with Tukey's *post hoc* test; df = 6,45; $F = 3.96$; $p = 0.003$), suggesting that excretion of the introduced titanium was very low during this time. However, after 90 days the concentration of titanium in the kidney was significantly lower than the concentration seen at 6 h, 12 h, and 21 days postinjection suggesting that there is some excretion of titanium after 90 days.

Low levels of titanium, approximately 15 times lower than that in the kidneys were detected in the liver (Fig. 1). The apparent changes in titanium content over the course of the exposure shown in Figure 1 were not statistically significant (ANOVA: df = 6,39; $F = 1.358$; $p = 0.256$). Very low levels of titanium were detected in the blood of some of the fish sampled at 6 and 12 h, postinjection. A relatively high level of titanium was detected in the blood of two of the fish sampled at 90 days, however, for the treatment group as a whole, there was no statistical difference compared with controls (Kruskal-Wallis $H = 8.841$, $p = 0.183$) (Fig. 1). Two preliminary experiments showed that titanium did not accumulate in the brain, gills or spleen (data not shown) of fish injected with the same dose of TiO_2.

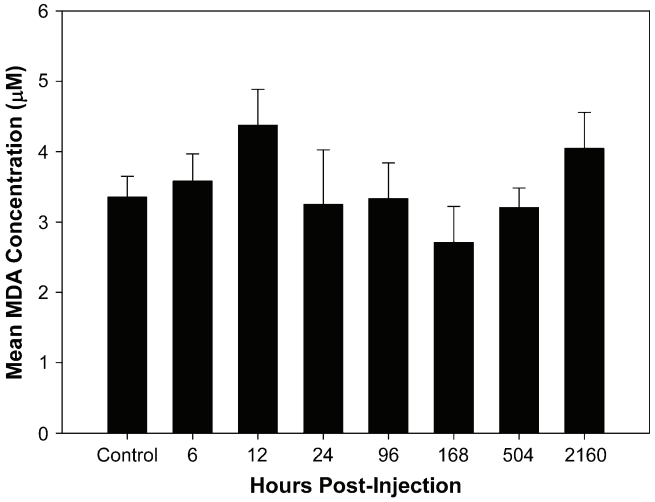

FIG. 2. TBARS in plasma of rainbow trout at sampling time points from 6 h to 3 weeks after intravenous injection of titanium dioxide nanoparticles (34.2 nm). Data are means ± SE. $n = 8$.

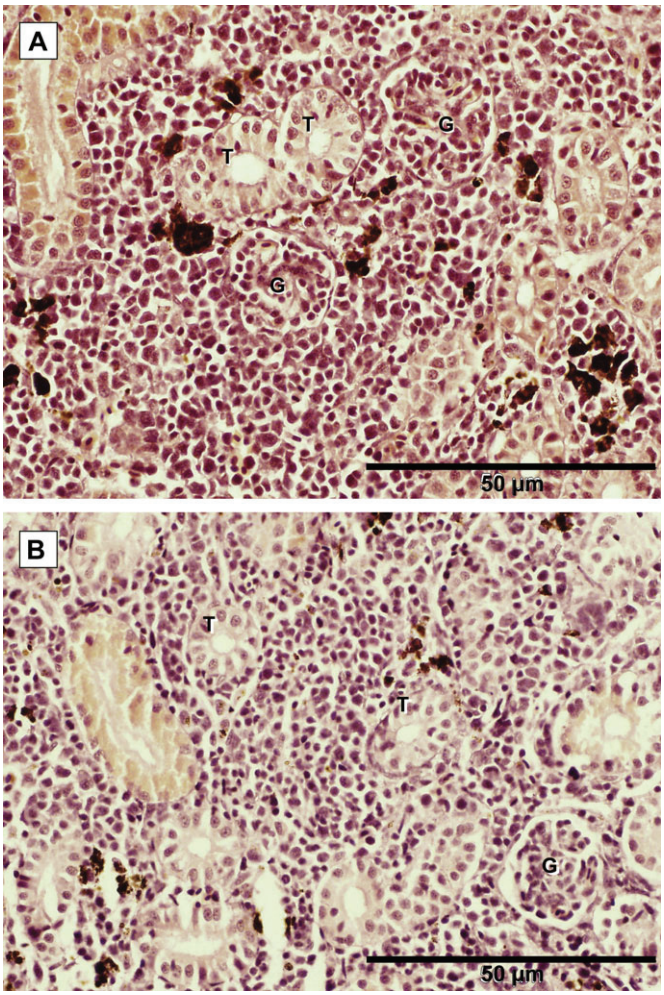

FIG. 3. Kidney morphology as visualized by light microscopy in trout 3 weeks after intravenous injection of (A) 0 µg and (B) 100 µg TiO_2 nanoparticles (34.2 nm) in 1 ml of trout ringer. G indicates glomeruli and T indicates kidney tubules. Scale bar 50 µm, sections were stained hematoxylin and eosin.

There was no significant change in TBARS in the blood at any time point compared with control fish sampled at 90 days (ANOVA: df = 7,51; $F = 1.458$; $p = 0.207$), therefore, giving no indication of lipid peroxidation (Fig. 2).

Examination of the kidney tissue by light microscopy showed no evidence of overt damage or trauma for nanoparticle injected fish (Fig. 3), however, TEM showed the presence of small aggregates of nanoparticle material in cells surrounding the kidney tubules (Fig. 4). The aggregates were between ~500 nm to 2 µm in diameter and appeared to be enclosed within the membranes of darkly stained vesicles, some of which were irregular and some rounded in shape. No aggregates were observed in the cells surrounding the kidney tubules of control fish.

There was no significant change in length or weight of the fish over the course of the exposure.

Second TiO_2 Injection Study

Plasma creatinine concentrations appeared to fluctuate in both treated and nontreated fish, with TiO_2 treated fish showing apparently lower, but not significantly different (Repeat measure ANOVA: $F = 1.383$, $p = 0.37$) concentrations, compared with control fish. Plasma creatinine concentrations decreased from 5.6 to 2.5 mg/l in control fish and from 5.0 to 1.7 mg/l in TiO_2 treated fish during the first 24 h. In control fish, the levels then gradually increased again to 4.4 by the end of the experiment, increased again to 4.4 mg/l by the end 7 days in TiO_2 injected fish, falling again to 2.9 mg/l by 21 days (Fig. 5.).

As observed in the first TiO_2 injection study, analysis of homogenates of kidney and liver tissues taken from fish that were sampled 504-h postinjection showed no significant changes in lipid peroxidation (TBARS) compared with controls (ANOVA kidney: df = 1,12; $F = 0.013$; $p = 0.913$; liver: df = 1,13; $F = 0.121$; $p = 0.733$) (Fig. 6). Titanium dioxide had no effect on the total protein content of the plasma (repeat measure ANOVA: $F = 2.630$, $p = 0.132$) suggesting that production of urine was not affected by the presence of TiO_2 nanoparticles in the kidney (Fig. 7). No significant change in length or weight of the fish was observed over the course of the exposure.

DISCUSSION

Previous studies investigating the effects of TiO_2 nanoparticles on rainbow trout have done so by exposure via the water column and via the diet (Federici *et al.*, 2007; Handy *et al.*, 2008c; Johnston *et al.*, in press; Moger *et al.*, 2008) (*manuscript submitted*). In some of this work, uptake of nanoparticles from the water into gills was shown to occur at very low levels using the imaging technique of Coherent Anti-Stokes Raman microscopy (Moger *et al.*, 2008). In another study moderately toxic effects were reported, which included decreases in Na^+K^+-ATPase activity in the gills and intestine, increases in TBARS in the gill, intestine and brain and increases in the total glutathione levels in the gills, but depletion of glutathione in the liver compared with controls with significant accumulation of TiO_2 in the liver and brain (Federici *et al.*, 2007). However, TiO_2 was not measured in the blood in any of these studies, making interpretations of the findings difficult. The main objectives of the present study were, therefore, to investigate the kinetics of TiO_2 nanoparticle distribution in rainbow trout and to determine any effects on those target organs accumulating the nanoparticles, using an intravenous injection approach.

In the first injection study, the kidneys were found to be the main target organ for accumulation of titanium, with a lower level of accumulation in the liver. Six hours after injection, 10–19% of the total burden of injected titanium was found in the kidneys and this level persisted up to three weeks after injection. TEM imaging of the kidneys of trout sampled at 21 days revealed

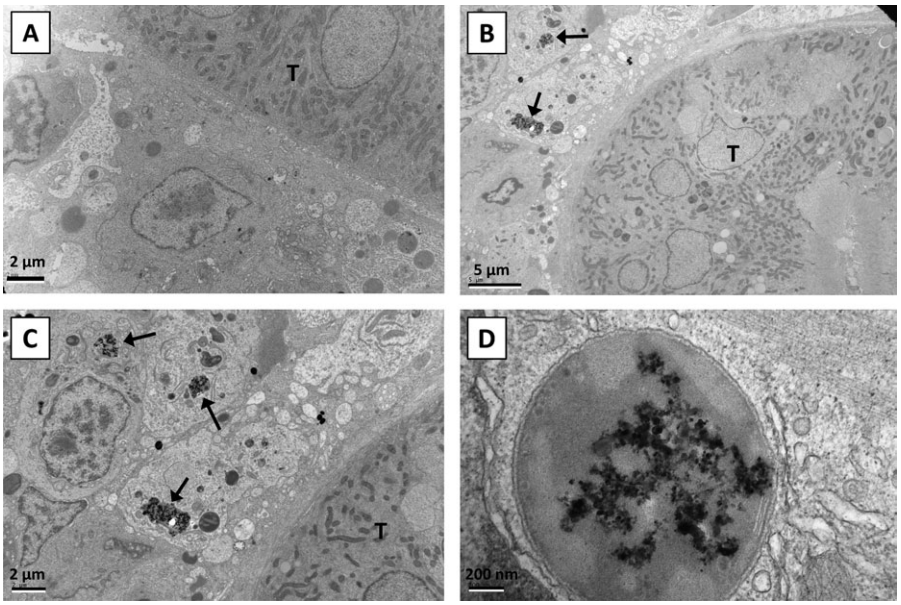

FIG. 4. TEM images of kidney tissues dissected from rainbow trout 3 weeks after intravenous injection of 1 ml of trout perfusion ringer (A) or 100 µg TiO_2 nanoparticles (34.2 nm) in 1 ml of trout ringer (B–D). (B, C) Clusters of nanoparticles, indicated by arrows, are visible in tissue surrounding the kidney tubule T. (D) A cluster of nanoparticles in a membrane-bound vesicle.

clusters of nanoparticles in the hematopoietic cells surrounding the kidney tubules and in some cases these clusters were compartmentalized in lysosomes. After 90 days, however, the level of titanium in the kidney was significantly lower (49–57%) than the concentrations seen at 6 and 12 h and at 504 h, suggesting that the titanium was beginning to be eliminated from the kidney. The level of titanium did not significantly change in the liver throughout the study period (6 h to 90 days). These data suggest that approximately 20% of nanoparticles persist in the hematopoietic tissue of the kidney for periods of weeks and do not reach the kidney tubules to be excreted in the urine. After a prolonged period, however (90 days), there was evidence of some elimination of the TiO_2. It is not clear whether this elimination was related to an active excretion of the TiO_2 or simply due elimination of the TiO_2 along with dead cells during normal tissue cell turnover. Little is known, however, about the turnover time for kidney cells in fish.

In the second study, plasma samples were analyzed for creatinine content to determine whether the presence of the

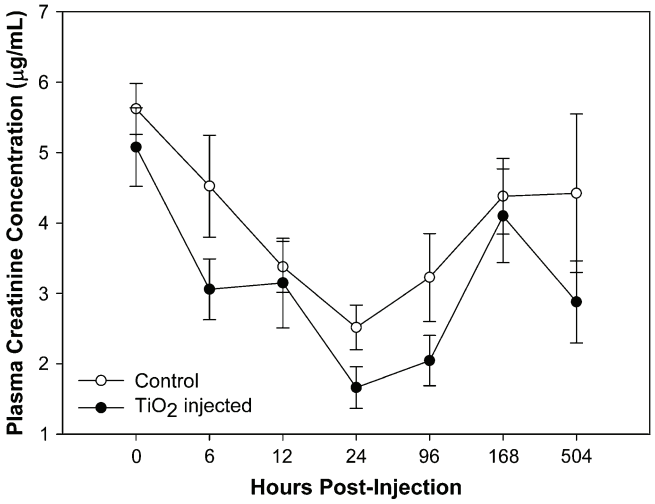

FIG. 5. Creatinine concentration in plasma of rainbow trout at sampling time points from 6 hours to 3 weeks after intravenous injection of titanium dioxide nanoparticles (34.2 nm). Data are means ± SE. $n = 8$.

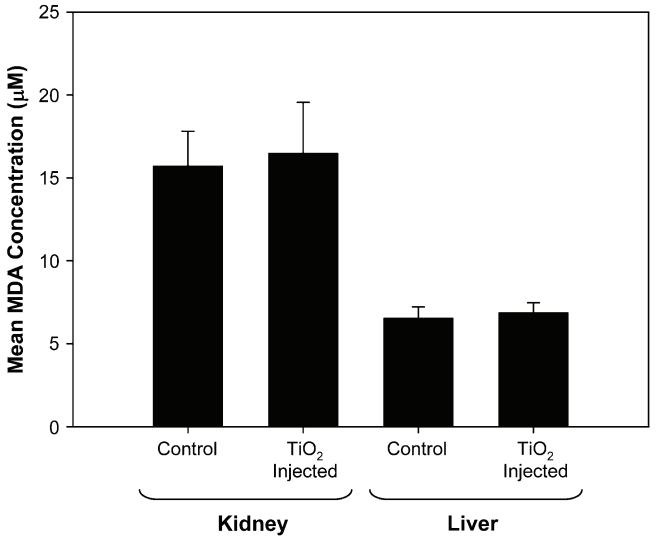

FIG. 6. TBARS in homogenates of kidneys and livers of rainbow trout 3 weeks after intravenous injection of titanium dioxide nanoparticles (34.2 nm). Data are means ± SE. $n = 8$.

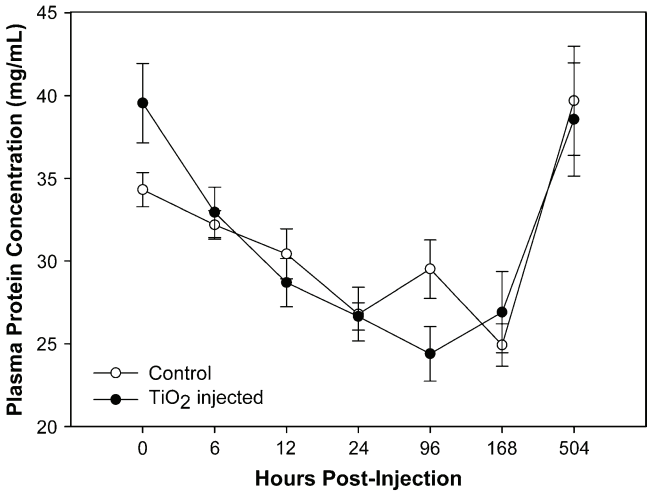

FIG. 7. Plasma protein concentration in plasma of rainbow trout at sampling time points from 6 h to 3 weeks after intravenous injection of titanium dioxide nanoparticles. Data are means ± SE. $n = 8$.

nanoparticles in the kidney caused any compromise in kidney function. Creatinine is a breakdown product of creatine phosphate and is usually produced by the body and excreted by the kidneys at a fairly constant rate (Bond, 1996). A change in plasma creatinine concentration can be used as an indicator of glomerular filtration rate, with a rise in creatinine levels indicating damage to nephrons. Creatinine levels in all fish were similar to documented normal levels (Manera and Britti, 2006; Rehulka and Minarik, 2003).

Impairment of kidney function may also be demonstrated by reduced urine production, which in turn is indicated by a rise in plasma protein concentration. Changes in the dilution of the plasma would also cause fluctuations in the plasma creatinine concentration. We therefore also measured the total plasma protein concentrations, so were able to compare the creatinine concentrations without the influence of possible changing plasma concentrations.

Plasma protein concentrations in control and TiO_2 treated trout were not significantly different and were within documented normal ranges (Manera and Britti, 2006; Rehulka and Minarik, 2003). Therefore, despite the presence of TiO_2 in the kidneys at up to 23 μg/g of tissue, there appeared to be little impact on glomerular filtration or urine production.

Our TEM images showed some of the retained TiO_2 nanoparticles were localized within the hematopoietic cells of the kidneys, so although no obvious impairment of renal function was observed, other effects such as changes in blood cell counts or decreased hemoglobin or other systematic issues (Amend and Smith, 1975) are possible, but these were not investigated for in this study.

Analysis of TBARS in the blood of fish at all time points showed no evidence of lipid peroxidation, and this was similarly so for the kidney and liver tissue of fish 3 weeks after injection where again there was no evidence of lipid peroxidation,. Previous *in vitro* work has shown that TiO_2 can generate reactive oxygen species and cause oxidative damage (Gurr *et al*., 2005; Long *et al*., 2007; Sayes *et al*., 2006). Federici *et al*. (2007) found that rainbow trout exposed via the water to TiO_2 showed evidence of lipid peroxidation in the gills, intestine and in the brain. However, in our study, although TiO_2 was shown to accumulate in the kidneys and liver, no lipid peroxidation was observed.

To date this is the only study of intravenous injection of TiO_2 nanoparticles into fish. A previous study where rats were injected with (5 mg/kg body weight) TiO_2 nanoparticles similarly found that TiO_2 was removed from the blood within 24 h after injection, however, in that study the major target organ was the liver, although TiO_2 was also found in the spleen, lungs and kidneys (Fabian *et al*., 2008). In the rat study, the levels of TiO_2 were maximal in the spleen, lung and kidney 24 h after injection, and decreased to control levels by the end of the experiment (28 days). In that study no detailed analyses were conducted on the localization of TiO_2 in the rat kidney. In contrast, the levels of TiO_2 in the liver were retained throughout the 28-day study. In agreement with our study, there were no obvious health effects or changes in organ function (including for immune response) in the rat study.

Likely routes of uptake of TiO_2 nanoparticles into fish include both the gill and the gut epithelia (Handy *et al*., 2008a) and there is *in vitro* evidence to show that these particles can be taken up into Chinese hamster ovary cells (Suzuki *et al*., 2007) and BEAS-2B cells (Park *et al*., 2008) and translocated between epithelial and subepithelial cells in rat tracheal explants systems (Churg *et al*., 1998). Other nanoparticle types have been shown to be taken up and translocated to other organs in rats including carbon nanoparticles into the liver via the blood (Oberdörster *et al*., 2002) and the brain via the olfactory nerve after inhalation (Oberdörster *et al*., 2004) and the translocation of gold nanoparticles in to the blood and kidney after inhalation (Yu *et al*., 2007).

The bioavailability and toxicity of TiO_2 nanoparticles to aquatic organisms may be limited due to the aggregation behavior in aquatic media (Lovern and Klaper, 2006), although there is a lot of uncertainty regarding bioavailability, in part due to deficiencies of our understanding of how manufactured nanoparticles behave in aquatic systems. There is little yet known about how aggregation is affected by water chemistry, including pH and ion concentration, or how interaction of nanoparticles with natural colloids and dissolved organic matter present in natural waters affects both the bioavailability and biological effects of nanoparticles (Handy *et al*., 2008b; Lead and Wilkinson, 2006).

Our data show that even high doses of intravenously injected TiO_2 nanoparticles cause very limited (if any) overt impairment of renal function or oxidative stress in the blood,

in rainbow trout, despite evidence of significant uptake into and retention in this tissue. Longer-term exposures to TiO_2 nanoparticles might result in continual uptake and accumulation of nanoparticles in these tissues and therefore give the potential for functional effects, however, considering what is known about the behavior of TiO_2 nanoparticles in the aquatic environment, it is likely that most pelagic fish species will have limited exposure only to these nanoparticles via the water column.

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CHAPTER 4

Effects of aqueous exposure to silver nanoparticles of different sizes in rainbow trout

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Effects of Aqueous Exposure to Silver Nanoparticles of Different Sizes in Rainbow Trout

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Abstract

Despite increasing application of silver nanoparticles (NPs) in industry and consumer products, there is still little known about their potential toxicity, particularly to organisms in aquatic environments. To investigate the fate and effects of silver NPs in fish, rainbow trout (*Oncorhynchus mykiss*) were exposed *via* the water to commercial silver particles of three nominal sizes: 10 nm (N_{10}), 35 nm (N_{35}) and 600-1,600 nm (N_{Bulk}) and to silver nitrate for 10 days. Uptake into the gills, liver and kidneys was quantified by inductively coupled plasma – optical emission spectrometry (ICP-OES), and levels of lipid peroxidation in gills, liver and blood determined by measurements of thiobarbituric reactive substances (TBARS). Expression of a suite of genes, namely *cyp1a2*, *cyp3a45*, *hsp70a*, *gpx* and *g6pd*, known to be involved in a range of toxicological response to xenobiotics was analysed in the gills and liver using real-time PCR. Uptake of silver particles from the water into the tissues of exposed fish was low, but nevertheless occurred for current estimated environmental exposures. Of the silver particles tested, N_{10} were found to be the most highly concentrated within gill tissues and N_{10} and N_{Bulk} were the most highly concentrated in liver. There were no effects on lipid peroxidation in any of the tissues analysed for any of the silver particles tested, and this is likely due to the low uptake rates. However, exposure to N_{10} particles was found to induce expression of *cyp1a2* in the gills, suggesting a possible increase in oxidative metabolism in this tissue.

Keywords: silver; rainbow trout; lipid peroxidation; nanoparticles; nanotoxicology; gene expression.

Introduction

Current growth in the nanotechnology industry and the increasing numbers of products making use of the unusual properties of engineered nanoparticles is becoming extremely important in the global economy. Increased production and use of nano-products will inevitably lead to increased levels of discharge of nanomaterials into the environment through their intentional and accidental releases or via weathering of products that contain them. The aquatic environment is particularly vulnerable as it is likely to act as a sink for many of these particles, as it does for many chemical discharges. The fate of nanoparticles in the aquatic environment, their interactions with biotic and abiotic components, and their potential to cause harm are all still poorly understood and these uncertainties are driving concerns on the risks they may pose to human and environmental health. Silver nanoparticles are already used in a variety of consumer products, notably for their antimicrobial properties, including in washing machines (Jung et al., 2007) and fabrics (Perelshtein et al., 2008), and prospective applications also include use in wound dressings (Arora et al., 2008), water treatment filters (Li et al., 2008), catalysts (Kumar et al., 2008), sensors (Schrand et al., 2008), inks (Wang et al., 2008) and pharmaceuticals (Chen and Schluesener, 2008; Sun et al., 2008).

Though their antimicrobial properties are well known (Choi et al., 2008; Jayesh et al., 2008), silver nanoparticles have also been shown to cause toxicity in vertebrate cell lines with findings typified by the generation of reactive oxygen species (Hussain et al., 2005; Schrand et al., 2008), apoptosis (Braydich-Stolle et al., 2005; Park et al., 2007), increased lipid peroxidation (Arora et al., 2008), reduced mitochondrial function (Braydich-Stolle et al., 2005; Hussain et al., 2005; Schrand et al., 2008) and depletion of oxidative stress markers (Arora et al., 2008; Hussain et al., 2005). Furthermore, a recent study by Larese et al., (Larese et al., 2009) demonstrated absorption of silver nanoparticles in the *stratum corneum* and the outermost surface of the epidermis in both intact and damaged human skin.

A few *in vivo* studies in fish have shown some evidence of enhanced toxicity compared with bulk counterparts (i.e. larger particulate silver material >100 nm). Concentration dependent mortality and developmental effects, including spinal deformities and cardiac arrhythmia, have been shown in zebrafish (*Danio rerio*) embryos exposed to silver nanoparticles (5-20 nm), at a threshold concentration of 50 μg mL^{-1}. (Asharani et a., 2008; Yeo and Kang, 2008). In adult zebrafish, exposure to 20-30 nm silver nanoparticles has shown a greater toxicity compared with dissolved silver (Ag^+ from silver nitrate), based on mass of metal (Griffitt et al., 2009). In that work, more silver was associated with the gills in exposures to nanoparticles (26.6 ± 8.8 nm; 1000 μg L^{-1}) compared with fish exposed to silver ions and there was a greater thickening of the gill filaments in the silver nanoparticle exposed fish compared with controls.

The mechanism of toxicity of silver nanoparticles in fish has not been determined. An enhanced toxicity of nanoparticulate silver, compared to silver ions (Ag^+), may result from their shape and/or size, the release of silver ions (silver ions are well known to be toxic to aquatic organisms) (Mayer et al., 2003; Walker et al., 2008; Wood et al., 1996a); or a combination of both. Navarro et al., (2008), examined the rate of photosynthesis in *Chlamydomonas reinhardtii* exposed to silver nanoparticles or silver ions in both the presence and absence of cysteine (which binds free silver ions), and showed that silver nanoparticles were more toxic than silver ions, based on the concentration of ions present, requiring a higher concentration of cysteine to eliminate the toxicity. These findings suggest that interactions between the algae and the nanoparticles may enhance the release of silver ions, in turn suggesting that the nanoparticles acted as an effective delivery vehicle for silver ions.

Environmental concentrations of silver nanoparticles have not been determined, but estimates in natural waters range between 0.03 to 500 ng L^{-1} (Luoma, 2008). Sock fabrics are potentially a major source of silver nanoparticles to the aquatic environment; (Benn and Westerhoff, 2008) showed that washing of socks impregnated with

nanoparticulate silver resulted in the release of up to 1300 µg silver L^{-1}, some of it nanoparticulate. Many factors will likely affect the relative toxicity of nanoparticles for exposed organisms in natural waters, through effects on aggregation behaviour and thus their bioavailability, including pH and ionic concentrations, and interactions between nanoparticles and organic material and natural colloids (Baalousha et al., 2008; Handy et al., 2008b). Solubility is an important consideration in the toxicity of silver nanoparticles too, and factors affecting their solubility, such as presence of algae (Navarro et al., 2008), are also likely to influence this. It is not known how, or indeed if, the release of the silver ions is influenced by the size of the silver particle.

Previous work examining the toxicity of silver nanoparticles has provided conflicting evidence on the influence of size of particle on toxicity. A study by (Carlson et al., 2008) exposing alveolar macrophages to silver nanoparticles found that the generation of reactive oxygen species was size-dependent. Inoue et al (Inoue et al., 2009) also found that particle size was a determining factor in the level of lung inflammation in mice exposed to latex nanoparticles. However, in a series of pulmonary instillation studies in rats with nanoscale TiO_2 rods and dots, (Warheit et al., 2006) did not find that toxicity to the pulmonary system was dependent on either particle size or surface area. Contrasting with this, however, a further study by this research group (Warheit et al., 2007) found that the surface characteristics of quartz particles were responsible for the differences in pulmonary toxicity. There may, of course, be particle type specific effects. That is, nanoscale may increase the inherent toxicity of some materials, but not others.

The purposes of our study were two-fold. Firstly, to investigate the fate and effects of silver nanoparticles in rainbow trout exposed *via* the water column, at a range of concentrations which included concentrations with likely environmental relevance (Benn and Westerhoff 2008; Luoma 2008), and secondly, to investigate any differences in the fate and biological effects due to the size of the particle. We exposed juvenile rainbow trout to three nominal sizes of silver particle: 10 nm (N_{10}), 35 nm (N_{35}) and 600-1,600

nm (N_{Bulk}) and to silver nitrate for 10 days and measured the silver concentration in the gills, liver and kidney. Thiobarbituric acid reactive substances (TBARS) were measured in the blood plasma and in gill and liver tissue homogenates to ascertain whether lipid peroxidation had occurred as a result of exposure. Further, expression of a suite of genes related to metabolism and response to xenobiotics, specifically glucose-6-phosphate dehydrogenase (*g6pd*), glutathione peroxidase (*gpx*), cytochrome P450 1A2 (*cyp1a2*), cytochrome P450 3A45 (*cyp3a45*), and heat shock protein 70a (*hsp70a*) were analysed in the gills and liver as additional biological effect measures reporting on specific mechanisms of toxicity. Previous studies have shown the expression of these genes to be upregulated in rainbow trout (Lee and Buhler 2003; Råbergh et al., 2000; Walker et al., 2007; Williams et al., 1996) and brown trout (Hansen et al., 2006; Hansen et al., 2007) in response to exposure to various metals.

Materials and Methods

Rainbow Trout Husbandry

Juvenile female rainbow trout with a mean total body weight of 19.52 ± 0.56 g (mean ± S.E.) and fork-length of 12.39 ± 0.14 g (mean ± S.E.) were obtained from Hatchlands Trout Farm, Devon, UK. Fish were maintained in the laboratory in 500 L tanks supplied *via* a flow-through system with dechlorinated tap water (pH 7.79 ± 0.01 (S.E.); conductivity 189.58 µS ± 0.49 (S.E)) on a 12 hour light/12 hour dark cycle and were fed on pelleted feed (Emerald Fingerling 30, Skretting, UK), at a rate of 1% of their body weight prior to exposure. Water temperatures were maintained between 9 and 11°C throughout.

Materials and Particle Characterisation

All chemicals were purchased from Sigma-Aldrich, UK unless otherwise stated.

Silver particles (designated as N_{10}, N_{35} and N_{Bulk}) were purchased from Nanostructured and Amorphous Materials Inc., Houston, USA. Based on the manufacturers specifications, N_{10} (average particle size measured by transmission electron microscopy, TEM) silver particles were spherical particles with a specific surface area of 9-11 m^2 g^{-1}, bulk density of 2.05 g cm^{-3} and a true density of 10.5 g cm^{-3} and had a purity of 99.9% based on trace metal analysis. N_{35} silver particles of average size 35 nm (measured by TEM; max < 100 nm) had a specific surface area of 30-50 m^2 g^{-1}, bulk density of 0.30-0.60 g cm^{-3} and a true density of 10.5 g cm^{-3} and had a purity of 99.5% based on trace metal analysis. N_{Bulk} silver particles had an average particle size of 0.6-1.6 µm and purity of 99.95%.

Our own characterization of all the silver particles was carried out in HPLC-grade water or on powders. Particle sizes (hydrodynamic diameters), polydispersity index and zeta potential were measured on a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd. Malvern, UK) operating with a He-Ne laser at a wavelength of 633 nm using back scattered light. Our results are the means of triplicate runs, and in each run 5

measurements were made and standard errors were determined from the replicate measurements. Particle sizes and particle numbers were also measured using a NanoSight NTA LM10 with a laser output of 30mW at 650 nm. Mean square displacements of single particles were determined by tracking the scattered light followed by analysis by the Nanosight software, and standard deviations of the mean values were calculated. All measurements were carried out at nanoparticle concentrations of 1 mg L^{-1} after sonication for 30 minutes. The adsorption method was used to prepare samples for AFM (atomic force microscopy) analysis. In this method, mica sheets were cleaved on both sides, and then immersed vertically into the sample solution (10 mg L^{-1}) for 30 min (in the case of N_{Bulk}, both 30 min and 4 hrs were tested). Following adsorption, the mica sheets were withdrawn from the solution and gently rinsed by immersion in deionised water to remove non-adsorbed sample. All AFM images were obtained using a XE-100 AFM (Park Systems). All scans were performed in air, at room temperature and AFM height measurements were recorded. Images were acquired in a true non-contact mode and recorded in topography mode with a pixel size of 256 x 256 and a scan rate of 0.5-1.0 Hz. 80 particles were counted in case of N_{10}, 40 particles were counted in case of N_{35} and 10 particles in case of N_{Bulk}, and mean particle sizes including standard errors are reported. The low counts reflected the absence of particles on the mica. X-ray diffraction (XRD) was performed using a Bruker AXS D8 Autosampler. EVA software programme was used for the assignment of reflections and analysis of the XRD patterns. Crystallite sizes were evaluated using the Scherrer equation and mean standard error values are reported. A JEOL JSM-7000F Field Emission Scanning Electron Microscope was employed to characterise particle size and back scattered electron images were recorded. Air-drying of a small drop of suspension directly onto a Formvar/Carbon 300 mesh Ni grid was employed.

An FEI TECNAI F20 Field Emission gun (FEG) coupled with an x-ray Energy Dispersive Spectrometer (X-EDS) from Oxford Instruments (Oxfordshire, UK) was used for TEM particle size analysis. The TEM was operating at an accelerating voltage of 200 keV, a field emission gun at emission 3, gun lens 2-3 (apparent size) and extraction voltage of

3800-4400 eV and spot size 2-3. The aperture of the second condenser lens was nominally 50 µm and the objective aperture was nominally 40 µm. TEM micrographs were collected on a Gatan TV camera and Digital Micrograph software was used to measure particle size. TEM samples were prepared by ultracentrifugation of nanoparticle suspensions on a TEM grid at 30000 rpm (150000g) using a Beckman ultracentrifuge (L7-65 Ultrcentrifuge) with a swing out rotor SW40Ti as described in (Wilkinson et al., 1999).

BET (Brunauer, Emmett and Teller) surface areas (m^2 g^{-1}) were determined on a Coulter™ SA3100™ series surface area and pore size analyser. Samples were outgassed for 10 hrs at 200 °C prior to analysis. Results are the means of three measurements and the standard errors are reported.

Experimental Design

Eight fish were deployed into experimental glass tanks measuring 60 cm x 30 cm x 38 cm (L x W x H) with a total volume of 36 L, and aerated with 10 cm airstones. Seven treatment regimes were run in duplicate and these were as follows: 10 µg L^{-1} N_{10} silver particles (N_{10} Low), 100 µg L^{-1} N_{10} silver particles (N_{10}High), 10 µg L^{-1} N_{35}silver particles (N_{35}Low), 100 µg L^{-1} N_{35} silver particles (N_{35} High), 100 µg L^{-1} N_{Bulk} silver particles (N_{Bulk}), 0.1 µg L^{-1} silver nitrate ($AgNO_3$) and water control. Fish were exposed for 10 days and were not fed for the duration of the exposure.

Dosing stocks of the silver particles were made by suspending 360 mg of each particle in 1 L ultrapure water (Maxima ultrapure water, Elga) and sonicating for 30 minutes, and diluting as required. The silver nitrate dosing stock (360 µg L^{-1}) was also made up in ultrapure water and sonicated similarly. Experimental tanks were dosed 24 hours prior to addition of the fish, then drained and re-dosed immediately before adding the fish to minimize reduction of nominal dosing concentrations through adhesion of the particles/chemical to the glass and airstones. Water changes of 75% (27 L) and

corresponding re-dosing was carried out every 48 hours. Dosing stocks were sonicated for 30 minutes prior to each dosing.

Water samples (9 mL) were taken immediately after addition of the fish, at 1, 2, 4, 8, 12 and 24 hours, before and after every water change, and at the end of the exposure for analysis of silver concentration by ICP-OES. Water pH and conductivity were monitored daily and ranged between 7.58-8.54 and 179-212 µS, respectively.

Experimental Sampling

At the end of the exposure period (10 days), fish were euthanised with an overdose of benzocaine. One mL blood was taken from each fish and centrifuged at 12,000 x g for 5 minutes at 4°C and the plasma removed and stored at -20°C for analysis of thiobarbituric reactive substances (TBARS). Both gill arches were removed and divided as follows: approximately 50 mg was taken and flash-frozen in liquid nitrogen and stored at -80°C for RNA extraction and subsequent gene expression analysis; approximately 100 mg was taken and frozen in liquid nitrogen and stored at -20°C for analysis of TBARS and the remainder was weighed, frozen in 50 mL falcon tubes and stored at -20°C before preparation for measurement of silver content using inductively-coupled plasma optical emission spectroscopy (ICP-OES). Additionally, from one fish in each tank, a 4 mm section of gill tissue was taken and fixed in formalin for histopathological analysis. A further 4 mm section was taken from one fish from the N_{10} High treatment and one control fish and fixed in 2% paraformaldehyde/2.5% glutaraldehyde for TEM analysis. The liver was dissected out from each fish and a 50 mg sample taken and stored at -80°C for subsequent RNA extraction. In addition, a 100 mg section taken and stored at -20°C for TBARS analysis, and a 4 mm^3 sample taken and fixed in formalin for histological processing and tissue effects analysis. The remaining liver tissue was weighed, frozen and stored at -20°C prior to preparation and analysis for silver content

by ICP-OES. Kidneys were dissected out, weighed, frozen and stored at -20°C for analysis silver content via ICP-OES.

Determination of Silver Concentrations in Fish Tissues

Tissue and blood samples were defrosted and digested at room temperature with a combination of 4 mL concentrated HNO_3 (AR grade, Fisher Scientific) and 1 mL hydrogen peroxide (Laboratory reagent grade, Fisher Scientific) for 24 hours before being heated for 24 hours on a Gerhardt Kjeldatherm digester unit at 125°C. The temperature was then increased to 190°C to evaporate the nitric acid and the samples subsequently re-dissolved in 10 mL 10% HNO_3 and 200 µL of 10% Triton-X 100. The samples were then analysed on a Vista-MPX CCD Simultaneous ICP-OES. Water samples were prepared for analysis by the addition of 1 mL HNO_3 and 200 µL 10% Triton-X 100. Calibration standards containing 500 µg L^{-1} and 1000 µg L^{-1} and a quality control standard containing 400 µg L^{-1} silver were made up from a stock solution of 157.5 mg L^{-1} silver nitrate in 10% HNO_3. Internal standards of 1, 10, 100, 500 and 1000 µg L^{-1} were made using all silver particle types suspended in 10% HNO_3 dispersed with 10% Triton-X 100 and used according to the exposure regime samples being measured, i.e. N_{10} internal standards for N_{10}exposure samples. All samples and standards were sonicated for 30 minutes prior to measurement. The detection limit of the ICP-OES for silver in the tissue and water samples was 10 µg L^{-1}.

Histological analyses

Trout liver and gill tissues were fixed in 10% neutral buffered formalin for 6 hours before being transferred to 70% industrial methylated spirits (IMS). The tissue samples were dehydrated in series in IMS (AR grade Fisher Scientific) in a Shandon Hypercenter XP Tissue Processor and embedded in paraffin wax. The samples were sectioned on a rotary microtome (Leica). Five microscope slides, with 5-6 sections per slide, were prepared for each tissue sample) and the slides were stained with haematoxylin and eosin in a Shandon automatic slide stainer (Thermo Scientific, UK). Tissues were examined and

assessed for any signs of histopathological changes using a Zeiss Axioskop 40 light microscope with an Olympus DP70 Digital Microscope Camera and AnalySIS Image Processing Software and examined for evidence of gill or liver injury.

Gill tissues were fixed for Transmission Electron Microscopy (TEM) adopting an in-house method. Briefly, tissues were fixed for 2hrs in 2% paraformaldehyde/2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4), washed three times in 0.1M phosphate buffer for 5 mins and fixed in 1% osmium tetroxide in phosphate buffer for 1 hour. The tissues were then washed in deionised water for 5 x 5 minutes, before being cut into 1 mm^3 pieces and subsequently suspended in 2% uranyl acetate for 1 hour. The tissues were then dehydrated in an ethanol series: 30%, 50%, 70%, 90% and 100% (x2), in each for 10 minutes and embedded in TAAB resin. The tissues were blocked in shallow planchets and placed in a 60°C oven for 20 hours. Tissues were sectioned on an ultramicrotome (Ultracut, Reichert) and examined for structural alterations and subcellular localization of silver using a Joel TEM 1400 transmission electron microscope.

Measurement of Lipid Peroxidation

Measurement of 1,1,3,3-tetramethoxypropane (malonaldehyde; MDA) using the TBARS assay is widely used as an indicator of lipid peroxidation. The protocol adopted was similar to that described previously (Conner *et al.*, 2006). Briefly, 8 standards of MDA at concentrations ranging from 0.625 µM to 100 µM were prepared from a 500 µM stock solution of MDA in 2% ethanol. Tissue samples were homogenized with a hand-held tissue homogenizer with 250 µL 100 mM HEPES. The samples were then centrifuged at 1600 x g for 10 minutes at 4°C and the supernatant reserved. 100µL of tissue homogenate/plasma and standards were mixed with 500 µL of 0.4% thiobarbituric acid (40 mg in 10 mL) in 10% acetic acid, pH 5.0.. The tubes were heated to 90°C for 1 hour and then cooled to room temperature under tap water. 600 µL of butanol was added and the mixture was then vortexed and then centrifuged at 3,100 x g for 10 min. The butanol phase was removed and 150 µL placed in duplicate in a flat-bottomed, 96-well microtiter plate and the absorbance measured at 532 nm on a Molecular Devices

SpectraMax 340pc microtiter plate reader. A standard curve was prepared and MDA concentrations were determined accordingly.

Gene expression analyses

Total RNA was extracted from each tissue sample using Tri Reagent (Sigma) following the manufacturer's instructions. Total RNA concentrations were determined by measuring the absorbance at 260 nm using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). The RNA was treated with DNase (Cat. No. M6101, Promega) and subsequently reverse-transcribed to cDNA using M-MLV Reverse transcriptase (Cat. No. M1701, Promega) and random hexamer primers as previously described (Filby and Tyler, 2005).

Real-time PCR assays were developed for the quantification of a selection of genes involved in the response to xenobiotics and their metabolism in rainbow trout. Primers specific for rainbow trout glucose-6-phosphate dehydrogenase (*g6pd*; Genbank Accession no. EF551311: 5′-CGGTTGTCTGTGTTCTTC-3′/5′-GGTGCTTGATGTTCTTGG-3′), glutathione peroxidase (*gpx*; Genbank Accession no. AF281338: 5′-GCTCCATTCGCAGTATTC-3′/5′-TCCTTCCCATTCACATCC-3′), cytochrome P450 1A2 (*cyp1a2*; Genbank Accession no. U62797: 5′-CTTCCGCCATATTGTCGTATC-3′/5′-CCACCACCTGCCCAAAC-3′), cytochrome P450 3A45 (*cyp3a45*; Genbank Accession no. AF267126: 5′-GTCCTCTCACCTTCCTTTACC-3′/5′-TCTGCCTGCTTCTTCATTCC-3′), heat shock protein 70a (*hsp70a*; Genbank Accession no. NM_001124228: 5′-CTGCTGCTGCTGGATGTG-3′/5′-GCTGGTTGTCGGAGTAAGTG-3′) were designed for use in real-time PCR with Beacon Designer 7.2 software (Premier Biosoft International, Palo Alto, CA, USA) according to manufacturer's guidelines and purchased from MWG Biotech.

Assays were optimized and validated for real-time PCR using SYBR Green chemistry as described previously (Filby and Tyler, 2005).

Assays had detection ranges of at least three orders of magnitude. Specificity of primer sets throughout this range of detection was confirmed by the observation of single amplification products of the expected size and melting temperature. All assays were quantitative with standard curve (mean threshold cycle [Ct] vs. log cDNA dilution) slopes of -2.754 (*g6pd*), -3.367 (*gpx*), -2.901 (*cyp1a2*), -2.722 (*cyp3a45*), -3.538 (*hsp70a*), translating to high PCR efficiencies (E) of 2.31 (*g6pd*), 1.98 (*gpx*), 2.21 (*cyp1a2*), 2.33 (*cyp3a45*), 1.92 (*hsp70a*). Over the detection range, the linear correlation (R^2) between the mean Ct and the logarithm of the cDNA dilution was >0.98 in each case.

Gene expression was measured for each gene in liver and gill samples using SYBR Green chemistry with the iCycler iQ Real-time Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) according to the protocols described previously (Filby and Tyler, 2005).

For each gene, samples were analyzed in triplicate. Relative expression levels were calculated using an efficiency-corrected version of the arithmetic comparative $2^{-\Delta\Delta Ct}$ method, as described previously (Filby and Tyler, 2005). The housekeeping gene ribosomal protein L8 (*rpl8*) was also measured in each sample and was used for relative quantification because its expression did not change for the tissues analyzed following any of the treatments.

Statistical analyses

Data are expressed as mean values ± standard error of the mean (S.E.) and statistical analyses were performed using SPSS version 16.0, with the significance value set at 0.05. All data were checked for conformity with the required assumptions for parametric tests (normality of error and homogeneity of variance). When these assumptions were not met, data were either transformed to meet these assumptions or equivalent non-parametric tests were conducted (see relevant sections of the results for further details).

Results

Particle characterisation

Particles with zeta potentials more positive than +30mV or more negative than -30mV are normally considered stable (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948) in the absence of steric stabilisation. If the particles have a higher density than the solution they will eventually sediment. Zeta potential measurements of all silver particle types in HPLC-grade water suggested that the particles had a high propensity for aggregation (Table 1.) which was confirmed by hydrodynamic diameter measurements of the particles. The N_{10}silver particles had the most negative zeta potential value (-12.5 mV), on average formed the smallest aggregate sizes in water (ca. 590 nm, measured by DLS) and had the lowest polydispersity index suggesting that aggregate sizes were more uniform compared to the two other particle types. The N_{35} silver formed the largest aggregates in water (ca. 2030 nm) and had a high polydispersity index suggesting that both larger and smaller aggregate sizes were present. N_{Bulk} silver had an average aggregate size of 940 nm and a polydispersity index of 0.69 suggesting aggregates were of a more uniform size than for the N_{35}silver particles. Aggregation, followed by sedimentation, would have removed much of the material from the water column limiting potential uptake of the NPs. Furthermore, it might be expected that diffusive uptake into gills would be limited from this aggregation.

Particle sizes determined from Nanoparticle Tracking Analysis (NTA) are lower compared to the values obtained from DLS technique (Table 1), as previously found (Domingos et al., 2009), possibly due to the bias towards large aggregates in DLS. The N_{35} and N_{10} silver particles show similar hydrodynamic diameters from NTA analysis. However particle number concentrations differ significantly: 1.07 x 10^8 for the latter and 0.21 x 10^8 for the former. The higher particle number concentration for the N_{10} silver partly explains the higher tendency for aggregation, as is also observed on back scattered electron images. It is also clearly seen that the number of smaller particles is higher for the N_{10} particles compared to the N_{35} giving good agreement with the TEM data (Fig. 1.

A-C and Table 1.). In the case of N_{Bulk} silver, single particles between 1 and 2 μm could be identified alongside smaller particle sizes.

Ag crystallite sizes were calculated using the Scherrer equation from XRD spectra. As expected and in agreement with the Bragg peak broadening of the XRD patterns (Fig. 1. D), the N_{10} silver showed the smallest crystallite size (21 nm), indicated by the peak broadening in the XRD data, not seen for the larger sized particles. However, N_{35} silver and N_{Bulk} silver showed similar crystallite sizes (68 nm and 60 nm, respectively) suggesting that the N_{35} silver contains mainly large particles similar to the N_{Bulk} particles (also observed by TEM imaging, Fig. 1. E-G). XRD analysis confirmed cubic crystal system (face-centred lattice) in case of all the three silver samples.

Semi-quantitative AFM analysis showed the presence of small particles with approximate sizes of 46 nm in the case of N_{10} silver, giving very good agreement with TEM data (Table 1). However, single large particles/aggregates between 140-190 nm were also identified in agreement with NTA data. In the case of N_{35} silver, particles with approximate sizes of 90 nm were identified together with larger particles/aggregates between 130-140 nm, giving good agreement with both TEM and NTA data. Smaller particles of approximate size 10 nm were also observed, again confirming the sample polydispersity and giving good agreement with the NTA data. In the case of N_{Bulk} silver (30 min adsorption time) only smaller particles of an approximate size of 18 nm were observed, suggesting that larger particles were not sticking to the mica slide support due to low diffusivities and low adhesion to the mica. However, the bulk particle sample clearly contained very small (nano)particles, although in much lower concentrations than the N_{10} and N_{35} samples. In addition, the 4 hrs adsorption time employed for N_{Bulk} silver identified particles with approximate sizes of 147 nm, but very small particles (~18 nm) were also observed in this sample.

The BET method was used for determining Ag specific surface areas. The measured BET surface area values are lower than those supplied by manufacturer (Table 1), but in agreement with all the measured size data. The low values observed are consistent with both the larger particles observed and the possibility of reduced specific surface areas as NPs become densely packed and aggregated during outgassing. As expected, the smallest surface area (0.6 m^2 g^{-1}) was obtained for the N_{Bulk}, while N_{10} and N_{35} showed similar specific surface areas (2.0 m^2 g^{-1} and 2.9 m^2 g^{-1}, respectively). Synthesis of all data indicates that the N_{10} particles were clearly smaller than the N_{35} particles and these were smaller than the N_{Bulk} particles. Nevertheless, there was considerable sample polydispersity.

TEM analysis suggests that the N_{10} and N_{35} particles are larger than reported by the manufacturer with an average diameter of 49±18.5 and 114±65.3 nm, respectively, whereas the N_{Bulk} particles are smaller than reported by the manufacturer with an average diameter of 137.4±62.2 nm (Table 1). The N_{10} sample contained only particles < 100 nm, whereas the N_{35} and N_{Bulk} samples contained a high proportion of large particles with 47% and 75.6%, respectively, being larger than 100 nm. Additionally, the N_{10} sample contained a high proportion (51%) of particles < 50 nm, whereas the N_{35} and N_{Bulk} samples contained a very small fraction (11.6% and 1.4% respectively) of these particles. TEM analyses indicate that the N_{10} sample was different from the other two samples, which are very similar in terms of their size as determined by TEM .

Exposure Media and Tissue Silver Content

Trace metal analysis of water samples taken from the exposure tanks by ICP-OES showed the measured silver concentrations in the water were considerably lower for all exposure conditions except for the N_{35} Low concentration treatment. Silver concentrations in the N_{10} Low and $AgNO_3$ tanks were below the detection limit of the ICP-OES machine for all samples. For the N_{10} High, N_{35} Low, N_{35} High and N_{Bulk} treatments, the mean silver concentrations in the exposure media were 35.5 µg L^{-1} ± 2.44 (S.E.),

9.4 µg L^{-1} ± 4.62 (S.E.), 35.3 µg L^{-1} ± 2.64 (S.E.) and 46.6 µg L^{-1} ± 4.73 (S.E.) respectively.

Trace metal analysis of the trout tissues determined by ICP-OES showed that there was significantly enhanced level of silver in/on the gills of fish in all treatments compared with controls, except for the $AgNO_3$ exposure, where the $AgNO_3$ dose to the tanks was between 100 and 1000 fold lower than the ENP concentrations (Fig. 2.). The highest concentration of silver was observed in the gills of fish in the N_{10} High treatment group with an average of 0.61 ± 0.07 µg g^{-1} tissue (mean ± S.E.) and this was significantly higher (Mann-Whitney with Holm's Sequential Bonferroni Adjustment $U(4) = 37.00$, $Z = -3.43$, $p < 0.001$) compared with in the gills of fish from the N_{10} Low treatment, where the concentrations were approximately 50% lower. Similar mean silver concentrations were observed in the gills of fish in the N_{35} Low (0.26 µg g^{-1} tissue ± 0.06 µg g^{-1}), N_{35} High (0.25 µg g^{-1} tissue ± 0.08 µg g^{-1}) and the N_{Bulk} treatments (0.32 µg g^{-1} tissue ± 0.21 µg g^{-1}).

Analysis of the liver revealed silver uptake in all four treatment groups: N_{10} High, N_{35} Low, N_{35} High and N_{Bulk}. The highest uptake occurred in the liver of fish exposed to N_{Bulk} silver particles at 1.63 ± 0.18 µg/g tissue, compared with 1.50 ± 0.30 µg/g tissue in the N_{10} High, and 0.92 ± 0.16 µg/g tissue in the N_{35} High treatment groups. The uptake of N_{Bulk} particles into the liver was significantly higher than the uptake for the N_{35} High treatment group (Mann-Whitney with Holm's Sequential Bonferroni Adjustment $U(3) = 64.00$, $Z = -2.414$, $p = 0.015$). There was hepatic uptake of N_{35} particles in the low exposure treatment, but this occurred in one fish only. No uptake was detected in the liver of fish exposed to $AgNO_3$ or the N_{10} Low treatment groups. Where uptake of silver into the liver occurred, the overall silver content was more than double the levels in the gill tissue in those fish. Levels of silver in the kidneys of exposed fish were below the detection limit for all treatments.

Lipid Peroxidation

Oxidative stress in the cellular environment may result in the oxidation of polyunsaturated fatty acids. This results in the formation of unstable lipid hydroperoxides which in turn decompose to form malonaldehyde (MDA). MDA reacts with thiobarbituric acid to form a coloured product which can be measured spectrophotometrically and is thus a commonly used biomarker for lipid peroxidation (Ohkawa et al., 1979; Rand, 1995).

Analysis of TBARS showed no evidence of lipid peroxidation in the gills of fish from any of the treatments, except for the N_{10} High treatment (that contained the highest gill silver content), with significantly lower levels of measured MDA compared with controls (ANOVA with Tukey's post-hoc test; df=6,104; F=2.629; p=0.021) (Fig. 3.). In the liver tissue homogenates, similarly there was no evidence of lipid peroxidation, except for MDA concentrations in the livers of the fish from the silver nitrate treatment which were significantly lower compared with those in the controls (ANOVA with Tukey's post-hoc test; df=6,105; F=3.993; p=0.001). Examination of TBARS in the blood plasma showed no evidence of an elevation in MDA concentration for any of the particle treatment groups. There was, however, evidence of lipid peroxidation in the plasma of fish from the silver nitrate treatment, where MDA concentrations were significantly higher than controls, and the N_{10} Low and N_{35} Low treatment groups (ANOVA with Tukey's post-hoc test; df=6,90; F=3.991; p=0.001).

Histology

Histological examination of gill tissue showed no evidence of physical damage or changes to gill filaments in treatments to silver for any of the particle sizes. Gills of fish exposed to $AgNO_3$, however, showed slight damage with deformation of the secondary lamellae observed (Fig. 4.). There was no evidence of tissue damage in the liver for any of the silver treatments compared with controls (not shown).

TEM Analysis

Micrographs of gill tissue from N_{10}High treatment show electron dense areas, which correspond to particles associated with the gill tissue. The particles are highly aggregated in clusters of approximately 600 nm, corresponding well with the hydrodynamic diameter size as measured by dynamic light scattering (ca. 590 nm) and appear to be associating with the pavement cells at the gill surface (Fig. 5.). There was no confirmation of the presence of silver in the gill tissue by EDX analysis (data not shown). However, Ag content of the tissue must be ~0.1% or greater in order to be detected using this method.

Quantitative Real-Time PCR

Analysis of the expression of five genes, that have been shown previously to be induced in rainbow trout gills and liver as a result of exposure to various metal ions, showed that their expression was unchanged as a result of exposure to silver particles, with the exception for *cyp1a2*, where there was a 3-fold induction in the gills of fish exposed to the high concentration of N_{10} particles (ANOVA: df=6,48; F=2.691; p=0.025) (Fig. 6.).

Discussion

Very few studies to date have investigated the effects of exposure to silver nanoparticles in fish and these have been focused on zebrafish embryos where mortality, notochord and heart abnormalities have all been described (Asharani et al., 2008; Lee et al., 2007; Yeo and Kang, 2008). Similarly, there is little in the literature regarding the uptake and biodistribution of silver nanoparticles into internal organs or the potential toxicity of silver nanoparticle in intact free swimming fish. Furthermore, there have been no studies in fish examining the differential uptake and toxicity of nano-sized versus bulk-sized silver particles. The main aims of this study were, therefore, to identify the target organs for silver particles of different sizes as a result of *in vivo* exposure and uptake from the water in fish, using rainbow trout as a study species, and to compare the patterns of biodistribution and toxicological effects seen for two different sized silver nanoparticles (nominally 10 nm and 35 nm) and bulk silver particles (nominally 600-1600 nm).

There was clearly a substantial difference between the manufacturer's information on material properties and those seen by our own investigations. This discrepancy has been observed by many workers and leads us to the strong recommendation that, as a minimum, commercially obtained NPs need to be fully characterised before any toxicology or other experiments are performed. The reasons for the differences are most likely to be a) incorrect manufacturer's information, possibly due to limited and/or inappropriate measurement or batch to batch variation or b) changes in material properties between synthesis and initial characterisation and use. The characterisation performed here in the silver particles is internally consistent, showing all three particles are polydisperse, with the NPs generally larger than their nominal values and existing, at least partially, as nanostructured aggregates. The larger particles were generally better characterised by the manufacturer, but it is noticeable that there were also some small particles present in those samples. Thus, we would expect that all samples would have low (mass) bioavailability and not to behave as well dispersed 10 or 35 nm particles.

Nevertheless, the NPs are likely to be representative of commercially available NPs in current commercial products. For all the measurements of silver concentration in the exposure media, except for in the N_{35} Low treatment group, the actual measurements were as much as 65% lower than the nominal dosing concentrations, even immediately after dosing. The silver concentration in the tank water were highest, as expected after the (re-)dosing of the tanks during the water changes, and then they gradually and progressively decreased over time until the next (re-)dosing event (data not shown). These data suggest that aggregation and sedimentation of the particles in the exposure media resulted in a lower silver NP concentration in the water column (and to which the fish were being exposed) compared with the nominal dosing concentration. The level of silver in the gills of rainbow trout exposed to the high dose of N_{10} silver particles was significantly higher than the uptake for all other particle sizes. Characterization of the silver particles by TEM and XRD, show that the N_{35} and N_{Bulk} silver particles were similar in terms of particle size measurements, whereas the N_{10} silver particles were markedly smaller suggesting that smaller particles show a greater propensity to associate with/enter into gill tissue than larger particles. TEM images on gill tissue from trout exposed to N_{10} particles showed the presence of silver particles. However, from the imaging alone, we cannot be definitive as to whether the particles detected were on the surface of the pavement cells or were incorporated into them. EDX analysis did not detect silver in the gills, likely because concentrations were not sufficiently high to be able to do so, but their presence was supported by the ICP-OES measurements (<1 μg g^{-1}). The fact that the liver content of silver was twice that of the gills would suggest transport across the gills (see also later discussion). Assuming that there is 1% solubility of Ag in the exposure media (Fabrega et al., 2009; Navarro et al., 2008), uptake of silver in gills of fish exposed to the low dose of Ag NPs, at least, cannot be explained by NP dissolution and uptake of dissolved Ag, further indicating that some uptake of Ag NPs must have occurred.

Despite the association/uptake of silver with/into the gill tissue, there was no obvious damage to the gill filaments as a result of the exposures to silver particles, although some low level damage was noted in the gills of fish exposed to $AgNO_3$. Griffitt et al. (Griffitt et al., 2009) similarly found that more silver associated with the gills of zebrafish exposed to silver nanoparticles compared with fish exposed to soluble silver ($AgNO_3$). In that study, significant thickening of the gill lamellae was observed in fish exposed to $AgNO_3$ whereas concentrations of 1000 µg 27 nm silver particles L^{-1} did not elicit any changes in gill lamellar thickness (for a 48h exposure). The effective doses of silver particles for inducing gill lamellar disruptions in the study by Griffitt et al. were, however, between 10 - and 100-fold higher than in our study, and this likely explains the differences in the effects observed.

The levels of silver present in the liver of fish exposed to the high concentrations of all the particle sizes were approximately twice the concentrations in the gills per gram of tissue, suggesting that transportation of silver occurs within the blood from the sites of uptake. Contrasting with findings in the gills (where the amount of silver measured was highest for the 10 nm high exposure regime), both N_{10} silver and N_{Bulk} silver particles showed the highest accumulation in the liver tissue. It is difficult to equate the tissue levels of N_{10} silver or N_{Bulk} silver in the liver with those in the gills, indicating a possible alternative uptake route. Indeed, it may be the case that uptake of N_{Bulk} silver (and nano silver also) was not principally *via* the gills, but rather via the gut as a function of drinking and/or feeding on aggregated material (the fish were not fed for the exposure period). The feeding on aggregated materials that accumulate on the bottom of the tank has reported previously for aqueous exposures to other nanomaterials (Johnston et al., 2010).

Although there was a two-fold difference in uptake of N_{10} Ag particles in the gills between the low and the high dose exposures, in none of the gill samples where uptake occurred, was the concentration of Ag in the gill 10 times higher for the high dose than for the low dose. This might be explained by lowered bioavailability of Ag ENPs in the

water column as a result of increased propensity for particle aggregation at higher nanoparticle concentrations as has shown in previous studies (Johnston et al., 2010). The difference in uptake of N_{35} Ag in the liver of fish between the high and low dose exposures is close to 10-fold however, further suggesting differences in route of uptake of Ag NPs between gill and liver tissue, and that uptake into the liver as a result of fish feeding on Ag NP aggregates from the tank floor may be an important consideration.

The mechanism by which electrolytes, metal ions and organic molecules are taken up across epithelial cell layers is well described (Handy and Eddy, 2004). *In vivo*, both the gill and gut surfaces are surrounded by aqueous media which contributes to an unstirred aqueous layer above the epithelium. This layer can exchange these molecules with the mucus layer covering the epithelium, which in turn can present the molecules to the apical surface of the epithelium where uptake may occur. It is not known, however, how well this model can be applied to nanoparticles and their uptake or how the physicochemical characteristics of the particles such as size, shape and surface charge and the interaction of particles with the aqueous media might affect uptake. Furthermore, it is not known how this may influence release of silver ions from the particles. Our results suggest, however, that distinct differences may exist between the interaction of different sized silver particles with epithelial membranes and also with the interaction of silver particles with different epithelial surfaces, i.e. the gut and gill. As Navarro et al. (2008) showed in their work with *C. reinhardtii* interaction with particles with biological membranes enhanced the release of Ag^+ from the particles, however whether or not this phenomena is also observed in interactions between silver particles and epithelial membranes in fish is not yet known.

Levels of MDA were shown to be significantly increased only in the plasma samples of fish exposed to $AgNO_3$, suggesting that some ionic silver was entering the blood stream via the gills and/or the gut epithelium and causing lipid peroxidation. Levels of MDA in the livers of these fish, however, were significantly lower than in control fish, and this

was in accordance with a lack of evidence of silver uptake into the livers for the $AgNO_3$ exposure. The apparent reduction of lipid peroxidation in the gills of fish exposed to the high concentration of N_{10} silver, despite a large accumulation of silver in the gills, is a surprising finding and is in marked contrast with several studies which demonstrate the potential for silver nanoparticles to generate reactive oxygen species (ROS) and cause oxidative stress. Rahman et al., (2009) found 25 nm silver particles induced the expression of oxidative stress-related genes in the mouse brain after intravenous injection 100 mg kg^{-1}, 500 mg kg^{-1} and 1000 mg kg^{-1} and *in vitro* studies have shown that silver nanoparticles have the capacity to generate ROS (Carlson et al., 2008; Hsin et al., 2008) and cause increased lipid peroxidation (Arora et al., 2008). Both liver and gill tissues have the ability to upregulate survival genes and DNA repair mechanisms (Diehl, 2000; Hansen et al., 1996) when an organism is exposed to environmental stressors which may also explain the reduced lipid peroxidation seen in response to $AgNO_3$ in the liver and to the high concentration of 10 nm Ag particles in the gills. However, if the solubility of Ag in water is taken to be 1% (Fabrega et al., 2009; Navarro et al., 2008) we would expect to see similar responses in the blood plasma of fish exposed to low concentrations of Ag NPs as in the exposure to $AgNO_3$. The absence of such a response may suggest that differences in dissolution rates exist between different preparations of Ag NPs.

It has been suggested that the mucus layer surrounding the gill epithelia may act as a barrier preventing nanoparticle uptake by the gills (Handy et al., 2008a) and a study by Smith et al. (Smith et al., 2007) demonstrated that carbon nanotubes readily associate and became trapped within mucus on the gill surface. If the silver nanoparticles are similarly associated with the mucus on the gills for aqueous exposures, reducing /preventing penetration into the gill epithelia, this would explain the lack of any lipid peroxidation in our exposures. Interestingly, Derksen et al. (Derksen et al., 1998) demonstrated under normal oxygen-level conditions (dissolved oxygen = 9.5 mg L^{-1}), that particulate matter associated with the gills was effectively cleared from the gills of

rainbow trout within 40 hours, however both elevated and reduced oxygen levels (25 mg L^{-1} and 4.5 mg L^{-1} respectively) caused significantly reduced clearance of particulate matter, in resulting altered behaviour normally associated with respiratory stress. It is quite possible therefore, that fish exposed to nanoparticles in environments with low oxygen concentrations may be more vulnerable to vesicular uptake or oxidative stress in the gill tissue.

The suite of five genes analysed by rtPCR were chosen as genes representing a range of toxicity mechanisms (principally for heavy metals) in living cells. The Cytochrome P450 monooxygenase system plays an important role in the detoxification of both endogenous and exogenous chemicals in animals and in fish they have often been used as biomarkers for environmental contamination (Råbergh et al., 2000). Both *cyp1a2* and *cyp3a45* have been shown to have roles in the oxidative metabolism of exogenous compounds in rainbow trout (Lee and Buhler, 2003; Råbergh et al., 2000). The expression of heat shock proteins is associated with a general shock response which is universally conserved throughout the animal kingdom. Measurement of heat shock protein induction, in particular *hsp70*, has been proposed as a useful technique in toxicological screening and environmental monitoring as a variety of stressors including, heavy metals, teratogens, anoxia and heat have been shown to induce synthesis. Williams et al. (Williams et al., 1996) showed that accumulation of HSP70 in the gills of juvenile rainbow trout occurred as a result of exposure to cadmium, copper, lead and zinc *via* the water and *via* the diet. Both glucose-6-phosphate dehydrogenase (G6PD) and the glutathione peroxidase (GPX) enzyme family play significant roles in protecting cells from oxidative damage and measurement of their activity is commonly used as markers for oxidative stress. Both genes have multiple metal response elements in their 5' flanking region which has been proposed as the reason for their responsiveness to metals (Walker et al., 2007). Exposure of brown trout (*Salmo trutta*) to both cadmium and copper *via* the water have been shown to induce expression of *gpx* mRNA (Hansen et al., 2007; Hansen et al., 2006) and both *g6pd* and *gpx* have been shown to be

responsive to zinc in the gills of rainbow trout (Walker et al., 2007). Thus, the genes selected for study represented a wide range of toxicological effect pathways. Interestingly, only the expression of *cyp1a2* was found to be significantly altered and this only occurred in the gills of fish exposed to the high concentration of 10 nm silver particles, correlating with the highest level of accumulation of silver in the gills in this treatment group. (Griffitt et al., 2009), adopting a transcriptomic approach, found none of our targeted genes were differentially expressed in the gills of zebrafish exposed to silver nanoparticles (26 nm, 1000 µg L^{-1}). A study by (Rahman et al., 2009) found that exposure of rats to silver nanoparticles caused significant down-regulation of *Gpx2* in the brain frontal cortex and up-regulation of *Gpx3* in the caudate nucleus. The doses of silver nanoparticles eliciting these responses, however, were between 500 and 1000 mg kg^{-1} and are approximately 1000-fold higher than the maximum concentration of nanoparticles adopted in our experiment.

Silver ions have been shown to be toxic to fish, inhibiting carbonic anhydrase activity leading to a net loss of Na^+ and Cl^- across the gills and inhibiting Na^+/K^+ATPase activity (Morgan et al., 1997). The toxic effects of silver ions are due to their interaction at the gill surface and not as a result of internal silver accumulation (Wood et al., 1996b). We, therefore, might have expected exposure to $AgNO_3$ to alter the expression of some of these genes in the gills. ICP-OES employed in this study, cannot distinguish between different valency states of metals. Although previous studies have established that Ag solubility in water is ~1% (Fabrega et al., 2009; Navarro et al., 2008) in our experiment, it is not known what proportion (if any) of the silver present in the Ag NP exposure media was in the form of Ag^+. Furthermore, it is not known in what form silver was associated with the gills or transported to the liver. The lack of any effect, however, is likely explained by the low exposure concentrations adopted (chosen to reflect a 10-fold lower concentration than the accepted 96h LC50 value (Wood et al., 1996a) for rainbow trout) and effective repair mechanisms in these tissues.

The known toxicity of silver ions has led to the proposal in a number of studies that release of silver ions (Ag^+) from silver nanoparticles could be in part responsible for toxic responses seen in exposures to silver nanoparticles (eg Navarro et al., 2008). This idea however, conflicts with the known stability of zero-valent silver in water. (Griffitt et al., 2009) conducted concurrent exposures of zebrafish to silver nanoparticles and Ag^+ ($AgNO_3$ at concentrations that matched the amount of silver ions released by the nanoparticles. Dissolved silver from nanoparticles was measured and found to be 0.07% of the silver nanoparticle mass. The method used by Griffitt and colleagues however, does not specifically elucidate whether the silver present was ionic or very small zero-valent silver nanoparticles. This may explain why gill damage was observed in Ag^+ exposures but not in silver nanoparticle exposures. Also the gill global gene expression patterns between these exposures differed markedly from each other, suggesting toxicity mechanisms differed between treatments.

Conclusion

Our results show that smaller nanoparticle sizes have a greater propensity to associate with the gills of rainbow trout, but that the mucus layer on the gills may be an effective barrier to entry of the nanoparticles into the gill cells. However, induction of *cyp1a2* in the gills of fish exposed to high concentrations of N_{10} silver particles may be indicative of oxidative metabolism in response to the exposure. At this time, it is difficult to speculate on the mechanism of action of *cyp1a2* in the gills as it is unknown whether the effects seen are as a result of exposure to the silver nanoparticles, silver ions or a combination of both. We show evidence of lipid peroxidation in the plasma of fish exposed to $AgNO_3$ however, there was an apparent decreased lipid peroxidation in the gills of fish exposed to high concentrations of N_{10} silver particles. Uptake of silver particles was demonstrated in the liver but with no differences in uptake relative to particle size. Liver burden of silver was approximately twice that seen in gills suggesting that the gut epithelium may be an important route of exposure for silver particles to fish. These results suggest that

both the size of the nanoparticle and the type of epithelium where nanoparticles are presented affect their uptake. The importance of Ag^+ as the mechanism of silver nanoparticle toxicity needs further investigation and accurate measurements of the proportions of zero-valent silver and Ag^+ (and Ag^+ sorbed to NP surfaces) for the different sized nanoparticles would be needed to do this. Care should also be taken when defining dissolved silver. Ag^+ in solution and stable suspensions of zero-valent silver nanoparticles are distinctly different, but have both been described in the literature as solutions. Our findings also revealed that for all three particle types, size and surface area measurements differed considerably to the information given by the manufacturers, emphasising the need for rigorous characterization of particles to ascertain the nature of the particles to which the test organisms are exposed. Our results show that exposure of silver nanoparticles to rainbow trout at concentrations close to current estimations of environmental levels can result in accumulation of silver in the gills and liver of fish and can affect likely oxidative metabolism in the gills.

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List of Figures

N_{35} silver particles, (E) 100 µg L^{-1} N_{Bulk} silver particles. Arrows indicate damage to secondary lamellae.

Figure 5. TEM images of gill tissue dissected from rainbow trout after waterborne exposure to 100 µg L^{-1} N_{10} silver particles for 10 days. Images B and C are higher magnification images of aggregates in image A.

Figure 6. Expression of *cyp1a2*, *cyp3a45*, *hsp70a*, *g6pd* and *gpx* in gills and liver as determined by real-time PCR. Results are represented as means ± S.E. expressed as fold-increase in relative mRNA expression (gene of interest: *rpl8*). Experimental groups consisted of eight fish and each fish was analyzed in triplicate. Statistically significant differences in the expression levels (compared to controls) are denoted by * (ANOVA $p<0.025$).

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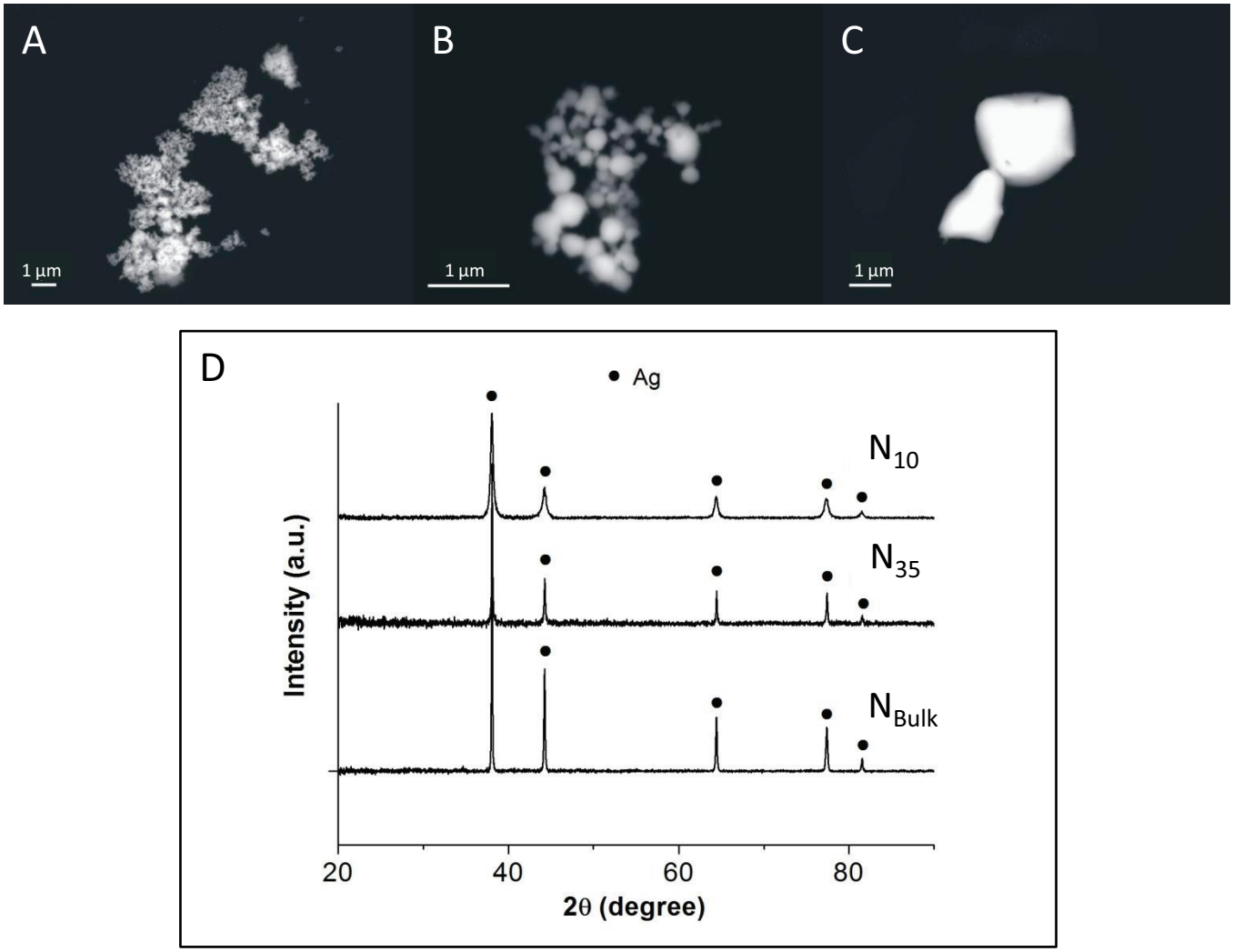
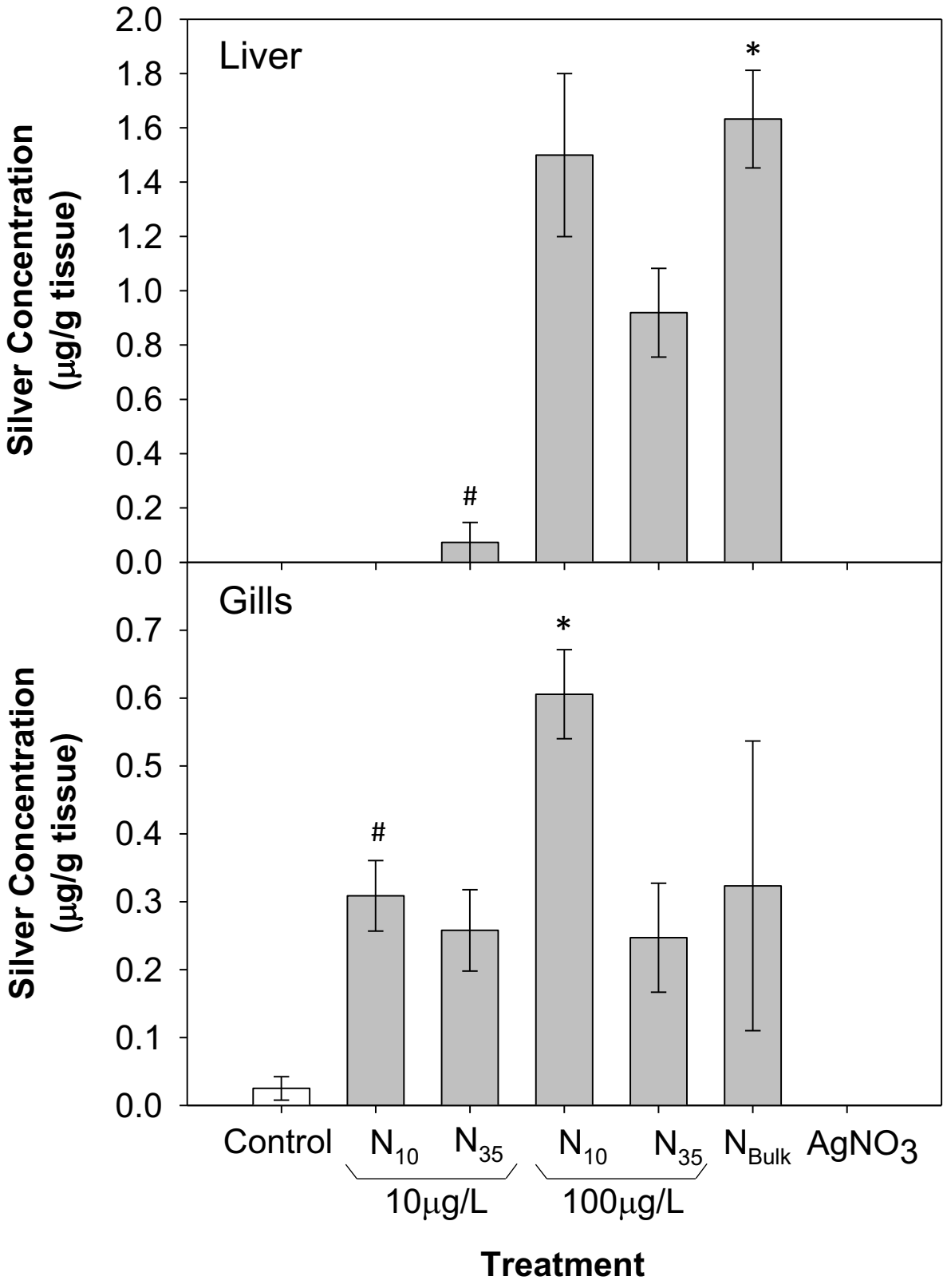


Scown et al. Figure 1.



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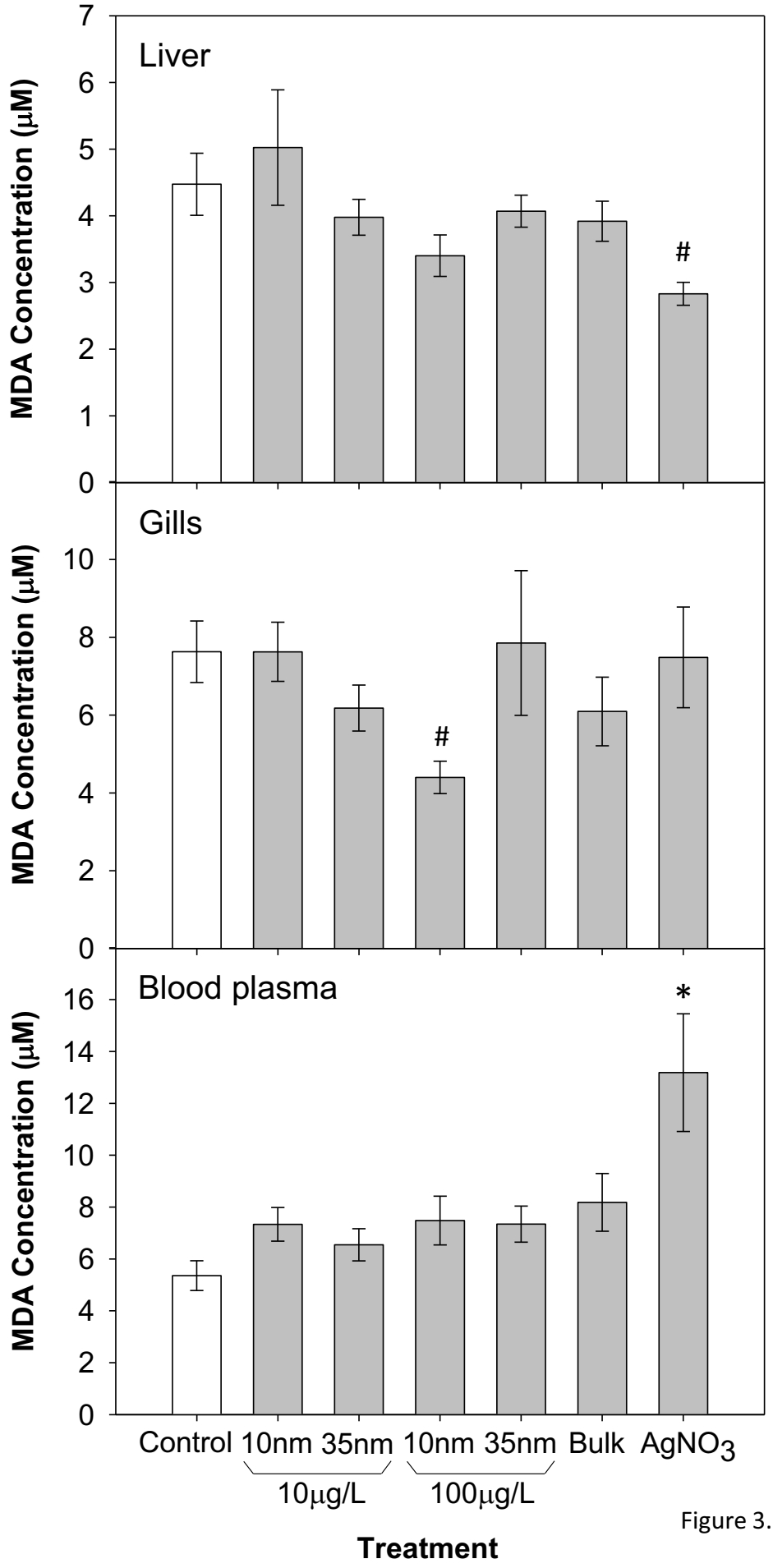

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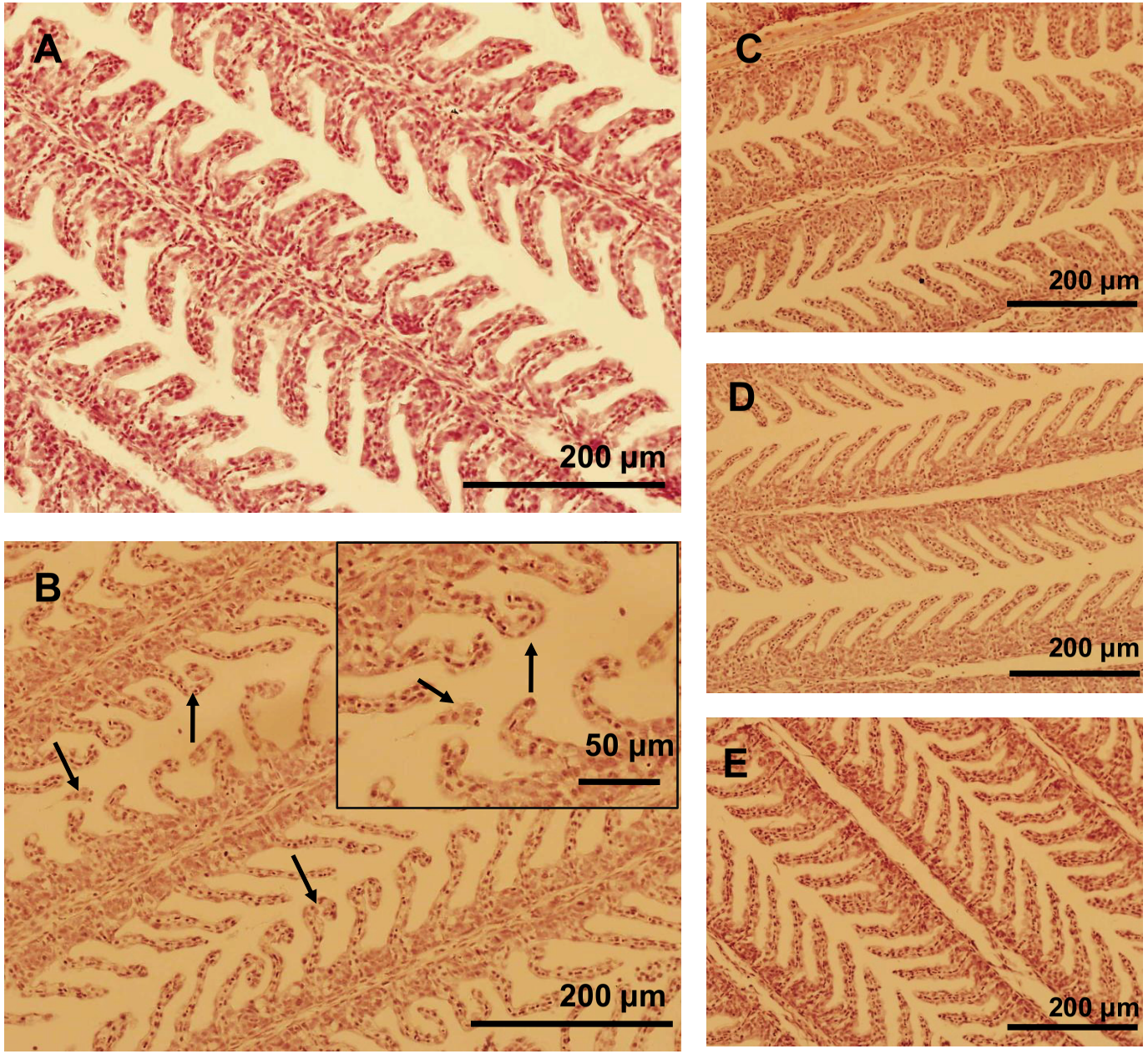

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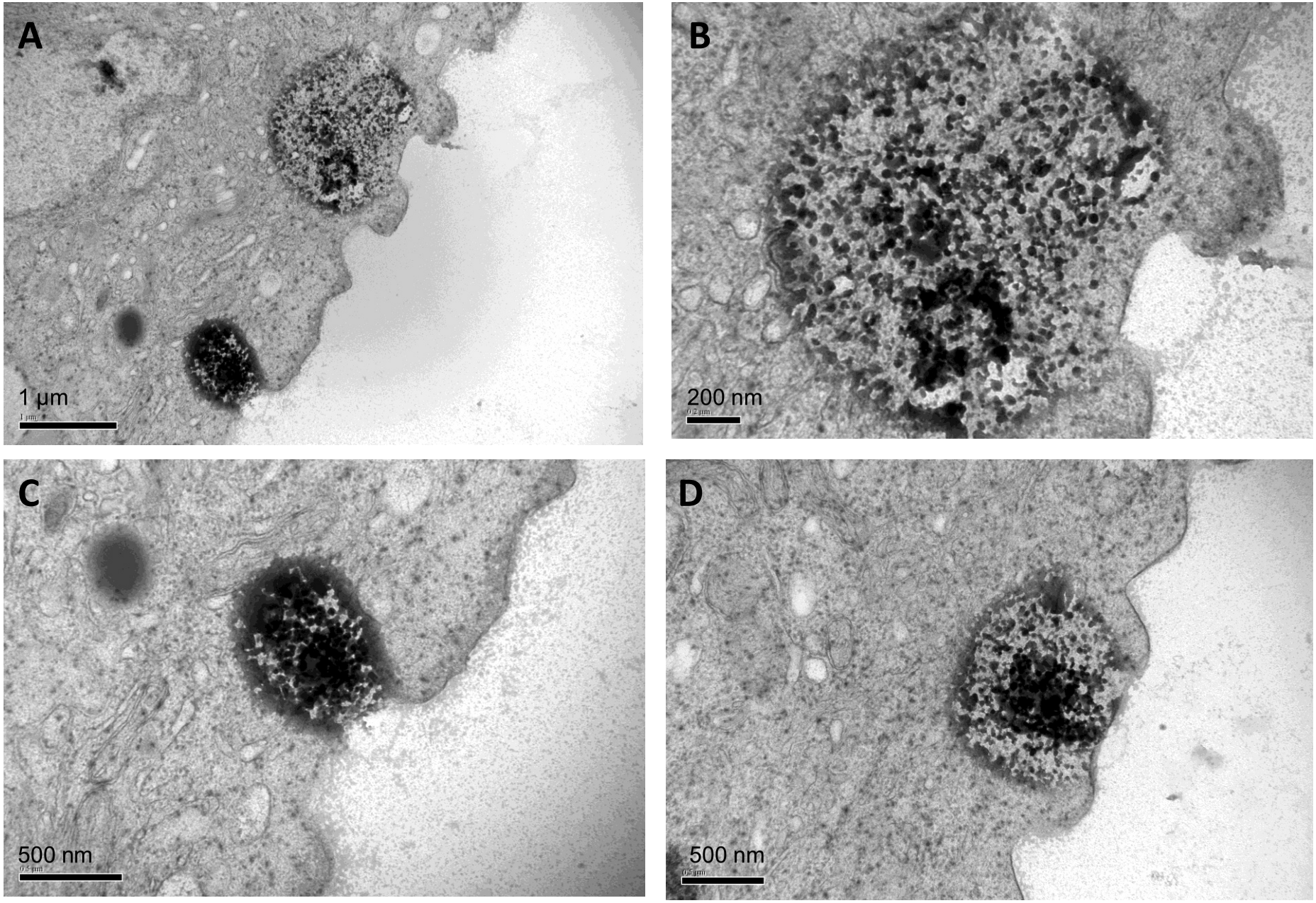

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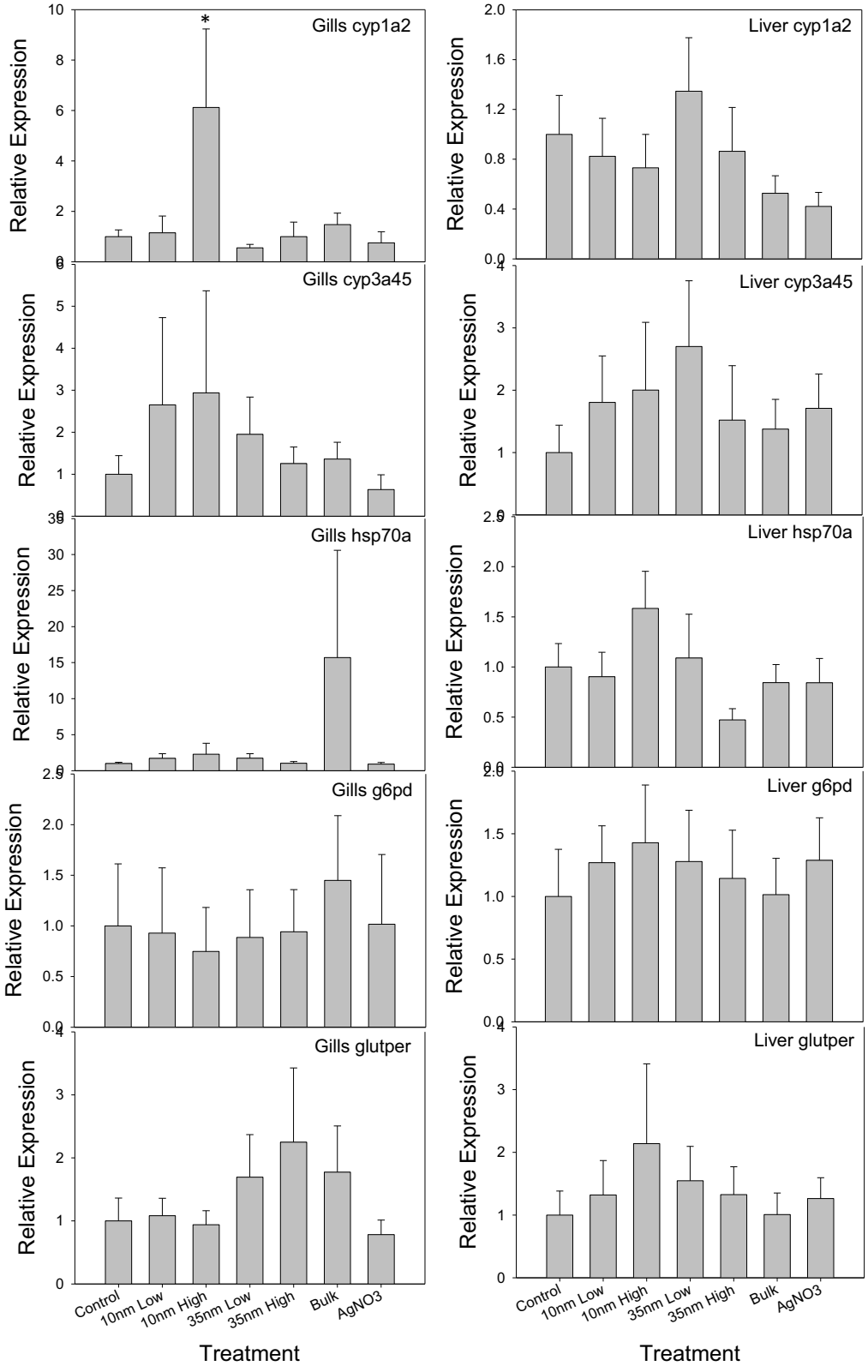

Scown et al. Figure 6

	N_{10} Silver	N_{10} Silver Manufacturers Specifications	N_{35} Silver	N_{35} Silver Manufacturers Specifications	N_{Bulk} Silver	N_{Bulk} Silver Manufacturers Specifications
Zeta potential (mV)	-12.52 ± 2.7	-	-6.50 ± 1.8	-	-2.8 ± 0.6	-
pH	7.11	-	7.34	-	6.40	-
Hydrodynamic diameter (nm) (DLS)	589 ± 101	-	2029 ± 524	-	938 ± 230	-
Polydispersity Index	0.54	-	0.93	-	0.69	-
Hydrodynamic diameter (nm) (NTA technique)	158 ± 76	-	166 ± 72	-	217 ± 130	-
Particle number mL^{-1} (NTA technique)	1.07×10^8	-	0.21×10^8	-	0.27×10^8	-
Mean primary particle size (nm) (TEM)	49 ± 18.5	10	114 ± 65.3	35 (max <100 nm)	137 ± 62.0	600-1,600
Mean particle size (nm) (AFM)	46.3 ± 10.7	-	90.0 ± 15.6	-	147.5 ± 82.3	-
Crystallite size (nm) (XRD technique)	21.2 ± 0.5	-	68.0 ± 2.0	-	60.0 ± 4.6	-
Surface Area ($m^2 g^{-1}$) (BET)	2.0 ± 0.2	9-11	2.9 ± 0.2	30-50	0.6 ± 0.1	-
Bulk Density ($g cm^{-3}$)	-	2.05	-	0.3-0.6	-	-
True Density ($g cm^{-3}$)	-	10.5	-	10.5	-	-
Purity (trace metal analysis)	-	99.9%	-	99.5%	-	99.95%

Table 1 Scown et al.

CHAPTER 5

Assessment of cultured fish hepatocytes for studying cellular uptake and (eco)toxicity of nanoparticles

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Assessment of cultured fish hepatocytes for studying cellular uptake and (eco)toxicity of nanoparticles

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Abstract

Nanotechnology is a rapidly growing industry of global economic importance and there are increasing concerns regarding the fate and possible toxic effects of engineered nanoparticles (ENPs) in the environment, yet relatively little is known in this regard. Aquatic organisms are likely to be particularly vulnerable to exposure to ENPs as our freshwater and marine environments act as a sink for most anthropogenically derived pollutants. Development and validation of *in vitro* models for nanoparticle toxicity screening to protect aquatic organisms is therefore essential to support the development and exploitation of a sustainable nanotechnology industry. We assessed the suitability of isolated primary trout hepatocytes for high through-put toxicity screening of ENPs, exposing them to a variety of well characterised metal and metal oxide nanoparticles and their bulk counterparts, and assessing their relative toxicity (lactate dehydrogenase (LDH) release, and glutathione-s-transferase (GST) activity) and effects on lipid peroxidation (Thiobarbituric reactive substances; TBARS). In parallel, uptake of the ENPs into the cultured hepatocytes was characterised using Coherent anti-stokes raman spectroscopy (CARS). None of the nanoparticle types studied affected cell viability (as determined by cell membrane integrity assessments using LDH release), at any of the exposure concentrations studied (up to 500 μg mL^{-1}). There were no effects of the ENPs on lipid peroxidation, with the exception for exposure to ZnO nanoparticles, or on glutathione-s-transferase levels. Established inducers of lipid peroxidation and GST activity in mammalian systems (hydrogen peroxide and silver nitrate) were also ineffective, suggesting a low responsiveness of these endpoints in trout hepatocytes in culture ENPs, however, were all shown to be internalised in the cultured hepatocytes using CARS. Our findings would suggest that fish hepatocyte cultures are suitable for studies investigating the cellular uptake of ENPs, but they are not sensitive to ENP exposure and are not a good *in vitro* model for nanoparticle toxicity screening.

Keywords: metal oxides; silver; titanium dioxide; cells; fish; lactate dehydrogenase; Thiobarbituric reactive substances; glutathione-s-transferase;.

List of Abbreviations

Engineered Nanoparticles (ENPs)

Titanium dioxide (TiO_2)

Cerium oxide (CeO_2)

Zinc oxide (ZnO)

Silver (Ag)

Lactate dehydrogenase (LDH)

Thiobarbituric reactive substances (TBARS)

Glutathione-s-transferase (GST)

Coherent anti-stokes raman spectroscopy (CARS)

Introduction

Nanotechnology exploits the unusual chemical and quantum properties of materials manufactured at or below the nanoscale. Over the past decade, the industry has expanded rapidly and engineered nanoparticles (ENPs) now have wide industrial applications and are incorporated into many consumer products [1-3]. As the number of nanoparticle types and applications increase, so does the likelihood that they will be released into the environment in significant quantities. The aquatic environment acts as a sink for most environmental pollutants and will thus likely receive ENPs from waste water and accidental release from factories and through degradation and wear of products containing ENPs. To date, however there is a lack of data on the environmental fate, bioavailability and biological effects of ENPs in aquatic organisms

There is a need to develop pragmatic and appropriate experimental approaches and techniques for the testing and screening ENPs for possible biological effects to assess potential risks. Given their very diverse nature (i.e. large number of types, shapes, forms and different functionalisations of ENPs) this ideally requires high throughput screening methods.

Most nanoparticles toxicity studies to date have been concerned with identifying the risks associated with inhalation of metal oxide nanoparticles and absorption through the skin. Many of the *in vitro* systems used for screening nanoparticle toxicity therefore have made use of terrestrial vertebrate cell lines such as alveolar macrophages [4], bronchial epithelial cells [5, 6] and pneumocytes and dermal fibroblast cell lines [7-9]. Uptake of nanoparticles by cells has been demonstrated [9, 10] as well as a range of cellular responses such as inflammation [6, 11], generation of reactive oxygen species [4, 6, 12], lipid peroxidation [5] and alterations to membrane integrity [8, 13].

Until recently little attention has been paid to potential risks associated with exposure of aquatic organisms to ENPs and the corresponding need for relevant *in vitro* models. Primary cultured fish hepatocytes have an established history for screening environmental contaminants, including endocrine disrupting chemicals, pharmaceuticals and heavy metals

for their potential impact on fish and other aquatic organisms [14-17] and potentially offer a suitable method for screening ENPs (eco)toxicity.

There are very few well-defined techniques for accurately imaging or characterising nanoparticles, especially when in biological samples. Dynamic light scattering (DLS) has been proposed as a useful technique for evaluating particle size and distribution in solution [18], however in terms of assessing uptake into organisms or cells there are fewer standard imaging methods. Gaining insight (through imaging) of the cell/subcellular localisation of particles can help to identify likely processes targeted and thus possible biological effects.

Early techniques such as ultra-high illumination light microscopy, lack accuracy at the nano-scale. A number of studies have turned to electron microscopy to determine nanoparticle uptake, distribution or characterisation, [19-21], with high resolution transmission electron microscopy offering the resolution capacity to identify crystalline structures of nanoparticles [22]. However, both TEM and SEM images require a dry sample and are acquired under high vacuum conditions, consequently prohibiting live imaging, therefore the characteristics of ENPs under such conditions are also not necessarily representative of their characteristics *in situ*. Furthermore, TEM is generally only able to visualise ENPs that are electron dense excluding visualisation of non-metal coatings or shells [23]. Energy filtered transmission electron microscopy (EFTEM) combined with electron energy loss (EEL) spectrum imaging however, has been used to successfully image single-walled carbon nanotubes (SWCNTs) in cells [24].

When considering uptake of ENPs into cells or whole organisms a number of techniques have been applied. Confocal microscopy has been used to determine the biodistribution of multi-walled carbon nanotubes (MWCNTs) in zebrafish embryos [25] however non-metallic ENPs must be fluorescently labelled which has ramifications when considering the toxicity potential of a novel material.

Dark-field microscopy has also been used to visualise uptake of silver nanoparticles into both zebrafish embryos and nematodes [26, 27] but the technique offers minimal penetration, thus places tight limits on the thickness of the sample that can be effectively examined. Samples must also be extremely pure and the high levels of illumination required

for visualization introduces the possibility of photodamage. The most recent techniques for visualising ENPs utilise intrinsic properties of various nanoparticles such as the plasma oscillations from gold nanoparticles, [28], or the low activation energy and subsequent high quantum yield from quantum dots, making their imaging and probing capabilities practical and effective.

Nonlinear optics (NLO) is a branch of optics that describes the behaviour of light in media. With the development of high pulsed lasers it has become possible to observe and take advantage of nonlinear effects and techniques developed to take advantage of the nonlinear optical phenomenon including second harmonic generation and two photon fluorescence. Multiphoton microscopy (MPM) is a type of laser scanning microscopy that derives its contrast from nonlinear optical properties of a sample (See [29] for review). The most recent nonlinear imaging modality is Coherent Anti-Stokes Raman Scattering (CARS) which derives image contrast from molecular vibrations within the sample.

Nonlinear imaging has many advantages over standard fluorescence confocal laser scanning microscopy, with the principle advantages being an increase in depth penetration and stain-free molecular contrast. CARS produces a strong directional signal, higher in frequency, shorter wavelength, than one-photon fluorescence that allows detection in presence of a strong fluorescent background and the Raman resonance enhancement provides chemical selectivity without the need for labelling It requires only moderate power intensity that is easily tolerable by biological samples, and, due to the low absorption of the near-infrared excitation beams, there is a significant reduction in photo damage, hence it is a minimally invasive technique. Nonlinear CARS signals are generated only at the focus point, where the excitation intensity is the highest, this leads to the inherent 3D resolution. The excitation wavelengths are near-infrared, which allows low scattering and thus increased penetration into the sample.

The aim of this study was to assess a rainbow trout hepatocyte primary cell culture, used routinely for toxicity assessments of conventional xenobiotics, for screening nanoparticle cellular uptake and toxicity. We exposed rainbow trout (*Oncorhynchus mykiss*) hepatocytes

to three metal oxide nanoparticles, that were well characterized: titanium dioxide (TiO_2), zinc oxide (ZnO), cerium oxide (CeO_2) and two sizes of the metallic nanoparticle silver (Ag) and their corresponding bulk counterparts, chosen because of their relatively high-volume industrial use, and evaluated lactate dehydrogenase (LDH) release, as a measure of overt toxicity (cell membrane integrity), lipid peroxidation and glutathione-S-transferase activity as endpoints for processes shown to be affected by some of these ENPs in other biological systems. Glutathione-dependent enzymes such as GST are involved in detoxification of a range of xenobiotic compounds including metals in cells and an increase in enzyme activity is often suggestive of cellular defence against oxidative stress [30]. We further applied coherent anti-stokes raman spectroscopy (CARS) to investigate the uptake capability of the selected ENPs into the cultured cells.

Materials and Methods

Fish Husbandry

Rainbow trout were obtained from Hatchlands Trout Farm, Devon, UK and were maintained in 500 L tanks supplied *via* a flow-through system with dechlorinated tap water on a 12 hour light/12 hour dark cycle Water temperatures were maintained between 9 and 11°C throughout and trout were fed maintenance food rations (Emerald Fingerling 30, Skretting, UK), at a rate of 1% of their body weight. Trout were starved for 24 hours prior to hepatocyte isolation.

Materials

All chemicals were purchased from Sigma-Aldrich, UK unless otherwise stated. Nanoparticle and bulk particle preparations of TiO_2, ZnO and CeO_2 were all purchased from Sigma-Aldrich, UK. Silver particles (10 nm, 35 nm and bulk particles) were purchased from Nanostructured and Amorphous Materials Inc., Houston, USA. Characterization information provided by the manufacturer is shown in table 1. Our own characterization of the particles included images of all particles in dry powder form by transmission electron microscopy, and measurements of hydrodynamic diameter, polydispersity index and zeta potential

performed on a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd. Malvern, UK) for all particle types in HPLC-grade water and in M199 cell culture media (Sigma Aldrich, UK). All measurements were carried out at nanoparticle concentrations of 1 mg L^{-1} after sonication for 30 minutes. The data from our characterization of the particles is discussed in the results section.

Hepatocyte Isolation

Hepatocyte isolations and cultures were based on the validated protocol of Bickley et al., (2009). All solutions were sterile filtered and all instruments and glassware were autoclaved before use to maintain aseptic conditions. Perfusions solutions (Table 2.) based on HEPES-buffered Hank's salts [31] were prepared as described in Bickley et al., (2009). Briefly, salts were dissolved in ultrapure water (Maxima ultrapure water, Elga) and the resulting solutions adjusted to pH 7.3 and sterile filtered through a 0.2µm filter (Millipore, USA).

Prior to liver perfusion, rainbow trout were anaesthetized by immersion in benzocaine until loss of equilibrium was achieved and injected with 200 µL heparin and re-immersed in the benzocaine until opercular movements ceased, at which time the brain was then destroyed. Under sterile conditions the body cavity was opened, taking care not to perforate the intestines, to expose the internal organs.

A 25 gauge needle was inserted into the hepatic portal vein and secured using a haemostat clamp. The liver was then perfused with 100 mL pre-perfusion solution (Hank's solution with 2.2 g L^{-1} Na_2-EDTA x $2H_2O$) and then 100 mL perfusion solution (Hank's solution containing 358 mg L^{-1} $CaCl_2$ x $2H_2O$ and 120 mg L^{-1} collagenase D) and then flushed with 100 mL (Hank's solution with 880 mg L^{-1} Na_2-EDTA x $2H_2O$). The liver was then excised from the body cavity into a petri dish, held on ice, teased apart using forceps and a razor blade in calcium and magnesium free (CMF) Hank's solution, and then the resulting suspension passed through a series of three mesh sizes: 250µm, 100µm and 50µm to further separate the cells. The suspension was then centrifuged at 75 x g for 5 minutes at 4°C and the resulting pellet re-suspended in CMF, centrifuged and washed a further two times. Finally the pellet was re-suspended in sterile M199 (Sigma Aldrich, UK), pH 7.3, supplemented with

10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin and 10µg mL^{-1} streptomycin. Cell viability was examined by a trypan blue exclusion test and the cell number counted using a haemocytometer. Flat-bottomed 96-well microtiter plates were seeded with 200µL cell suspension per well at 1.5 x 10^6 cells mL^{-1} (optimum cell density cited by CytoTox 96® Non-Radioactive Cytotoxicity Assay kit, Promega, UK) for the lactate dehydrogenase assay and for the thiobarbituric acid reactive substances assay and glutathione-S-transferase assay a 200µL cell suspension at 2 x 10^7 cells mL^{-1} was used (optimum cell density cited by TBARS Assay kit, Cayman Chemical, USA). The cells were maintained for 24 hours in a humidified incubator at 11°C to allow cells to become confluent prior to nanoparticle exposure.

Nanoparticle Exposures

Trout hepatocytes were exposed to nanoparticles 24 hours after isolation. For LDH determinations, nanoparticle and bulk powder suspensions of TiO_2, ZnO, CeO_2 and Ag with concentrations of between 20 pg mL^{-1} and 1 mg mL^{-1} in M199 in 10% FBS were made and were sonicated for 30 minutes prior to dosing. Cells were dosed by 100 µl media replacement with the nanoparticle suspensions, at concentrations of between 10 pg mL^{-1} and 500 µg mL^{-1} in quadruplicate wells and exposed for 24 hours in a humidified chamber at 11°C. For lipid peroxidation and GST assessments dosing suspensions were prepared in a similar way with a single dosing concentration of 500 µg mL^{-1} used for both analyses.

ENP Toxicity Assessment -Lactate Dehydrogenase Assay

The viability of hepatocytes 24h after isolation, prior to ENP exposure, and after 48h (post ENP exposure) was assessed visually under an inverted microscope (M40-5818, Wild Heerbrugg, Switzerland). Toxicity of the ENPs to the cultured hepatocytes was assessed using LDH release. The LDH assay was carried out using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, UK), following the manufacturers guidelines. Briefly, half of the control cells (4 wells) were lysed by incubating with 20 µL 9% (v/v) Triton® X-100 for 45 minutes at 37°C. The plates were then centrifuged at 250 x g for 4 minutes at room temperature. 50 µL aliquots of cell supernatant from control and ENP treated wells was then

transferred to a fresh 96-well flat bottom microtitre plate. 50 µL of the assay solution, reconstituted in phosphate buffered saline + 1% bovine serum albumin (BSA) was then added to each well, after which the plate was incubated in the dark at room temperature. During this time the reduction of NAD^+ to NADH is catalysed by LDH released from cells. NADH in turn reacts with a tetrazolium salt to form a red formazan product. The reaction was stopped by the addition of 50 µL 1M acetic acid and the resulting absorbance measured at 490 nm on a Molecular Devices SpectraMax 340pc microplate reader. The absorbance readings for exposed cells were compared against readings for spontaneous cell release and 100% LDH release from hepatoctes lysed with Triton® X-100.

Lipid Peroxidation and Glutathione-s-Transferase Activity

Thiobarbituric Acid Reactive Substances Assay

Metal-induced toxicity in cells is often mediated by the production of reactive oxygen species such as the superoxide ion, hydrogen peroxide, and hydroxyl radical [32, 33]. Measurement of 1,1,3,3-tetramethoxypropane (malonaldehyde; MDA) using the TBARS assay is widely used as an indicator of lipid peroxidation. The protocol adopted was similar to that described previously [34]. Briefly, 8 standards of 1,1,3,3-tetramethoxypropane (malonaldehyde; MDA) at concentrations ranging from 0.625µM to 100µM were made up for the TBARS assay using dilutions of a 500µM stock solution of MDA in 2% ethanol prepared in M199 media. Cells were also exposed to 500 µg mL^{-1} H_2O_2 as a positive control. After exposure, cells were homogenized using a Teflon pestle and handheld homogenizer and the homogenate centrifuged at 1,600 x g for 10 minutes at 4°C. 100µL of supernatant and standards were then mixed with 500 µL of 0.4% thiobarbituric acid (40mg in 10mL) in 10% acetic acid, pH 5.0 in 1.5mL microcentrifuge tubes. The tubes were heated to 90 °C for 1 hour and then cooled to room temperature under a stream of flowing tap water. 600 µL of butanol was added and the mixture vortexed vigorously mixed for 5 seconds. The mixture was then centrifuged at 3,100 x g for 10 min and the 150 µL of butanol phase removed and placed in duplicate in a flat-bottomed, 96-well microtiter plate and the absorbance measured at 532nm on a Molecular Devices SpectraMax 340pc microtiter plate reader. A

standard curve was prepared and MDA concentrations were determined accordingly. The TBARS assay was conducted for each ENP treatment in three separate hepatocyte isolations.

Glutathione-S-Transferase Assay

Glutathione–s–transferases (GSTs) play a major role in the detoxification of xenobiotic chemicals within cells and measurement of increased activity is often used as a biomarker for exposure to environmental contaminants [35-37]. Following exposure to nanoparticle and bulk particle types for 24 hours, GST activity was assessed in the hepatocytes. 100 mM phosphate buffered saline pH 7.3 (PBS), 10 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 10 mM reduced L-glutathione (GSH) solutions were warmed to 25°C in a water bath. Cells and liver tissue were sonicated with a sonic probe (Sonicprep 150 MSE Scientific Instruments) over ice for 20 seconds. The resulting homogenate was centrifuged for 20 minutes at 4°C at 10,000 x g. 40 µL of the resulting supernatant was added to microtiter plates in quadruplicate over ice using PBS as a blank. 200 µL PBS, 20 µL GSH and 20 µL were then quickly added to all wells and the reaction followed spectrophotometrically at 340 nm at 25°C for 3 minutes. Cells exposed to 10 ng mL^{-1} and 500 ng mL^{-1} $AgNO_3$ and 500 µg mL^{-1} H_2O_2 were set up as positive controls. In order to ascertain whether the response of primary hepatocytes differed compared with dissected whole liver tissue, 100 mg of rainbow trout liver tissue was dissected from an untreated fish was run in parallel with the treated hepatocytes. GST activity was expressed in nmoles of conjugated reduced glutathione min^{-1} mg $protein^{-1}$ (extinction coefficient, ε_{340} = 9600 L mol^{-1} cm^{-1}).

GST activity in the cells was quantified in cells and tissue relative to the total protein content of the sample. Protein concentration was determined in cell homogenates using the Bio-Rad Reagent Protein Assay (Bio-Rad, USA) following the manufacturers guidelines. Briefly, cell homogenates were diluted by 1 in 10 with ultrapure water (Maxima ultrapure water, Elga). Eight BSA standards of between 0 and 1 mg mL^{-1} were made. 5 µL of diluted homogenate and standards were then added to a microtiter plate in duplicate. Bio-Rad Protein Assay Reagent was diluted 1 in 5 with water and 200 µL added to each well. The absorbance was measured at 595 nm on a Molecular Devices SpectraMax 340pc microtiter

plate reader. A standard curve was prepared and protein concentrations were determined accordingly.

Coherent Anti-Stokes Raman Scattering

Coherent Anti-Stokes Raman Scattering (CARS) microscopy was adopted to visualise the internalisation of the ENPs into the hepatocytes. This is a technique that enables label-free imaging of ENPs within tissue [24]. The technique uses the vibrational frequencies of molecular bonds within a sample to derive chemical-specific contrast [for a review see 38] and eliminates the chemical perturbation seen when using fluorescent labelling. CARS can provide unrivalled contrast of metal oxide nanoparticles within biological tissues. Briefly**,** imaging was performed using a modified commercial inverted microscope and confocal laser scanner (IX71 and FV300, Olympus UK), as described previously [39]. Dual wavelength laser excitation was achieved using an optical parametric oscillator (Levante Emerald, APE Berlin) pumped with a frequency doubled Nd:Vandium picosecond oscillator (High-Q Laser Production GmbH). The optical parametric oscillator (OPO) produced signal and idler beams with pulse duration of 6 ps and a repetition rate of 76 MHz. A 60X, 1.2 NA water immersion objective (UPlanS Apo, Olympus UK) was used to focus the laser excitation into the sample. Due to the directional nature of CARS, simultaneous forwards- and epi- detection is desirable [40], and as described previously provides excellent contrast of sub-wavelength particles in biological tissues [39]. 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 using 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. The anti-Stoke signal was isolated at each photodetector by a single band-pass filter cantered at 750 nm (HQ750/210, Chroma Technologies). Three-dimensional data to prove cellular internalisation of the exposed ENPs were acquired by taking stacks of 2-dimensional images in the x-y plane each separated by an increment in the z-direction, which was achieved by alteration of the objective focus.

Forward and epi images were recompiled into 2D images using OsiriX open-source imaging software.

Statistical analyses

The data are expressed as mean values ± SE and were analyzed using SPSS version 16.0, with α set at 0.05. All data were checked for conformity with the assumptions of normality (homogeneity of variance and normality of error). If these assumptions were not met, data were transformed to meet these assumptions.

Results

Particle Characterisation

Particles dispersed in aquatic media with zeta potential values that are more positive than 30 mV or more negative than -30 mV are considered to be stable [41, 42]. Zeta potential measurements for all particle types in both HPLC-grade water and M199 cell culture media were within these values suggesting that all particles will show a propensity form aggregates when in suspension (Table 1.). This is confirmed by large hydrodynamic diameter measurements compared to average particle size. TiO_2 nanoparticles formed the smallest aggregates in water (189 nm), approximately 8 times larger than average particle size, however other particle aggregates were up to 60 times larger than average particle size as was the case for Ag 35 nm particles (hydrodynamic diameter 2029 nm). When suspended in M199 media, the average aggregate sizes were markedly larger (between 4 and 16 fold) for all particle types than when suspended in water with the exception of ZnO bulk particles forming aggregates of approximately the same size. The polydispersity index of the aggregates in general increased when particles were suspended in M199 media compared to when suspended in water suggesting that there was less uniformity in aggregate size.

ENP Toxicity and Biological Effects Measures in Cultured Hepatocytes

Triplicate trout hepatocyte cultures were conducted to assess the toxicity of the metal and metal oxide nanoparticles, Ag, TiO_2, ZnO, CeO_2 and their bulk counterparts.

Examination of the trout hepatocytes at 24h and 48h revealed the cells were confluent in the controls, ENP and bulk exposures and showed no abnormalities in their physical appearance. There was no evidence for any overt cytotoxicity as determined by the measurement of membrane integrity (LDH leakage), for any treatment compared with the controls (Fig. 1.). There was also no significant difference in LDH release between bulk materials and their corresponding nano-sized counterparts.

Investigations into the ability of nanoparticles to induce intracellular oxidant production (using TBARS as a reporter of MDA) found significant lipid peroxidation only upon exposure to zinc oxide nanoparticles, where MDA concentrations were approximately three times higher than levels in the controls (ANOVA with Tukey's post-hoc test; df=11,51; F=2.945; p=0.006) (Fig. 2.). Cells treated with 500 μg mL^{-1} H_2O_2 as a positive control, however failed to elicit a response significantly different from the control cells in culture. The MDA concentration in an equivalent samples of untreated liver tissue homogenate was over four-fold higher (4.47 μM) than the MDA concentration in the in the isolated hepatocyte controls (0.68 μM) suggesting a diminished capability of the hepatocytes to respond to cellular stress from xenobiotic compounds.

A significant increase in GST activity was only measured in cells exposed to zinc oxide bulk particles at an exposure concentration of 500 μg mL^{-1} (ANOVA with Tukey's post-hoc test; df=13,59; F=5.276; p<0.001) (Fig. 3.). Treatment of cells with 500 μg mL^{-1} H_2O_2, 10 ng mL^{-1} and 500 ng mL^{-1} $AgNO_3$ did not cause a significant increase in GST activity compared with control cells in culture. GST activity in untreated liver tissue homogenate was two orders of magnitude higher than enzyme activity in the cultured control hepatocytes again suggesting a reduced capacity for responses to cellular stress from xenobiotics for trout hepatocytes in culture.

Imaging uptake of ENPs into cultured trout hepatocytes (CARS)

E-CARS and F-CARS images of the isolated hepatocytes over different wavelengths and incubated with the various ENPs are shown in Figure 4. The figure highlights the differences between the two signals and wavelengths which are used in the interpretation of the images acquired. Images A and C are generated in the epi-direction which allows detection of small features due to its filtering out of signals from objects larger than the excitation wavelength, whilst B and D are taken in the forward direction, required for imaging objects with an axial length comparable to, or larger than, the excitation wavelength. Images A and B (Figure 4) were taken at 924nm, whereas images C and D were taken at 919nm. These differences between the two wavelengths are critical to the identification of the ENPs dosed to the hepatocyte cells. At 924nm C-H bonds are specifically excited and thus C-H rich structures such as lipids within the cell fluoresce (Fig 4 A,B). At 919nm lipids are negatively contrasted and appear as dark areas within the cell (Fig 4 C,D). The importance of these differences and their application in analysing ENP uptake are discussed below.

The incorporation of ENPs to cultured cells was visually assessed for various metal and metal oxide ENPS (Figure 5). Here we show signals from CARS images derived from primary hepatocytes dosed with CeO_2, Cu, TiO_2 and Ag nanoparticles. The origin of the signal from the ENPs appears to be on the same plane as the nucleus, suggesting uptake of ENPs into the cell. To confirm this, by taking a series of 2-dimensional images in the x-y plane, each separated in the z-direction by an increment of 0.25 μm, we created a z-stack and allowed the production of a 3 dimensional interpretation of the image, clearly placing the location of the ENPs within the hepatocyte cells (Figure 6), however no quantitative information on uptake can be derived from these images.

Discussion

Isolated hepatocytes have been used for many years for studying liver uptake, excretion, biotransformation and toxicity of drugs and other xenobiotics and they have provided an effective system for screening chemicals for toxicological effects, including in fish [14, 17,

43-45]. Furthermore, hepatocytes are relatively easy to culture, their basic cell functions are well characterised and they have enzyme systems that are representative of many other cell types [46, 47]. Cultured human and rat hepatocytes have been used to investigate the toxicology of various ENPs [48, 49], however, despite the fact that the aquatic environment will likely receive ENPs from a variety of sources in the near future, very few studies have used hepatocyte cultures derived from aquatic organisms to investigate the effects of ENPs At the time of writing, the authors are aware of only one other study using fish hepatocytes to assess the toxicity of cadmium telluride quantum dots [50].

Here we applied a rainbow trout hepatocyte culture system to assess its suitability for the screening of a range of metal and metal oxide nanoparticle types. We applied a suite of assays to determine toxicity of the nanoparticles as well as general responsiveness of the cells under standard culture conditions. CARS was employed to investigate uptake of the nanoparticles into the cultured cells.

Nanoparticle particokinetics within a cell culture system are influenced by a range of factors including pH, ionic strength and viscosity of the culture media as well as particle size, shape and charge density [51]. All of these factors influence the agglomeration and sedimentation rate of the particles within the media, which in turn determines the bioavailability of particles to the cells and resultant toxic effects.

Characterisations on our ENPs established zeta potential values close to zero point charge when the ENPs were suspended in HPLC-grade water. This meant there was a propensity for the particles to aggregate and when suspended in M199 cell culture medium, this effect was exacerbated, likely due to the high cation concentration resulting in the formation of larger aggregates, suggesting that cells were exposed to large aggregates rather than discrete particles.

Limbach et al. [52] found that although smaller sized cerium oxide nanoparticles (25-50 nm) were able to penetrate human lung fibroblasts by diffusion, they underwent rapid agglomeration in the exposure media leading to sedimentation which limited their transport into cells. Larger sized particles (250-500 nm) however, remained more discrete in the

exposure media and were taken up more readily. Teeguarden et al. [51] emphasised that measurement of the relative toxicities of nanoparticle types must take into account bioavailability and uptake into cells (effective dose), as well as the nominal dose, as smaller nanoparticle agglomerates remaining in suspension in the exposure media may not physically come in contact with cell layers on the bottom of culture vessels. These studies highlight the need not only for thorough characterization of nanoparticles in the exposure media, but also examination of their agglomeration, sedimentation and uptake behaviour within the culture system to gain any understanding on the uptake dynamics of nanoparticles to cells.

Membrane integrity as measured by LDH release was not shown to be affected by any nanoparticle or bulk particle type at any of the exposure concentrations, showing a distinct lack of evidence for any overt toxicity. These findings are in contrast with a number of other studies which have shown an increase in LDH leakage in cells exposed to metal and metal oxide nanoparticles at concentrations comparable to those used in our exposures. ZnO nanoparticles have been shown to cause LDH leakage in mouse embryo fibroblast and neuroblastoma cells at concentrations of between 10 and 100 μg mL^{-1} [53, 54], TiO_2 nanoparticles caused LDH leakage in mouse neuroblastoma cells and BRL 3A rat liver cells at concentrations of between 100 and 250 μg mL^{-1} [49, 53] and Ag nanoparticles were found to cause LDH leakage in BRL 3A cells at concentrations of between 10 and 50 μg mL^{-1} [49]. In theory, this disparity could be due to a low level of responsiveness of the trout hepatocytes to ENPs or because levels of uptake of ENPs to the cells were too low to elicit a response.

Despite the lack of overt toxicity, CARS imaging showed that the selected ENPs were taken up into the culture trout liver cells. All types of metal oxide and metal NPs generated a significantly large CARS signals to be detected in both epi- and forward-directions and at both 919 and 924 nm. This is because metal oxides are wide band gap semi-conductors and thus can be visualised across a broad wavelength spectrum. A strong signal at both

wavelengths was a requirement for positive identification of the ENPs against signals produced by the presence of lipid rich structures. Figure 4 also demonstrates the difference between images generated by the E- (A,C) and F-CARS (B,D) signals, where larger cellular structures appear in the forwards images and the smaller particles in the epi-image.
Another advantage of the two signals is the elimination of background noise, present in the forward image, arising from non-resonant electronic contribution (eg from surrounding media or water) to the CARS signal [39].

The CARS z-stacks (3-d images) of cells provided strong evidence to suggest that TiO_2, CeO_2, Ag and Cu nanoparticles were all taken up from the culture medium into the liver cells. This implies that nanoparticles enter the cells either by active endocytotic mechanisms or passively by diffusion through the membrane without compromising membrane integrity. A recent review [55] has suggested that diffusion of nanoparticles through cell membranes is improbable due to the propensity of nanoparticles to aggregate in aqueous media and proposed that uptake through vesicular transport was more likely. We were unable to identify the sub-cellular localisation of the ENPs/their aggregates using CARS because the resolution of the system is limited compared with other techniques such as electron microscopy.

The cultured hepatocytes contained aggregates of particles of various sizes, however it is not possible to determine the size of the smallest resolvable aggregate due to the point-spread-function of the imaging system exceeding the size of an individual nanoparticle [38]. This is best illustrated in Figure 5C where particles appearing to be of similar size have uneven brightness, indicating the presence of different sized aggregates. The effect is further exaggerated by the nonlinear concentration dependence of Raman scatterers on the CARS signal.

In our studies significant lipid peroxidation was only found only as a result of exposure to ZnO nanoparticles. Other studies have demonstrated ZnO nanoparticle exposure caused

lipid peroxidation-mediated genotoxicity in human epidermal cells at concentrations of 0.08-0.8 μg mL^{-1} [56] and ROS-mediated toxicity in exposures to zebrafish embryos at concentrations of 1-10 mg L^{-1} [57]. It has been suggested that observed toxicity as a result of exposure to some metal or metal oxide nanoparticles, including ZnO and Ag, may be a result of both nanoparticle exposure and release of metal ions from the nanoparticles into the exposure media [57-61]. It was surprising therefore to find that neither silver nanoparticles or any of the other ENPs induced a detectable induction of lipid peroxidation, as oxidative stress is a common finding as a result of metal oxide and metal nanoparticle exposure [62-70]. Silver nanoparticles have previously been shown to cause lipid peroxidation in human carcinoma and sarcoma cells at a concentration of 6.25 μg mL^{-1} [71] and toxicity and apoptosis mediated by generation of ROS in alveolar macrophages and mouse embryonic fibroblasts at a concentration of 50 μg mL^{-1} [72, 73]. Both TiO_2 and CeO_2 nanoparticles have also been found to induce an increase in ROS in human bronchial epithelial cells at concentrations of 5-40 μg mL^{-1} [6, 74] and in goldfish skin cells, oxidative DNA damage as a result of hydroxyl radical formation was observed at TiO_2 concentrations of 1-100 μg mL^{-1} [75]. However, in a study using the rainbow trout gonadal cell line RTG-2 oxidative damage as a result of TiO_2 nanoparticles was not observed at concentrations up to 50 μg mL^{-1} [76].

Interestingly, however, in our studies exposure of isolated trout hepatocytes to 500 μg mL^{-1} hydrogen peroxide failed to elicit an oxidative response, suggesting an extremely low responsiveness for oxidative damage and that they may not be a good nanoparticle toxicity model for studying these endpoints.

The glutathione-S-transferase enzyme family play an important role in the defence of cells against xenobiotics [77]. They are involved in detoxification both through catalysing the conjugation of reduced glutathione, via a sulfhydryl group to electrophilic centers on a wide variety of substrates, and through direct binding of compounds including free radicals and peroxides [36, 44, 78]. Few studies have so far investigated the effect of nanoparticle exposure on GST activity. Park et al., [6] , however, found that expression of the GST gene

was induced in a human bronchial epithelial cell line as a result of exposure to TiO_2 nanoparticles. In contrast, *in vivo* exposures of the terrestrial isopod *Porcellio scaber* to TiO_2 nanoparticles via the diet have shown conflicting results. In one study, a dose-dependent increase in GST was observed at doses of 0.5-3000 μg TiO_2 per gram of food [79], but in the other by the same research group, no effect was seen [80]. Increased GST activity has been associated with metal exposure in a number of fish species [35, 81-83]. In our study significant increases in GST activity were only observed in exposure to ZnO bulk particles. As for our assessments on lipid peroxidation however, there was no increase in GST activity on exposure to 500 μg mL^{-1} H_2O_2, 10 ng mL^{-1} and 500 ng mL^{-1} $AgNO_3$. Furthermore, GST activity in homogenised liver tissue was two orders of magnitude higher per mg of protein compared to the hepatocyte controls, suggesting that GST activity was considerably diminished in the cultured cells. Previous published material provides an array of contrasting data on the suitability of rainbow trout hepatocytes for assessing toxicity endpoints. Some studies have demonstrated isolated fish hepatocytes to be good models for measurement of toxicological endpoints such as GST activity [37, 44], but others have shown lower activity in hepatocytes than whole liver samples [84] and both liver cell mono-oxygenase activity and conjugating activities can be rapidly lost in hepatocyte cultures [46, 85].

The TBARS assay shows that significant lipid peroxidation occurred as a result of exposure to ZnO nanoparticles. If the mechanism of ZnO nanoparticle-induced lipid peroxidation is via the dissociation of Zn^{2+} ions into the culture media, arguably, GST activity would be expected to be elevated since GST catalyses the conjugation of zinc ions to glutathione sulfhydryl groups. GST also functions in the detoxification of ROS, meaning the absence of raised GST activity as a result of ZnO nanoparticle exposure was unexpected. Only ZnO bulk nanoparticles showed significantly elevated levels of GST activity, but were not shown to cause lipid peroxidation, suggesting that the elevated activity was not due to detoxification of ROS, but as a result of conjugation of Zn^{2+} ions with glutathione. The ability of zinc ions to cause oxidative stress in the livers of exposed fish however is well known [86-88] suggesting either other mechanisms are acting to increase GST activity, or that ROS are

being generated, but are not inducing lipid peroxidation. Dissociation of silver ions into aquatic media from silver nanoparticles has also been demonstrated and been implicated causing toxicity in zebrafish embryos [61] and oxidative stress in the mouse brain after intraperitoneal injection [89], therefore the lack of response in both the TBARS and GST activity assays in response to silver was unexpected and further suggests that the ability of the hepatocytes to respond to exposure to ENPs is diminished.

Despite a lowered functional capacity/capability of the isolated trout hepatocytes, visually, there were no signs of abnormality and the cell membranes were intact as assessed by LDH release). The protocol used in these studies was identical to that developed by Bickley et al.,[90] in our laboratory where the isolated fish hepatocytes are responsive to both cyp1A induction on toxicant challenge and vitellogenin mRNA induction after 24 hours exposure to oestrogen. The absence of responses of the positive controls in the TBARS and GSH assays indicated that the isolated hepatocytes however, were not suitable for signalling effects of ENP on lipid peroxidation or GST activity.

Conclusions

In conclusion, we have shown that some metal and metal oxide ENPs are bioavailable to fish hepatocytes in culture, and isolated trout hepatocytes thus offer a model for studying the cellular uptake of ENPs. None of the metal and metal oxide ENPs were found to be overtly toxic to trout hepatocytes in culture, however, and the hepatocytes were not found to be sufficiently responsive on the measures of for lipid peroxidation or GST activity to be developed as an effective screening system for ENPs.

Although rainbow trout hepatocytes have previously been shown to be suitable for examining a wide range of physiological parameters including responses to conventional xenobiotics [47], it has also been shown that some liver functions may be lost upon culturing of the cells [46, 85]. Even if a certain level of responsiveness is maintained, subtle responses may not be measureable suggesting hepatocytes may not be suitable for

investigating the toxic responses that may be induced by novel compounds such as nanoparticles.

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structure and outline and the E- CARS displays the very strong ENP signal. With the nucleus in focus these images are interpreted as cross section through the middle of the cell.

Figure 6. A 2-dimensional representation of a 3-dimensional z-stack showing a cluster of hepatocytes dosed with Cu NPs at 1000 ng L^{-1}. Frames A-D are representative images of a 180^{o} rotation, illustrating the source of the ENP signal as within the cell membrane in all three dimensions.

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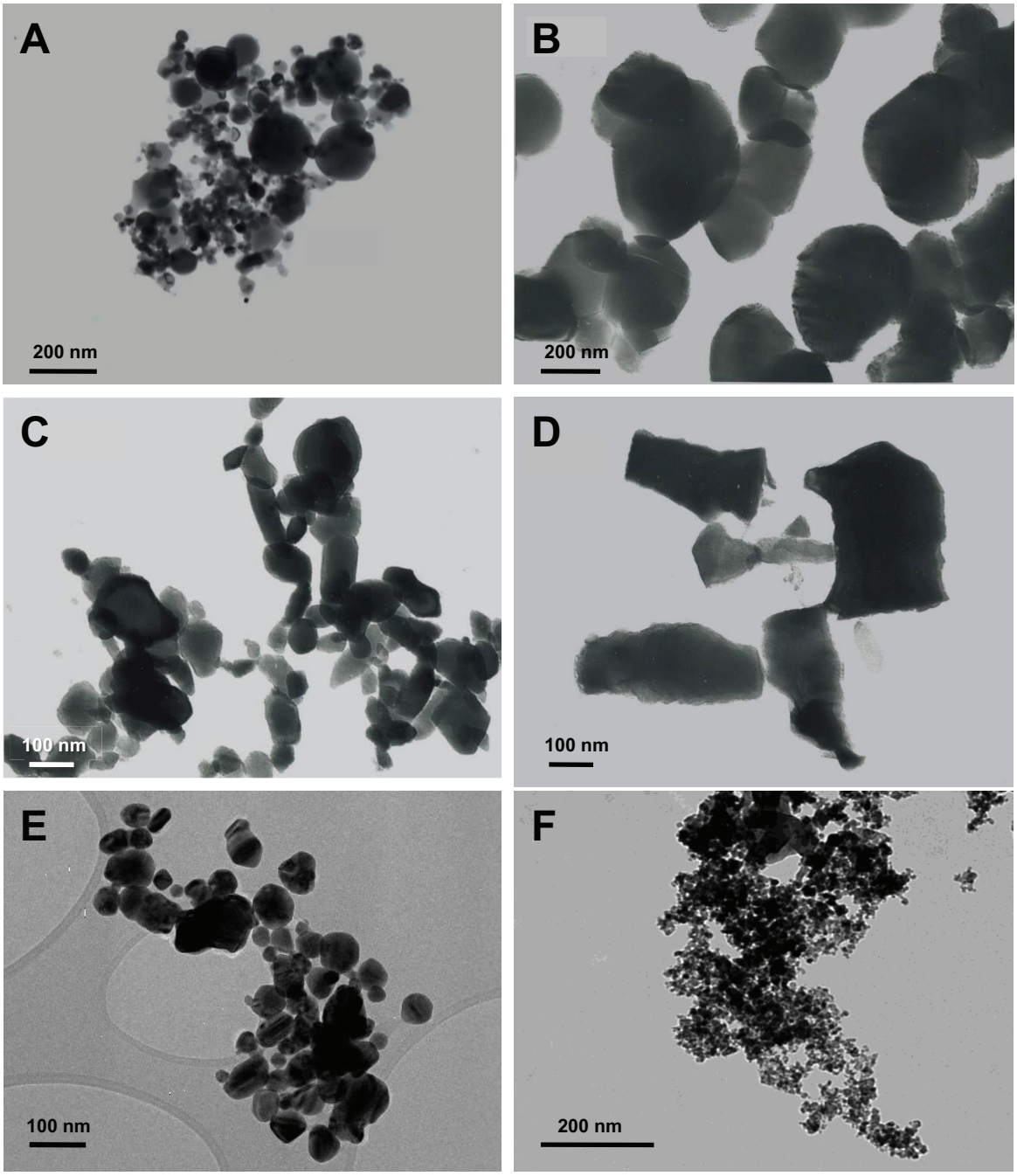
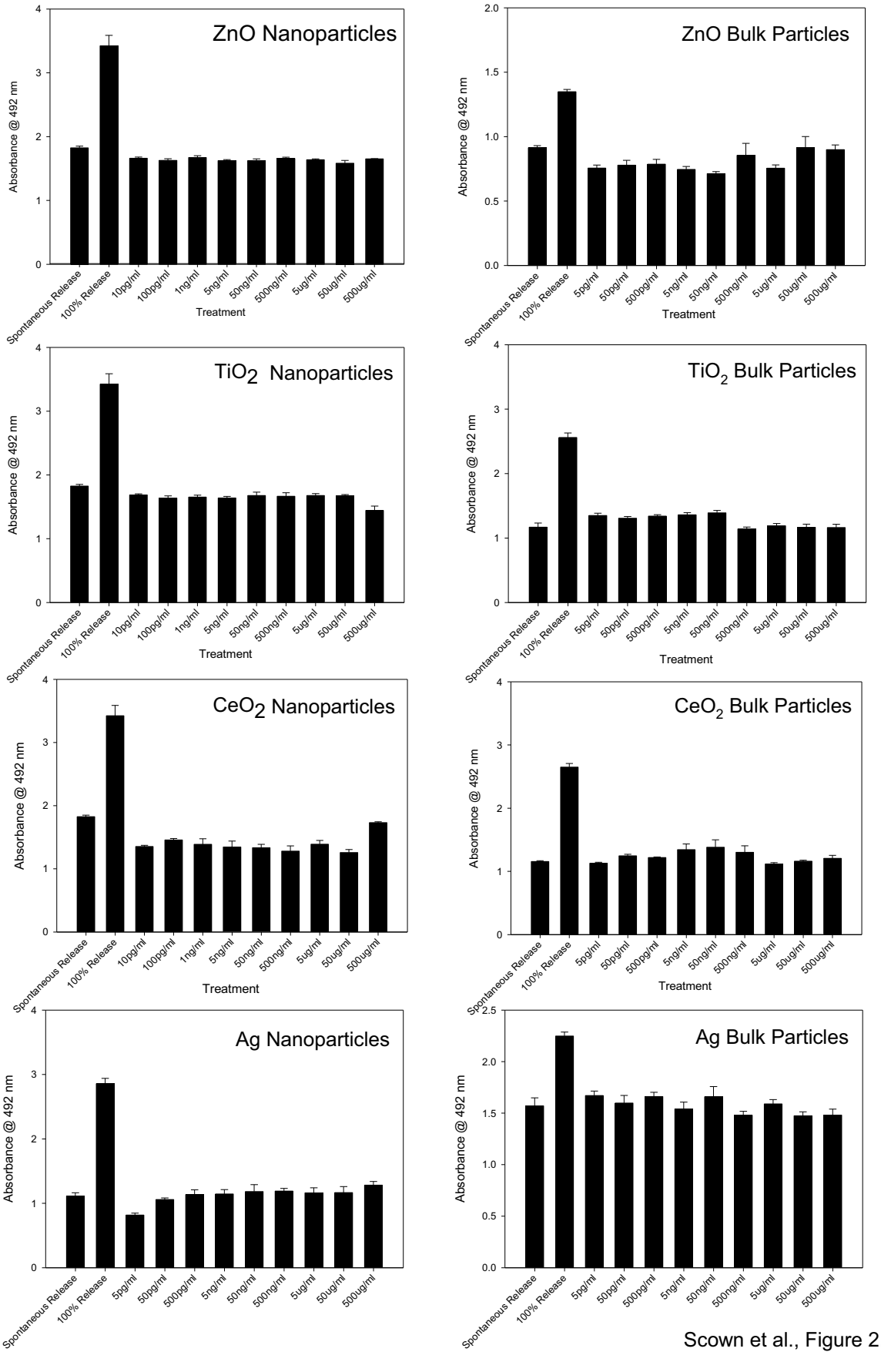


Figure 1. Scown et al



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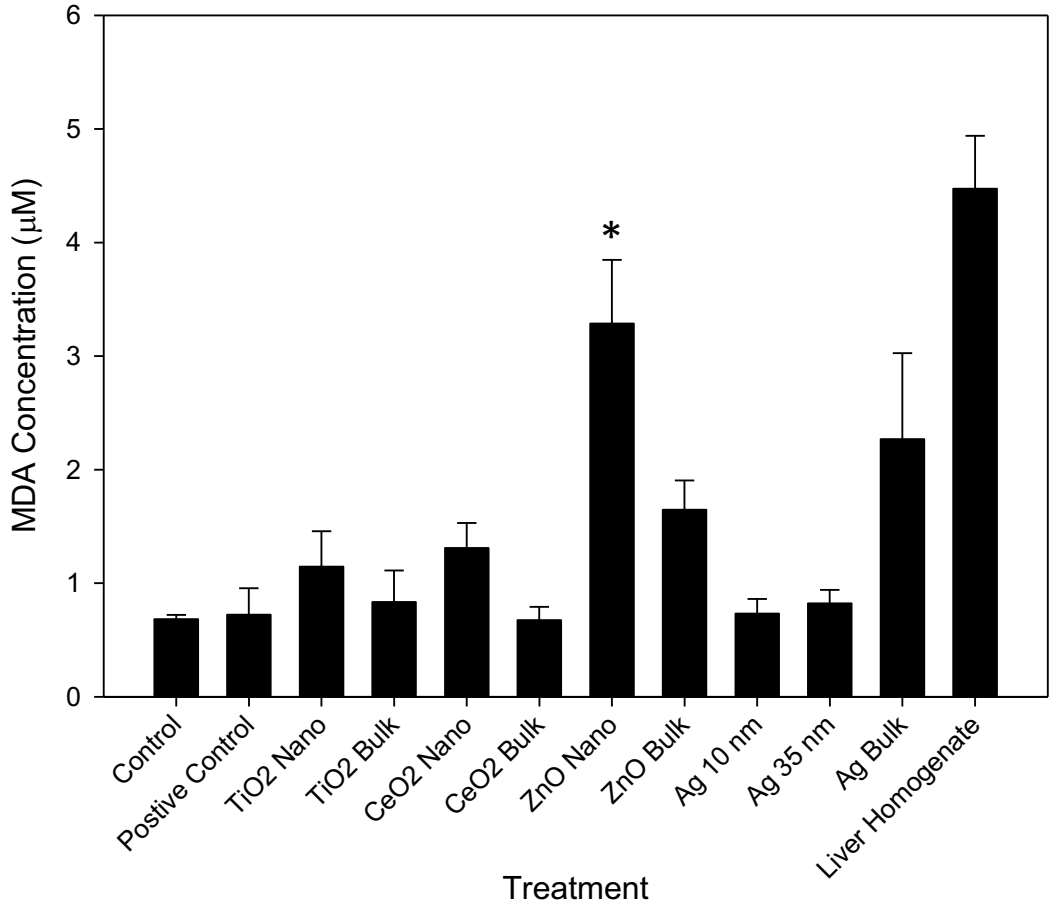

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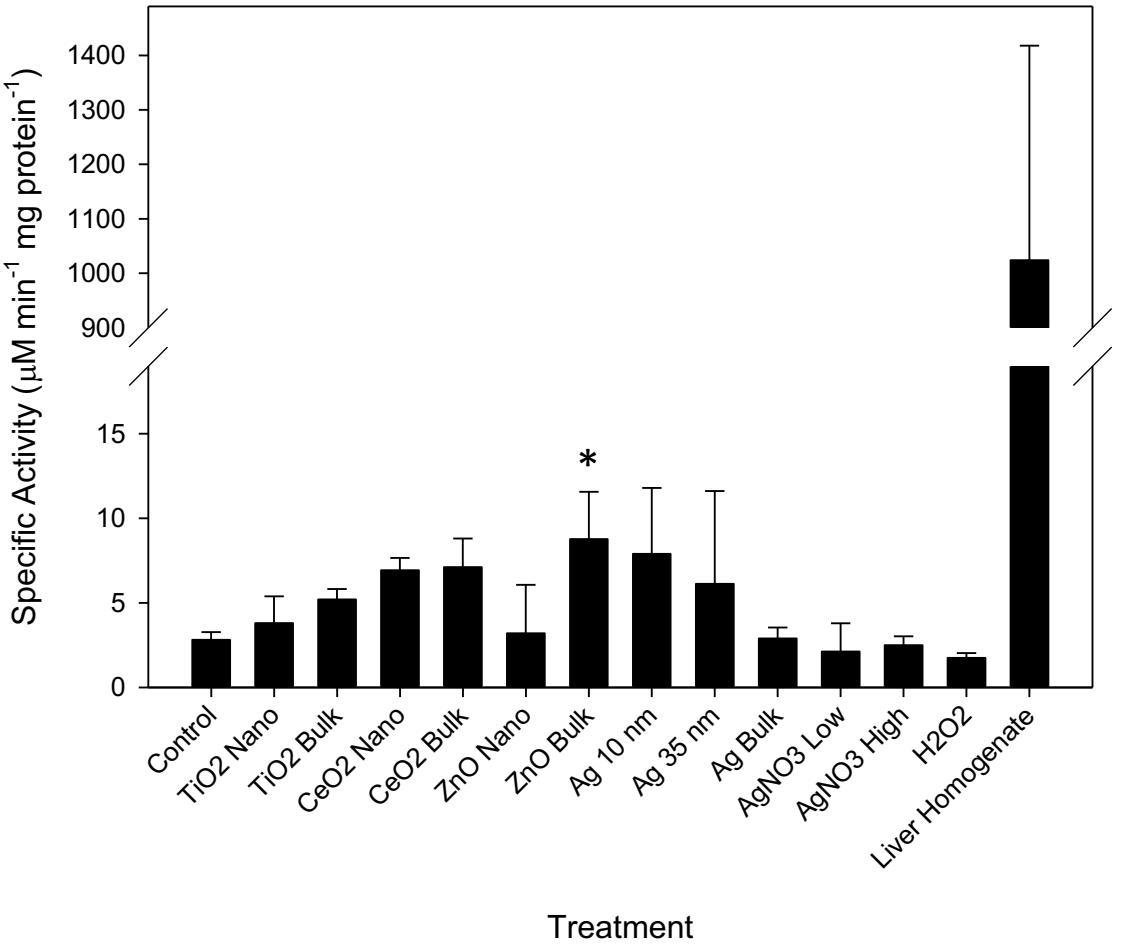

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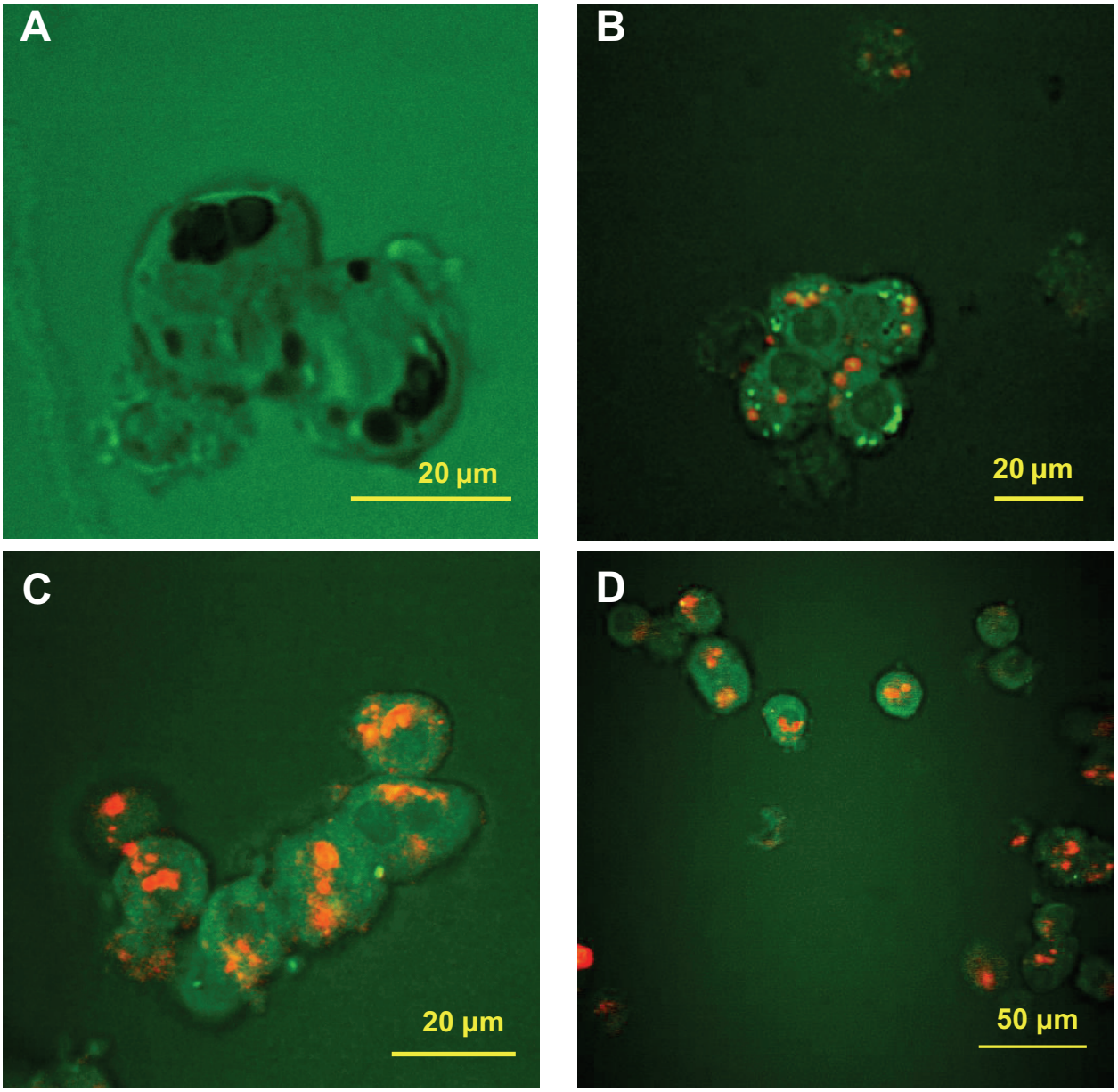

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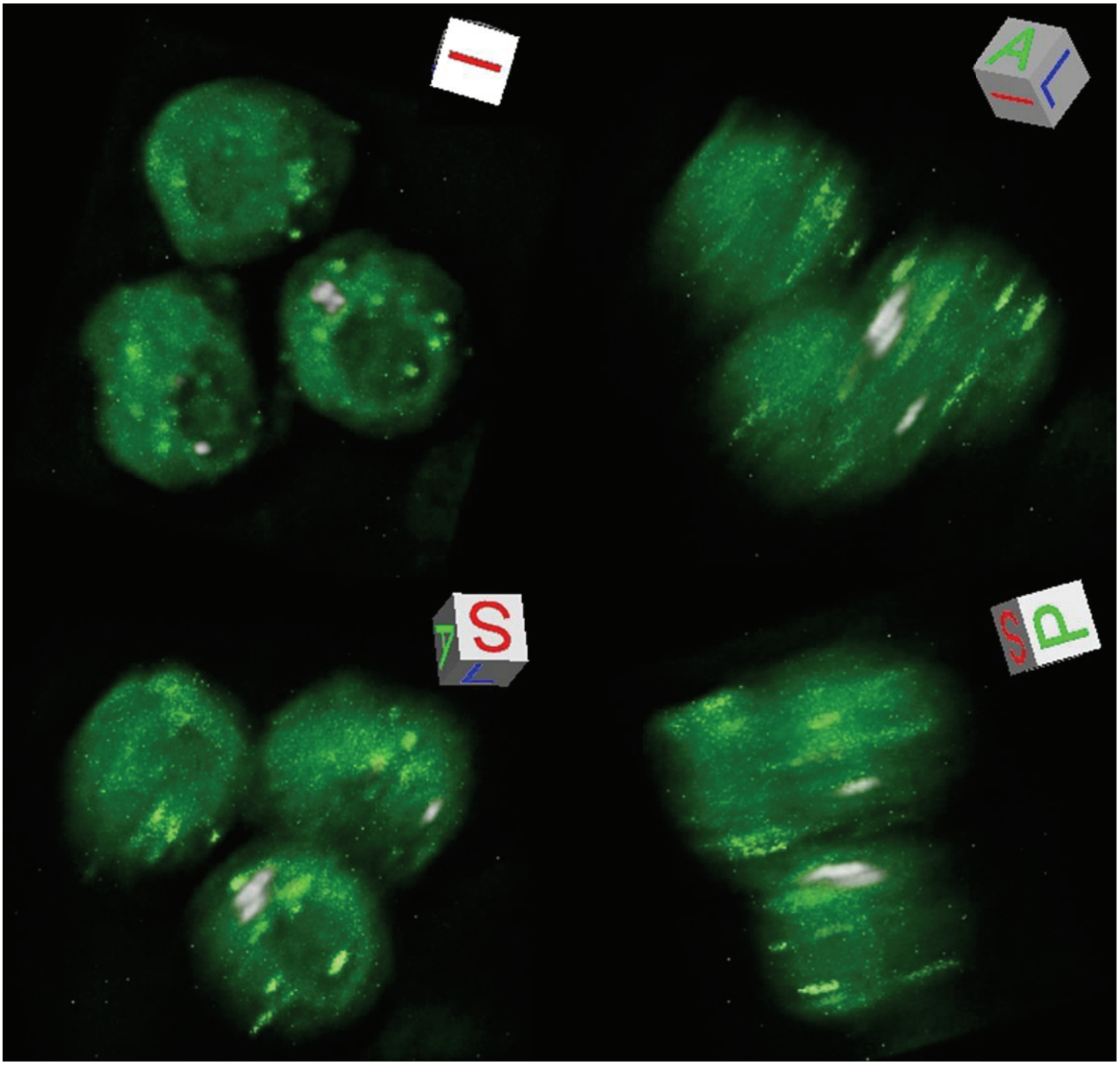

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		Manufacturers Specifications			Characterization Measurements			
Dispersion Media	**Particle Type**	**Purity (based on ICP trace metal analysis)**	**Average particle size (nm)**	**Specific Surface Area (m^2 g^{-1})**	**Hydrodynamic diameter - z-average (nm)**	**Polydispersity Index**	**Zeta-potential (mV)**	**pH**
HPLC Grade Water								
Nanopowder	TiO_2	>99.9%	23.2	46.3	189	0.27	+16.38	6.44
	ZnO	-	71	15	1207	0.72	-17.49	7.20
	CeO_2	-	10.6	76.0	338	0.40	+13.52	7.10
	Ag 10nm	99.9%	10	9-11	589	0.54	-12.52	7.11
	Ag 35nm	99.5%	35	30-50	2029	0.93	-6.50	7.34
Bulk Powder	TiO_2	99.4%	-	-	801	0.67	+19.96	6.99
	ZnO	>99.9%	480	-	1335	0.38	-28.50	6.71
	CeO_2	>99.9%	750	-	14724	0.90	-0.31	7.35
	Ag	99.95%	600-1600	-	938	0.69	-2.77	6.40
M199 Cell Culture Media								
Nanopowder	TiO_2	>99.9%	23.2	46.3	1393	0.62	-9.54	7.08
	ZnO	-	71	15	5697	1.00	-4.41	6.97
	CeO_2	-	10.6	76.0	2974	0.91	-7.78	7.34
	Ag 10nm	99.9%	10	9-11	9866	0.95	-2.60	7.35
	Ag 35nm	99.5%	35	30-50	9088	0.52	-0.27	7.58
Bulk Powder	TiO_2	99.4%	-	-	1533	0.77	-7.23	7.31
	ZnO	>99.9%	480	-	1303	0.87	-0.23	7.21
	CeO_2	>99.9%	750	-	18852	0.69	-0.24	6.99
	Ag	99.95%	600-1600	-	1602	0.69	-4.79	6.93

Table 1. Scown et al

CHAPTER 6

GENERAL DISCUSSION

6. GENERAL DISCUSSION

The science of the ecotoxicology of nanoparticles and nanotoxicology are still in their infancy, but with the size and importance of the nanotechnology industry rapidly increasing globally, the lack of knowledge and understanding about the potential impact it may have on the environment and human health urgently needs to be addressed.

Despite the vulnerability of the aquatic environment to exposure to anthropogenic compounds, the fate and behaviour and biological effects of ENMs in freshwater and marine environments have received relatively little attention. There is therefore an urgent need to increase the understanding of the potential impact of ENMs on aquatic organisms. In order to tackle this adequately, various issues relating to nanoparticle characteristics, their behaviour in aquatic media, and the differences between conducting exposures with ENMs and conventional xenobiotics need to be addressed. 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 ENM discharges into the aquatic environment.

In this chapter a critical synopsis of the findings of this work will be provided, placing them in context with the present, and rapidly emerging, state of the art. The shortfalls in the experimental approaches adopted and technological challenges faced will also be described and the current and future needs in the science will be highlighted, specifically with respect to the development of methods and techniques in order to allow for the field to progress.

6.1 OVERVIEW OF FINDINGS

The principal objective for my PhD studies was to examine the uptake and effects of a number of metal and metal oxide nanoparticles in fish. In order to do this, it was first necessary to investigate whether uptake of ENMs occurred, and if it did, in which organs the ENMs were localised. Once the target organs had been established, the resultant effects of the ENMs in these organs could be studied, as well as any differences in effects between particles of different sizes.

In the first study, the uptake of CeO_2 and ZnO nanoparticles in the bodies of zebrafish following exposure *via* the water, and the uptake of TiO_2 nanoparticles in the bodies of rainbow trout following exposure *via* the water and the diet were investigated in a series of experiments to test the following null hypothesis: fish exposed to metal oxide nanoparticles *via* the water column or the diet, do not accumulate metal oxides in their tissues as a result of exposure.

Analysis of zebrafish tissues by ICP-OES revealed significant uptake of cerium in the livers of fish exposed to 500 µg L^{-1} CeO_2 nanoparticles *via* the water column, however significant uptake was not seen in fish exposed to the higher concentration of CeO_2 nanoparticles (5000 µg L^{-1}). It is likely, therefore, that the presence of cerium in the liver was as a result of contamination from gut tissue during the sampling process as these tissues are closely associated *in vivo* and not due to actual uptake of CeO_2. Analysis of zebrafish tissues after exposure to ZnO nanoparticles *via* the water revealed no measureable uptake of ZnO in any tissues, although the high background levels of zinc occurring naturally in fish tissues may have masked low levels of uptake. In rainbow trout exposed to TiO_2 nanoparticles and bulk particles *via* the water CARS imaging revealed that there was limited uptake of TiO_2 nanoparticles across the gill epithelial membrane after waterborne exposure, there was little or no uptake in terms of measureable concentrations of TiO_2 using inductively coupled plasma – optical emission spectrometry/mass spectrometry (ICP-OES/MS) in any of the tissues examined following exposure *via* the water or the diet.

Chemical dispersants were not used to help achieve stable dispersions of the nanoparticles in the tank water to avoid introduction of any possible additional toxicity or mixture effects and dosing stocks were prepared solely by sonication. As a result of this, rapid aggregation of the TiO_2 nanoparticles in the exposure tanks occurred, especially on the introduction of fish, leading to a reduced concentration of nanoparticles in the water column and settling of TiO_2 aggregates on the tank floor. This therefore resulted in low bioavailability and limited uptake of the TiO_2 nanoparticles into the fish. It should also be realised however, that the techniques for quantifying TiO_2 uptake were limited in their

detection capabilities, possibly preventing measurement of low levels of uptake into the tissues. In spite of this, limited uptake of TiO_2 into the gill tissue after waterborne exposure was observed by CARS and the null hypothesis is therefore rejected.

In light of these findings, an intravenous injection study sought to investigate the kinetics and effects of TiO_2 nanoparticles introduced directly into the bodies of rainbow trout to test the null hypothesis TiO_2 nanoparticles introduced intravenously to rainbow trout are excreted rapidly and are not retained within the body. The findings of that study led to the null hypothesis being rejected as the kidneys were found to be the main target organ for accumulation of injected TiO_2, with clusters of nanoparticles, often compartmentalized in lysosomes evident in the hematopoietic tissue surrounding the kidney tubules. TiO_2 was also found to accumulate in the liver but at levels approximately 15 times lower than that in the kidney. Despite retention of TiO_2 in the kidneys for up to 21 days, no adverse effect on function and no evidence for lipid peroxidation were found in the kidney.

In the third study, rainbow trout were exposed to silver particles of varying size *via* the water column in order to test the null hypothesis that neither nanoparticulate, bulk or ionic silver show differences in uptake in rainbow trout exposed *via* the water column and observed toxic effects do not differ between particle type or ion.

Again, the dosing stocks were prepared without the aid of solvents or dispersants. The results of the study led to the rejection of the null hypothesis, as although aggregation and decreasing concentration of silver in the water column was observed, uptake in the gills and liver of trout occurred, unlike in the exposures to TiO_2, suggesting that the silver remaining in the water column was bioavailable to the fish. Furthermore, whereas smaller nanoparticles showed a greater propensity for association with gill tissue, there were no significant differences in uptake between particles of different sizes in the liver, possibly suggesting the route of uptake for silver particles to the liver was *via* the gut and not the gill epithelium. The dietary route cannot be ruled out in waterborne exposures as, although the fish were not fed for the duration of the exposure, fish may be exposed through drinking the water and in some cases were observed eating

aggregates of nanoparticles that had settled on the tank floor. A three-fold induction of *cyp1a2* in the gills of fish exposed to 100 µg L^{-1} 10 nm silver particles was also observed suggesting increased oxidative metabolism in the gills as a result of exposure.

In an attempt to develop an effective high through-put *in vitro* screening assay for nanoparticles, and building on the information from the preceding studies regarding characteristic effects in target organs, the suitability of isolated rainbow trout hepatocytes were examined for this purpose. Hepatocytes are a commonly used cell system for screening conventional xenobiotic compounds and the liver was shown to be a major target organ for silver particles entering into fish following waterborne exposure. Cultures of primary trout hepatocytes were exposed to a variety of metal and metal oxide nanoparticles at a range of concentrations to test the null hypothesis: primary trout hepatocytes are not a good model for assessing toxicity of ENMs so are not suitable for use in the development of high through-put screening assays for ENM toxicity.

Membrane integrity was not found to be affected by exposure to any nanoparticle type tested, however increased lipid peroxidation was observed in cells exposed to 500 µg mL^{-1} ZnO nanoparticles and increased GST activity was observed only in cells exposed to 500 µg mL^{-1} ZnO bulk particles, although the cells were unresponsive to positive controls in lipid peroxidation assays and GST assays despite remaining functional in culture as demonstrated *via* oestrogen induction of vitellogenin (VTG) synthesis. The hepatocytes, therefore, did not appear to be a good model for investigation of subtle toxic effects that may be elicited as a result of nanoparticle exposure, leading to the null hypothesis being accepted.

This said, CARS did provide evidence of uptake by the cells, suggesting hepatocytes may provide a useful model for studying uptake of ENMs into cells.

6.2 SHORTFALLS AND LIMITATIONS OF MY DATA AND TECHNOLOGICAL CHALLENGES FACED

A prominent challenge for the *in vivo* uptake studies was ascertaining the best way of tracing and measuring nanoparticles in fish to establish biodistribution in the tissues, and

this was an issue that was carefully considered before undertaking these studies. Although the use of fluorescent tags or coatings on the nanoparticles coupled with the use of confocal microscopy may have helped in identifying low levels of uptake in tissues, considering that the surface coating on a nanoparticle has the capacity to significantly alter its behaviour, it would be difficult to establish whether the uptake and distribution behaviour observed resulted from the nanoparticle itself or the applied coating. Furthermore, this method has less capacity for making quantitative measurements of tissue burden. Another option explored that would allow for quantitative uptake measurements to be carried out was the use of stable isotopes of the nanoparticles, however this option also proved problematic. Stable isotopes of the compounds to be used in these experiments were prohibitively expensive and at the time were also not yet available in nanoparticulate form. Since this time, stable isotopes have been proposed as a practical method for tracing nanoparticles (Gulson and Wong, 2006) and have been used to measure dermal absorption of zinc oxide (Gulson et al., 2008). Logistical and safety concerns regarding carrying out waterborne exposure studies using stable isotopes were also a major restriction for water born and dietary exposure studies in fish.

Analysis *via* ICP-OES and ICP-MS was employed for measurement of tissue burden of the metal and metal oxide nanoparticles in our studies, however there were also inherent problems in using these techniques. 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. Considerable time was spent optimizing the digestion methods both in terms of the tissues and the nanoparticle types used in order to maximise the percentage recovery. Whilst adequate methods were established for most of the nanoparticle types, the technique proved unsuitable for measuring ZnO nanoparticle uptake due to the relatively high background levels of zinc present in fish tissues. TiO_2 proved extremely difficult to digest due to its low reactivity and consequently, recovery levels were low (approximately 30%) and may have prevented detection of low levels of uptake in the tissues. Another disadvantage of using

ICP-OES/ICP-MS was that it does not give information about the form of the metal present in the sample before preparation. This is a particular issue for nanoparticles that may undergo some dissolution in water. In the silver exposure, it was therefore not possible to determine the proportion of dissolved ions released from the nanoparticles into the media, or the form of silver in the gills and liver tissues, so the relative importance of these ions in terms of uptake and mediating toxic response could not be determined.

The issue of whether or not to employ chemical dispersants or solvents in order to achieve monodispersed suspensions of nanoparticles in the exposure tanks was also given much thought. 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 ENMs 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.

It is likely that not using any dispersants in our exposures resulted in more limited bioavailability of the nanoparticles to the fish, especially in the case of TiO_2, therefore limiting our ability to examine toxic effects.

Despite this, the results of these studies reveal that although various types of raw nanoparticles have a propensity to aggregate in aquatic media, bioavailability in the water column and uptake of nanoparticles by trout is highly dependent on particle composition, as evidenced by uptake of silver into both gills and liver of trout.

Rapid aggregation behaviour was also exhibited by the nanoparticles in the cell culture media in the *in vitro* studies, suggesting cells were exposed to nanoparticle aggregates, as opposed to monodisperse nanoparticles. Although uptake of the nanoparticles into the cells was observed this aggregation behaviour is likely to have influenced their bioavailability, uptake and the resultant effects seen.

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 ENMs will enter the aquatic environment. From this perspective it could be argued that coated particles are of more direct environmental relevance. There is as yet very little information in the literature, regarding present or projected loads of ENMs 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. It was therefore difficult to design exposures with direct environmental relevance. In addition, although pH and cation content were monitored in our exposures, other water chemistry parameters such as the presence of humic and fulvic substances which are ubiquitous in natural waters may increase their stability and bioavailability in aquatic systems and these were not accounted for in our experiments. Nevertheless, understanding the fate and behaviour of the nanoparticles themselves before considering other factors is of fundamental importance and these studies still provide useful data to this context.

6.3 KEY ISSUES FOR THE PROGRESSION OF NANOTOXICOLOGY

It is clear that the differences in the way ENMs behave in aquatic media compared to conventional xenobiotics means a radically different approach to conducting aquatic exposures to ENMs is required.

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 ENM 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, as due to the less than optimal water quality and frequent disturbances, organisms will incur increased levels of stress that may make them more susceptible to uptake of compounds in the water column.

The 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 fish are necessarily exposed and in addition, the possible interactions of ENMs with NOM present in the water such as mucus exudates and faecal material from the fish means 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 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, both from the studies conducted, and from the increasing number of studies in the literature, that understanding exactly what organisms are exposed to during an aquatic exposure to ENMs is a dominant issue for the progression of nanotoxicology. An essential element of future nanotoxicity studies should therefore be a rigorous characterization of ENMs, experimental exposure media and characterization of the ENMs 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 characterization 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 ENMs and ENM aggregates through interactions of nanoparticles with organic elements in the exposure media (eg fulvic and humic substances) should also be studied as these are likely to have an impact on the aggregation and uptake behaviour.

This represents another major challenge in nanotoxicology and will be a complex task, as by necessity, it requires a multi-disciplinary approach involving many different analytical techniques and expertise across many scientific fields and further development of analytical tools and methodologies for both imaging and measuring nanoparticle parameters. Nevertheless, a firmer knowledge base in this area 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 provide 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 ENM 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 media would be neither a time nor cost effective approach to tackling the problem. Significant efforts should therefore be directed towards developing standard aquatic test methodologies for examining ENM 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 ENMs) 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 environmental levels of ENMs. Recently, attempts have been made to model the potential exposure of silver and TiO_2 nanoparticles to the environmental as a result of production of use of consumer products that contain them (Benn and Westerhoff, 2008; Boxall et al, 2008; Mueller and Nowack, 2008) and transport of TiO_2 nanoparticles from painted exterior facades to the aquatic environment has been demonstrated (Kaegi et al., 2008), however, data regarding actual environmental burden of any ENM type are not yet available. This is due in part to the fact that for most ENMs, current environmental levels are still likely to be extremely low but also the techniques and equipment needed to make such measurements are still being developed. For ecotoxicologists, this means conducting environmentally relevant exposures to ENMs is not yet possible. 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 ENMs and naturally occurring nanoparticles which may be similar in chemical composition. This again reiterates the importance of collaboration between scientific fields with differing specialities.

6.4 CONCLUSIONS

At this time, it is unknown to what extent nanotechnology is likely to be a big issue in terms of environmental health. Although the science is gradually becoming more data

rich, there is still a knowledge shortfall in terms of definitive information. It is imperative that studies become more integrative utilising multi-disciplinary approaches in order to push the science forward. Globally, steps are being taken to achieve this with funding initiatives encouraging both multi-disciplinary and multinational collaborative efforts. In 2008, the European Commission Seventh Framework Program budgeted approximately €600 million for research and development in nanotechnology, and invited calls for proposals in areas including development of risk assessment methodology for ENMs and the impact of ENMs on health and the environment, and a major collaborative research effort in environmental nanoscience between the United States of America and the United Kingdom is currently inviting proposals and aims to investigate fate and bioavailability of nanomaterials in biological and environmental systems with a budget of approximately £6 million.

It is likely that in between five to 10 years time, we will have a better idea of the potential impact of nanotechnology on the environment. This said, nanoparticles that are currently being developed for use in biomedical applications such as in medical imaging, pharmaceuticals and drug delivery are designed specifically to be able to penetrate cells and induce biological effects are likely to be a major future issue. It is therefore imperative that a reliable and applicable framework for conducting such aquatic toxicity tests is put in place now so that appropriate testing can be done when these problems arise.

CHAPTER 7

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CHAPTER 8

APPENDIX

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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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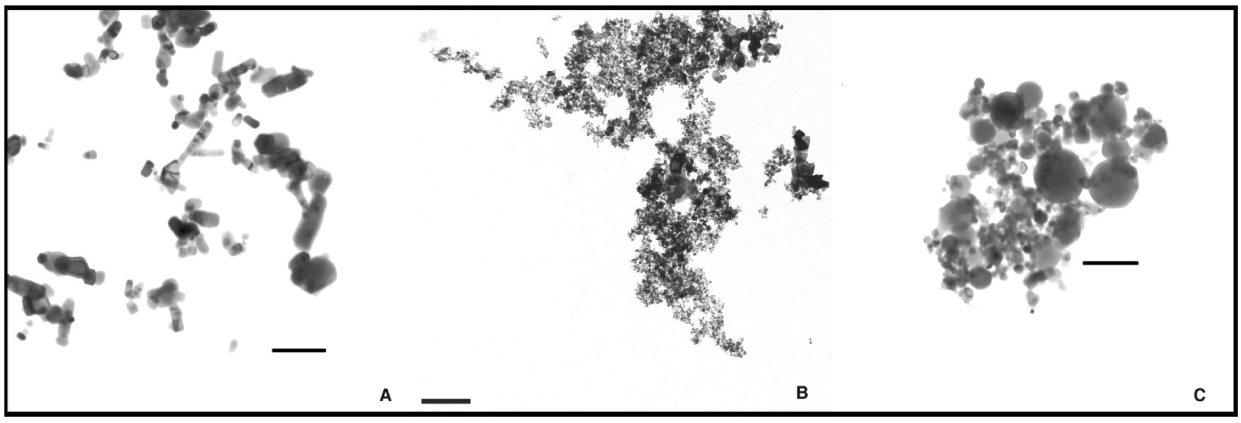

FIGURE 1. TEM micrographs of nanoparticle suspensions: (A) zinc oxide, (B) cerium dioxide, and (C) titanium dioxide. Scale bars represent 200 nm.

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 μg 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 μg CeO_2 L^{-1} but no significant uptake in fish exposed to 5000 μg 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.

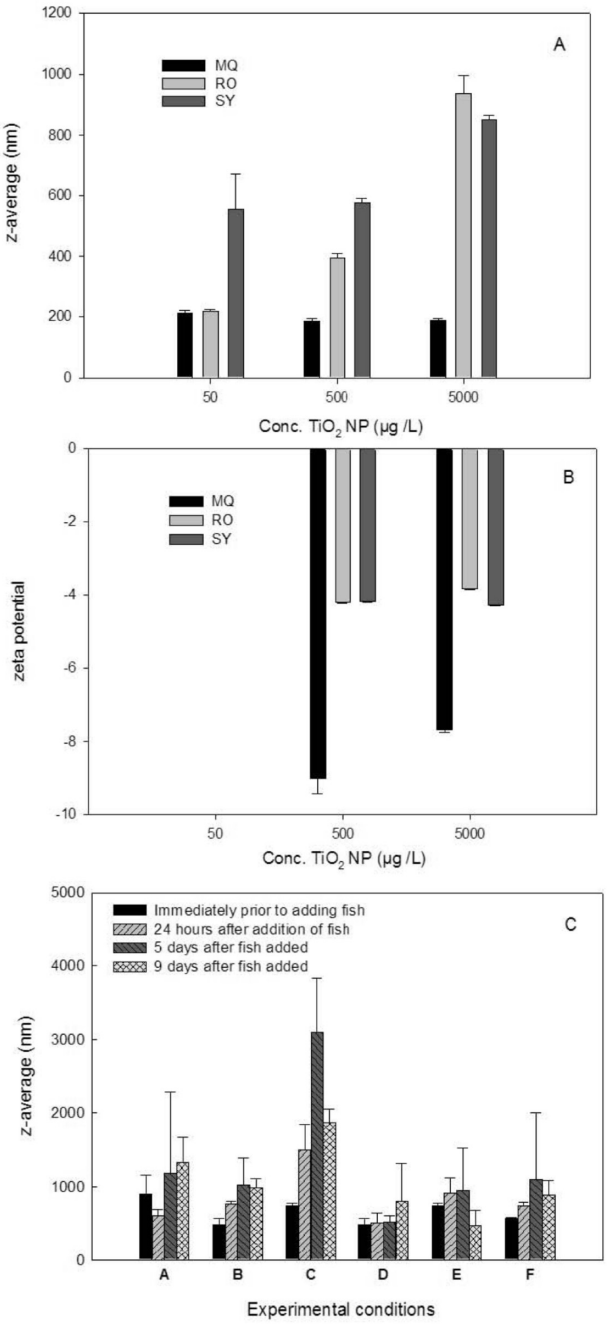

FIGURE 2. Hydrodynamic diameter and ζ potential of nanoscale titanium dioxide under different water conditions and exposure regimes. (A) Particle size vs concentration and water type. MQ, Milli-Q ultrapure water; RO, reverse osmosis-treated city water; SY, synthetic water (containing high ion concentrations; 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 μg L^{-1} TiO_2 NPs; group C, control, no fish, 5000 μg L^{-1} TiO_2; group D, fish added, 500 μg L^{-1} TiO_2 NPs; group E, fish added, 5000 mg L^{-1} TiO_2 NPs; group F, fish added, 5000 μg 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

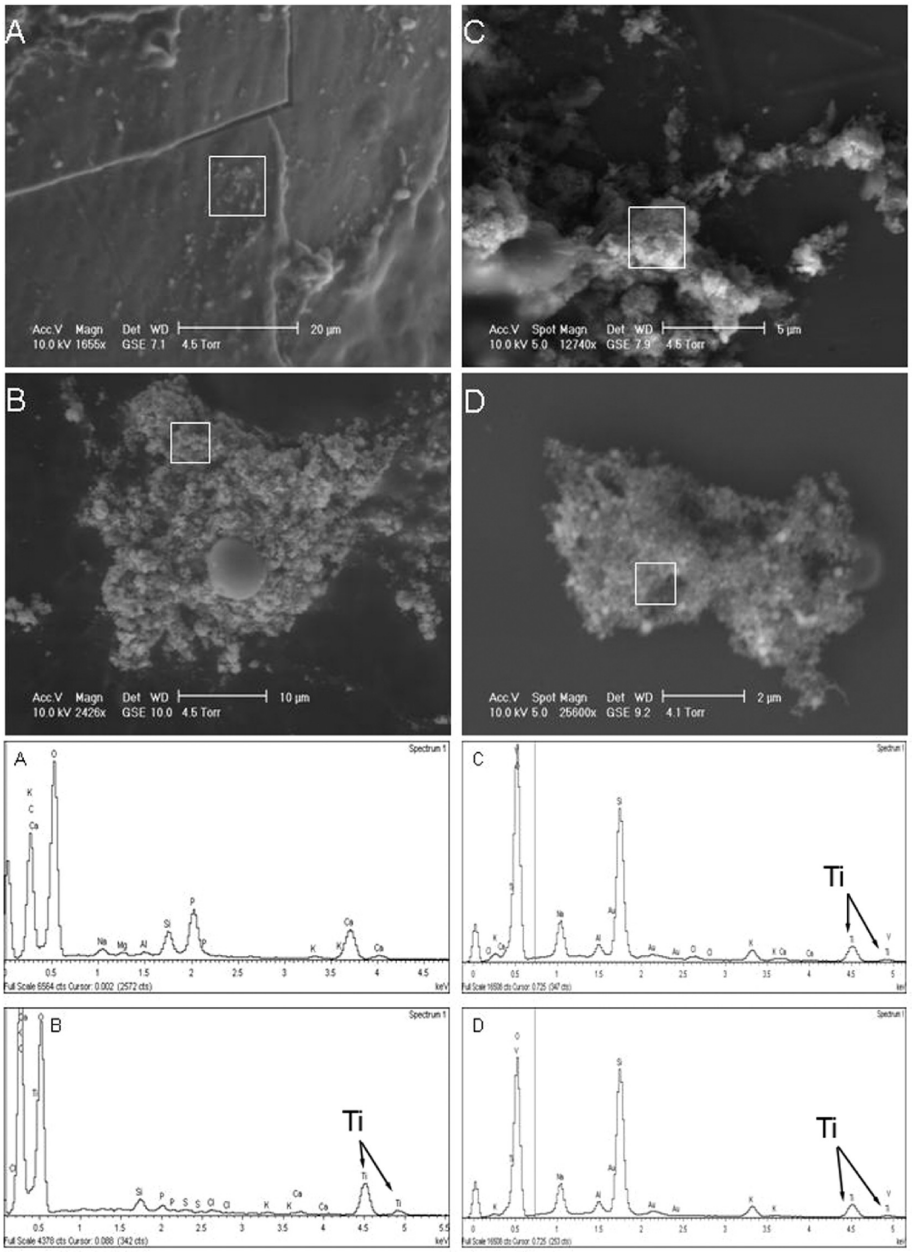

FIGURE 3. Environmental scanning electron micrographs (ESEM) of water samples and the corresponding EDX spectrum analysis (white square) at day 9 for water samples taken from tanks containing (A) fish with no particles, (B) fish with 5000 μg L^{-1} bulk TiO_2, (C) fish with 5000 μg L^{-1} TiO_2 NPs, 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; $n = 16$.

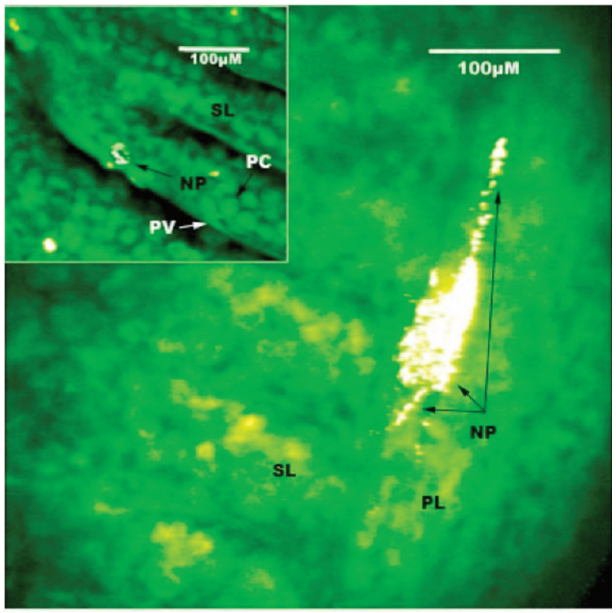

FIGURE 4. CARS image of TiO_2 nanoparticles on a section of the primary lamellae (main panel) and three-dimensional projection showing a nanoaggregate on the secondary lamellae (inset). PL, primary lamellae; SL, secondary lamellae; PC, pillar cell; PV, pavement cell (epithelium); NP with arrow, TiO_2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

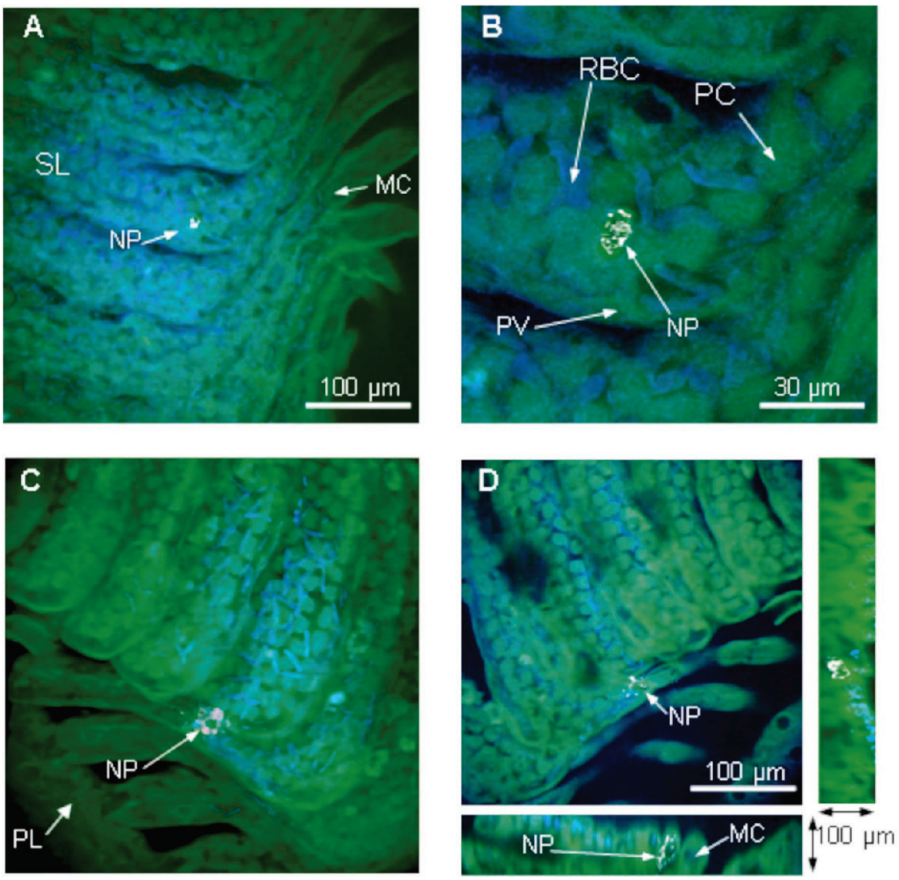

FIGURE 5. CARS images of gill tissue of rainbow trout, *Oncorhynchus mykiss*, following a waterborne exposure to TiO_2 nanoparticles (NPs). The cellular structure of the primary (PL) and secondary (SL) gill lamellae, composed of pillar cells (PC) and pavement cells (PV), was obtained by epidetection 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.

Acknowledgments

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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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SUPPORTING INFORMATION

Bioavailability of nanoscale metal oxides, TiO_2, CeO_2, and ZnO to fish

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1.0 Objective

The purpose of this study was to provide a comprehensive evaluation of the fate of metal oxide nanoparticles, such as zinc oxide, cerium dioxide, and titanium dioxide, in the aquatic environment using fish as a model organism.

2.0 Exposures

2.1 Experimental animals and water conditions

Zebrafish (*Danio rerio*, n = 200) were obtained from Exeter Aquatics (Devon, UK). Fish were kept in 60 L tanks supplied with mains tap water filtered by reverse osmosis (RO) and reconstituted with Analar grade mineral salts to standardized synthetic freshwater (SY): Na^+ = 19.50 mg L^{-1} (34.16 µS), K^+ = 1.94 mg L^{-1} (1.33 µS), Mg^{2+} = 6.98 mg L^{-1} (6.04 µS), Ca^{2+} = 27.62 mg L^{-1} (15.84 µS), pH 7.20 ± 0.1 SE. Prior to exposure, fish were maintained *via* a flow-through system and fed to satiation twice daily, each morning on freshly hatched *Artemia nauplii* and each afternoon on TetraMin® dry tropical flake food (Tetra Werke, Melle, Germany). Water temperature was maintained between 28 and 29°C. Fish were starved for 1 day prior to the experiments.

Rainbow trout (*Oncorhynchus mykiss*, n=80), ~200 g (~25cm) were obtained from Houghton Springs Fish Farm (Dorset, UK). Prior to exposure, fish were maintained in 500 L tanks supplied *via* a flow-through system with reconstituted RO water: Na^+ = 8.27 mg L^{-1} (14.07 µS), K^+ 2.07 mg L^{-1} (1.48 µS), Mg^{2+} = 4.38 mg L^{-1} (3.82 µS), Ca^{2+} = 24.50 mg L^{-1} (14.13 µS), pH 7.56 ± 0.04 S.E. and were fed on a maintenance ration of food (Emerald Fingerling 30, Skretting, UK) at a rate of 1% body weight per day. Water temperature was maintained between 9 and 11°C. Fish were deprived of food for 3 days prior to the experiments. A summary of all exposure regimes can be found in Figure S1.

2.2.1 Zebrafish exposed via the water to zinc oxide or cerium dioxide

Three 50 L tanks were filled with RO water and two were dosed with 10ml and 100ml zinc oxide or cerium dioxide nanoparticle stock suspension (2.5 g L^{-1}) respectively to give final concentrations of 500 µg L^{-1} and 5000 µg L^{-1} nanoparticles in the tank. Controls tanks included two tanks dosed with 500 µg L^{-1} bulk zinc oxide or cerium dioxide and two 500 µg L^{-1} zinc oxide or cerium dioxide tanks without fish.

After dosing, the water in each tank was agitated with a glass rod and a 10 mL water sample taken *via* glass pipette from the middle of the tank for analysis of nanoparticle concentration. Zebrafish (n=20 for zinc oxide; n=25 for cerium dioxide) were added to the first three tanks and exposed to the nanoparticles for 7 days. Airstones in the tanks provided continual movement of the water column throughout the exposure and water samples were taken from the middle of the exposure tanks daily. Fish were deprived of food for the duration of the experiment and were observed daily for 10 minutes to examine their behavior. At the end of the exposure fish were euthanized with benzocaine according to UK Home Office guidelines. The brain, gills, skin and liver were removed and frozen in liquid nitrogen, stored overnight at -80°C, and freeze-dried for 24 hours before weighing.

2.2.2 Rainbow trout exposed to TiO_2 via the water column

Glass experimental tanks (60 L) were set up for five treatment regimes in duplicate as follows: 500 µg L^{-1} TiO_2 NPs, 5,000 µg L^{-1} TiO_2 NPs, 5,000 µg L^{-1} TiO_2 bulk particles, 5,000 µg L^{-1} ionic titanium and water controls. Particle stock suspensions and ionic Ti solutions were sonicated for 30 minutes in a Decon F51006 ultrasonic waterbath to break up particle aggregates prior to dosing and re-dosing. The tanks were left for 24 hours with air stones providing continual movement of the water column then half drained and re-dosed to allow for nanoparticles adhering to the glass, which may affect the actual tank concentration. Eight rainbow trout were then added to each of the tanks.

Two 1 mL water samples were taken from each of the ten tanks one hour before addition of the fish, immediately after the fish were added, at 1, 2, 4, 12 and 24 hours and every 24 hours subsequently for 8 days (total exposure time = 9 days). Water changes of 50% were carried out on days 2, 4 and 6 and the tanks filled and re-dosed accordingly. Fish were euthanized at the end of the exposure and blood, brain, gills, skin, liver, gut and gall bladder were dissected as in previous exposures and stored at -20°C until further analysis.

2.2.4 Rainbow trout exposed to TiO_2 via the diet

Eight trout were placed into each of three pairs of 60L tanks supplied with flow-through RO water and allowed to acclimatize for one week before the start of the dosing. Three batches of pelleted feed containing differing amounts of TiO_2 nanoparticles were used for the feeding experiments. The feed base was made by grinding 500 g of the standard feed (Emerald Fingerling 30, Skretting, UK) in a blender and passing through a 1mm sieve. Low and high dose feed batches were made by thoroughly mixing 50 mg and 500 mg TiO_2 nanoparticles respectively with 50 g wheat flour. The control batch contained only 50 g wheat flour. The flour mixture was then added to the feed base and mixed thoroughly before addition of 0.5-1 L water and mixing to form a smooth paste. The paste was then passed through a piping bag (Ø 3 mm) and dried over night in a drying cabinet at 45ºC. The dried food was then broken up in to 10mm pieces. Fish in paired tanks were fed 16g of the control, low or high dose food daily based on a 1% body weight maintenance ration for 21 days *via* an automatic feeder.

Water samples of 1ml were taken daily from the top of each tank to ascertain the level of leaching from the food and feces into the water. Fish were euthanized at the end of the exposure and blood, brain, gills, skin, liver, gut and gall bladder were dissected as in previous exposures and stored at -20°C until further analysis.

2.3 CARS Microscopy

2.3.1 Light source

An Optical Parametric Oscillator (Levante Emerald, APE Berlin) pumped with a frequency doubled Nd:Vandium picosecond oscillator (High-Q Laser Production GmbH) was used. The pump laser generates a 6 ps, 76 MHz pulse train of 532 nm laser light with adjustable output power up to 10 W. The Optical Parametric Oscillator (OPO) uses non-critically phased matched optical parametric generation to produce signal and idler beams which exit the laser cavity collinearly with a perfect temporal overlap. The OPO provides continuous tuning over a wide range of wavelengths; from 670 nm to 980 nm for the signal, which is used as the pump, and between 1130 nm and 1450 nm for the idler output, used for the Stokes beam. The maximum combined output power of the signal and idler is approximately 2 W.

2.3.2 CARS microscope

Imaging was performed using a modified commercial inverted microscope and confocal laser scanner (IX71 and FV300, Olympus UK) suitable for conversion to multiphoton microscopy [1, 2]. To maximize the NIR throughput the standard galvanometer scanning mirrors were replaced with silver galvanometric mirrors and the tube lens was replaced with a MgF2 coated lens. A 60X, 1.2 NA water immersion objective (UPlanS Apo, Olympus UK) was used to focus the laser excitation into the sample. The scanning confocal dichroic was replaced by a silver mirror with high reflectivity throughout the visible and NIR (21010, Chroma Technologies). Due to the directional nature of the CARS generation, simultaneous forwards- and epi- detection is desirable [3]. The redundant internal detectors were replaced by external, non-descanned, detection, which has the advantage of increased efficiency for deep tissue imaging.

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 using 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. The anti-Stoke signal was isolated at each photodetector by a single band-pass filter cantered at 750 nm (HQ750/210, Chroma Technologies). Three-dimensional data was acquired by taking stacks of 2-dimensional images in the x-y plane each separated by an increment in the z direction, which was achieved by alteration of the objective focus.

3.0 Results and Analysis

3.1 *Analysis of aquarium water*

3.1.1 *Size & zeta potential of TiO_2 nanoparticles in experimental aquaria*

From Figure 2c in the main text of the manuscript, it can be seen that in the tanks where there were no fish, the particle size of the aggregated TiO_2 increased with time. In tanks that contained fish, the particles never reached a z-value over 1100 nm. A possible reason for this was a mechanism which aggregated the nanoparticles (i.e. fish mucus) and caused them to settle out of solution. With the higher concentration (5000 μg L^{-1} TiO_2) the particle sizes increased more before sedimenting to the bottom of the tank. The net result was that by T= day 9, only smaller particles were left in solution. Figure 2a in the main text also shows that increased particle concentration had an effect on aggregate size by increasing the rate of particle collision due to Brownian motion and thus increasing the chance of aggregation. This process is also controlled by charge (e.g. less charge, more aggregation).

Figure S2 shows the electrophoretic mobilities for particles in unfiltered aquarium water samples. Although no size-dependent or treatment dependent effects could be ascertained from the zeta potentials, it appears that the lowest charge occurs in tanks with the highest particle concentrations (including fish). This also supports the conclusion, that higher

concentrations of nanoparticles, or the presence of exudates from the fish, was increasing the aggregation of the nanoparticles and sedimentation of larger aggregates to the bottom of the tank. This effect was confirmed by visual observation, as large aggregates of TiO_2 formed shortly after introduction of the fish to the experimental aquaria.

3.2 Fluorescence EEM scanning

3.2.1 *FEEM scanning methods & peaks identified*

Fluorescence EEM (excitation-emission matrix) scans were analyzed for all fish tank samples using a Varian Cary Eclipse fluorescence spectrophotometer. Analysis was temperature controlled in 4 mL capacity cuvettes with 1 cm pathway at 20^{o}C. Excitation scans were between 200 and 400 nm and emission between 280 and 500 nm in 2 nm steps. Results were standardized to a mean Raman intensity of 20 units collected at ex 348 nm. DOC analyses were with a Shamadzu 5050 total carbon analyser. Two peaks were identified at Ex/Em 225-230/324-342 and at Ex/Em 280-285/324-354 both of which can be attributed to the protein, tryptophan [4], possibly related to the presence of faecal matter. Other peaks were present, presumably from the TiO_2 nanoparticles, but were not identified. Raman corrected intensities for peak 1 were 10 – 172.9 and for peak 2, 3 – 72.6 units. One sample,

(Figure S5 below) also showed fluorophores for fulvic and humic-like fluorescence.

3.2.2. FEEM scanning results for experimental aquaria (TiO_2 exposure)

There was a higher fluorescence signal for all the tanks containing the fish compared with tanks with no fish that we attribute to the tryptophan-like protein fluorophore (Figure S5). For the tanks containing the bulk material and the nanoparticles there was slightly less (but not significantly so) intensity which may be attributed to one or two factors: 1) the signal was quenched slightly by the TiO_2 particles – this has yet to be investigated fully, 2) the fish did not produce as much tryptophan, 3) The nanoparticles aggregated to the proteins and fell out

of solution. We did not find a fluorophore which could exclusively be attributed to fish mucus. The results compare favorably to the dissolved organic carbon DOC results and show a reasonable correlation to the tryptophan intensities (data not shown).

3.4 Fate of nanoparticles in fish

Analysis of the tissues of fish exposed to ZnO nanoparticles showed there was no significant uptake of zinc into any of the tissues examined. ICP-OES cannot distinguish between zinc oxide and any other form of zinc present in the tissues and as endogenous levels of zinc in fish tissue are relatively high, any zinc taken up must be measured against the background level of zinc already present.

There was no significant uptake of cerium in the brains, gills or skin of zebrafish exposed to CeO_2 NPs, however, there was a significant uptake of cerium to liver tissues of zebrafish exposed to CeO_2 NPs. In contrast to zinc, relatively small concentrations of cerium taken up into the tissues can be detected using ICP-OES.

Inter-individual variation in liver concentration was high, a few livers contained large amounts of cerium and some contained none at all.

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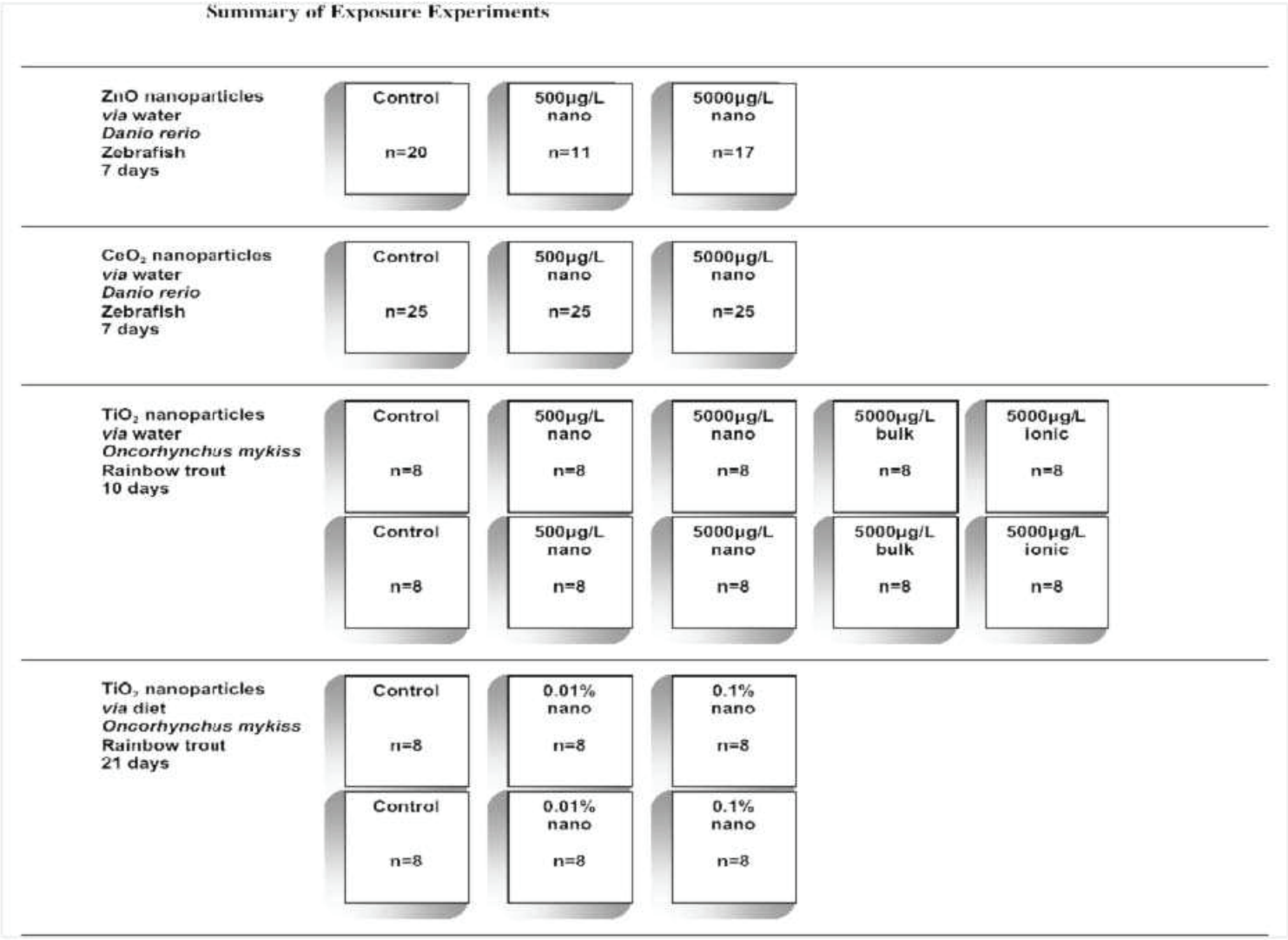

Figure S1. Summary of exposure regimes for all experiments for this study including zebrafish (*Danio rerio*) exposures to ZnO and CeO_2 *via* the water column and rainbow trout (*Oncorhynchus mykiss*) exposures to TiO_2 *via* the water column and *via* the diet.

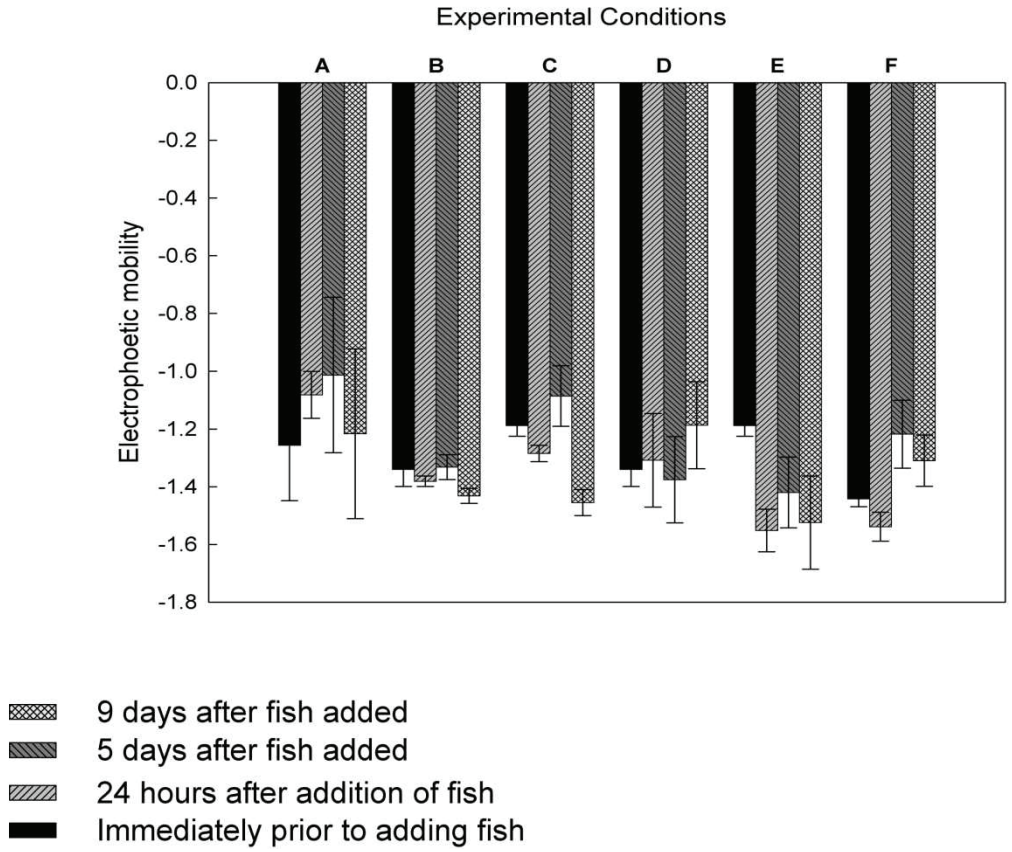

Figure S2. Electrophoretic mobilities of particles in exposure tank water; just prior to adding fish; 24 hours after fish were added; 5 days and 9 days: Where A is control, fish added, no nanoparticles (NPs); B is Control, no fish, 500 μg L^{-1} TiO_2 NPs; C is Control, no fish, 5000 μg L^{-1} TiO_2 NPs; D is fish added, 500 μg L^{-1} TiO_2 NPs; E is fish added, 5000 μg L^{-1} TiO_2 NPs; and F is fish added, 5000 μg L^{-1} bulk TiO_2.

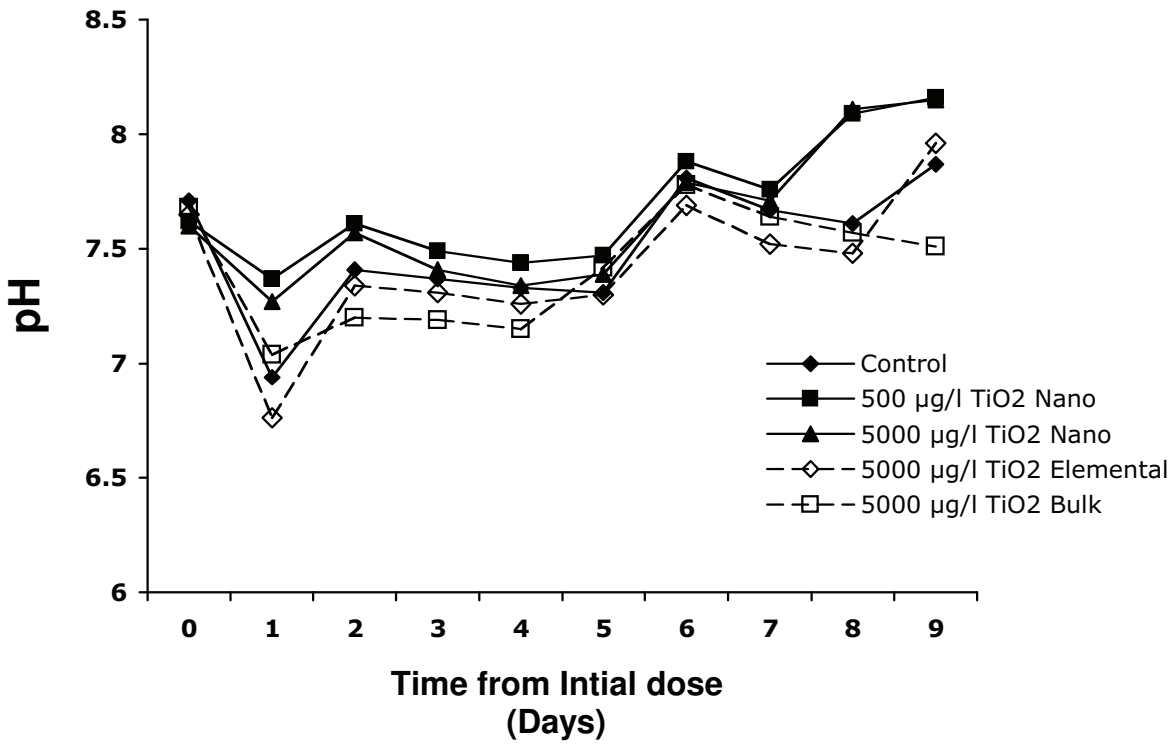

Figure S3. pH of experimental aquaria dosed with TiO_2 (NPs, bulk, or elemental) over the course of a water-borne exposure to rainbow trout, *Oncorhychus mykiss*. Data points indicate mean concentrations taken daily from two replicate tanks at mid-tank level.

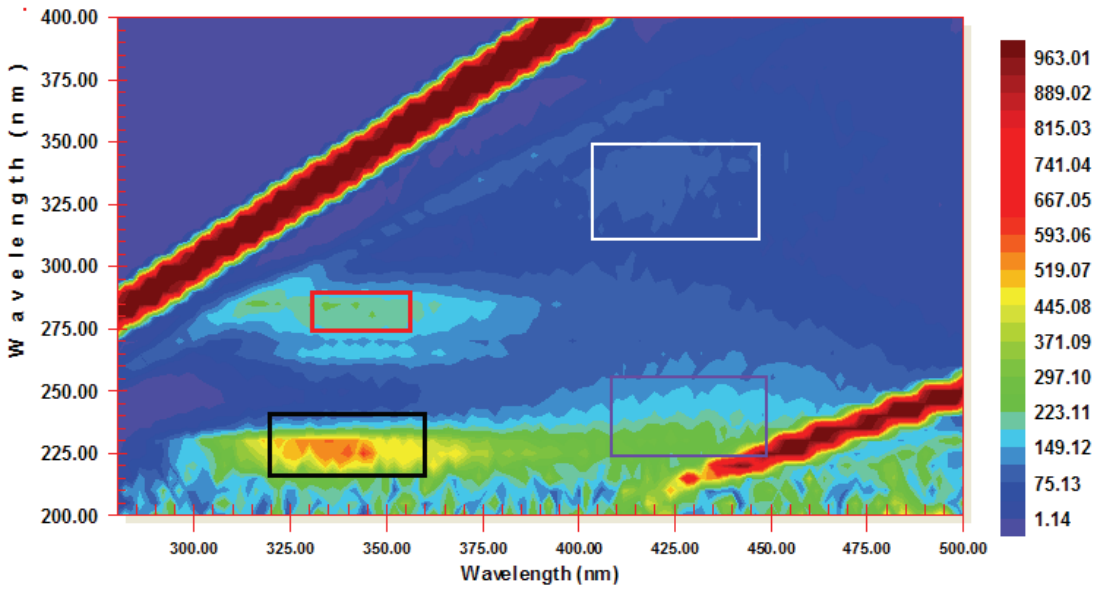

Figure S4. Fluorescence EEM image (Tank D: 500 µg L^{-1} TiO_2 NPs with fish on day 5) identifying the locations of 2 main peaks for tryptophan, where the black rectangle is peak 1 and the red rectangle is peak 2.

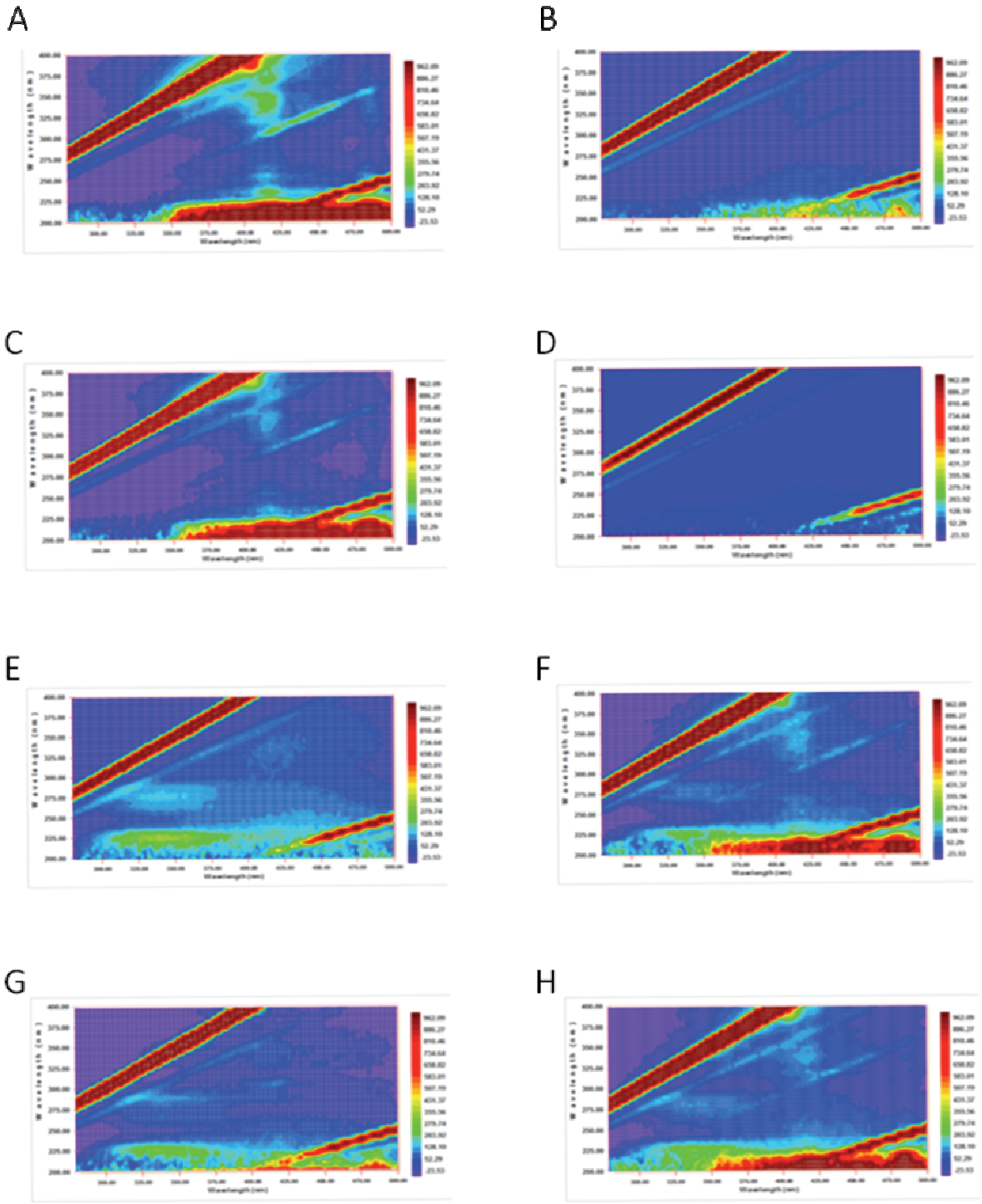

Figure S5. Fluorescence EEM images from the fish tanks before and after exposure to fish (a) 5000 µg L^{-1} TiO_2 NPs, time = Prior to fish introduction ; (b) 500 µg L^{-1} TiO_2 NPs, time = Prior to fish introduction; (c) 5000 µg L^{-1} TiO_2 bulk particles, time = Prior to fish introduction ; (d) Tank water only, no particles; (e) Fish only, day 9; (f) 5000 µg L^{-1} TiO_2 bulk particles, with fish, day 9 (g) 5000 µg L^{-1} TiO_2 NPs with fish, day 9 (h) 500 µg L^{-1} TiO_2 NPs, with fish, day 9.

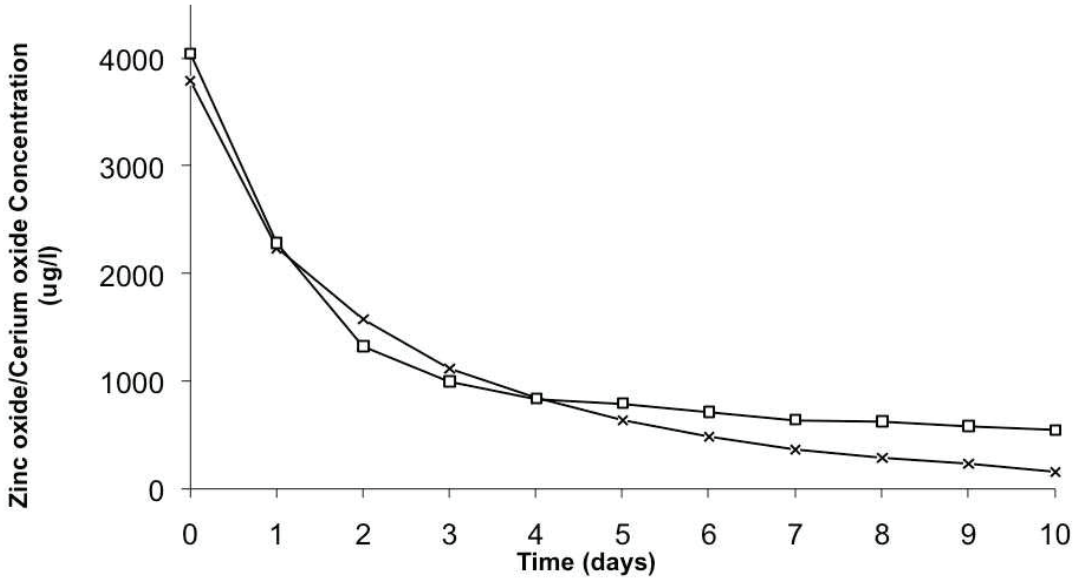

Figure S6. Concentration of cerium oxide (x) and zinc oxide (□) in tank water dosed at 5,000 μg L^{-1}. Data points indicate mean ± SE of concentrations taken daily from two replicate tanks at mid-tank level

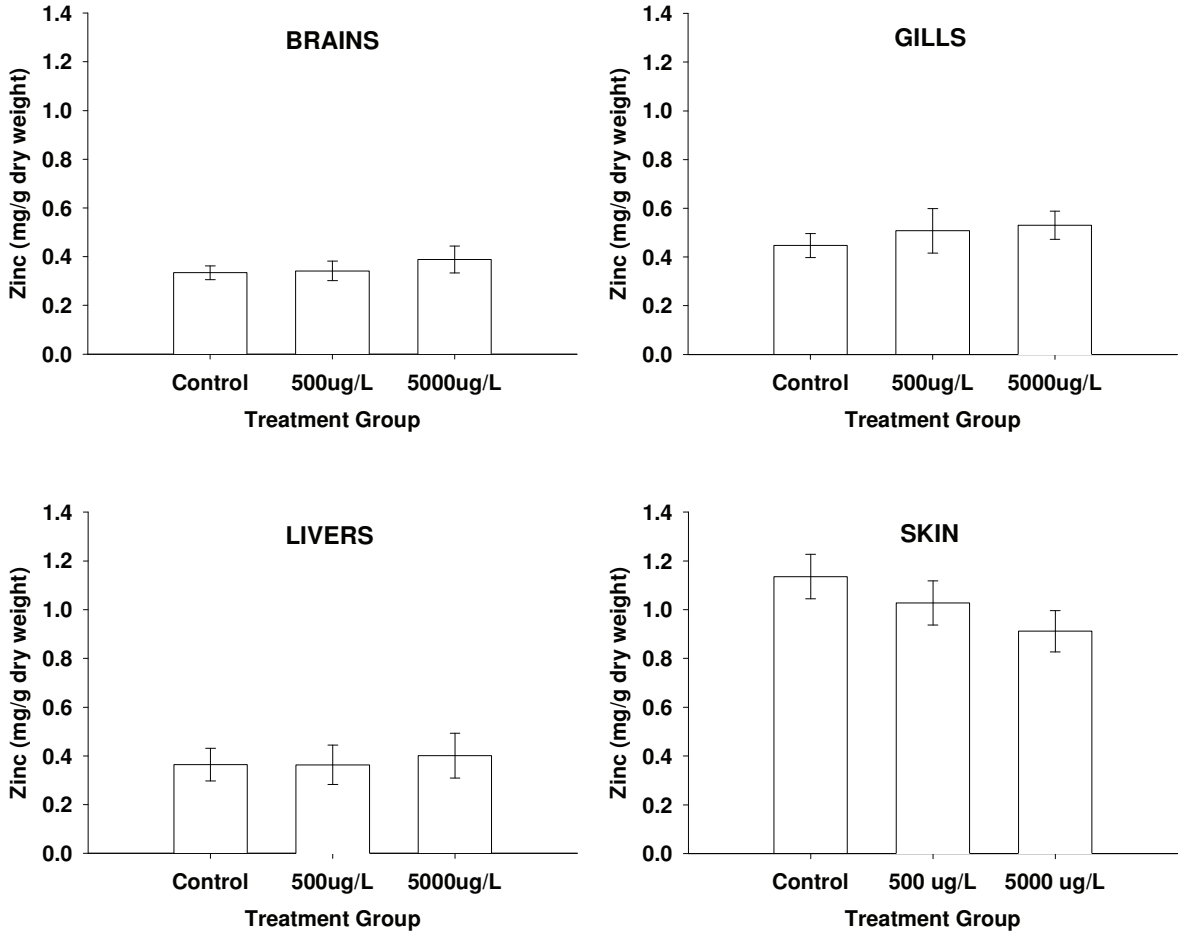

Figure S7. Zinc concentration in brains, gills, livers and skin of zebrafish, *Danio rerio*, exposed to two concentrations of zinc oxide nanoparticles. Error bars indicate ± S.E. (Numbers of fish sampled: control n=19; 500 μg L^{-1} n=11; 5000 μg L^{-1} n=17).

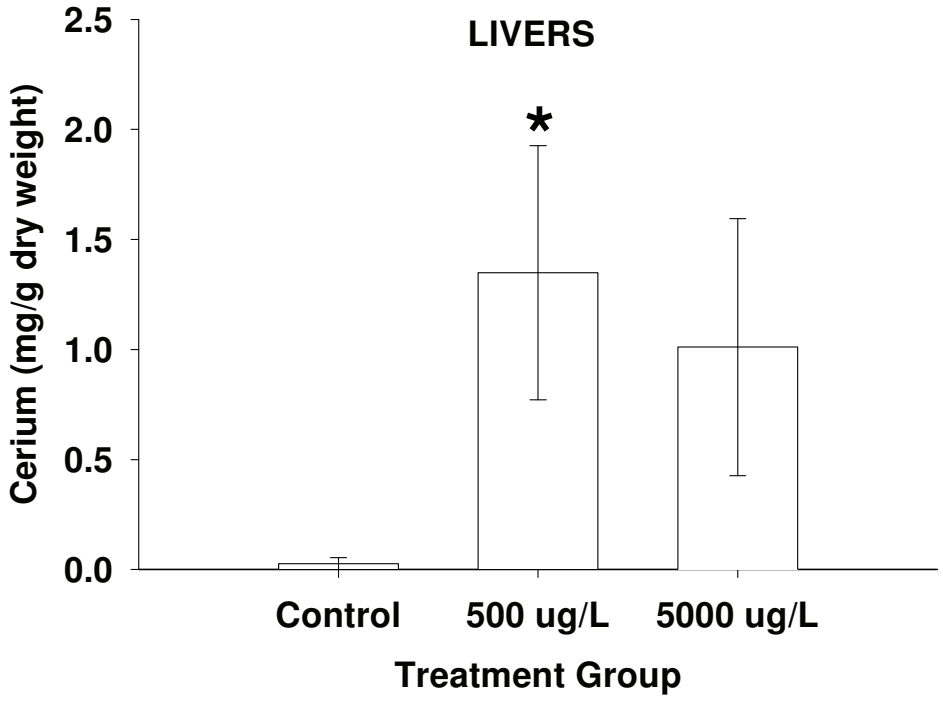

Figure S8. Cerium concentration in livers of zebrafish, *Danio rerio*, exposed to two concentrations of cerium oxide nanoparticles. Error bars indicate ± S.E. (n=25). * indicates significant uptake of cerium (Mann-Whitney, $p<0.0001$).

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Effects of Aqueous Exposure to Silver Nanoparticles of Different Sizes in Rainbow Trout

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Abstract

Despite increasing application of silver nanoparticles (NPs) in industry and consumer products, there is still little known about their potential toxicity, particularly to organisms in aquatic environments. To investigate the fate and effects of silver NPs in fish, rainbow trout

(*Oncorhynchus mykiss*) were exposed *via* the water to commercial silver particles of three nominal sizes: 10 nm (N_{10}), 35 nm (N_{35}) and 600-1,600 nm (N_{Bulk}) and to silver nitrate for 10 days. Uptake into the gills, liver and kidneys was quantified by inductively coupled plasma – optical emission spectrometry (ICP-OES), and levels of lipid peroxidation in gills, liver and blood determined by measurements of thiobarbituric reactive substances (TBARS). Expression of a suite of genes, namely *cyp1a2*, *cyp3a45*, *hsp70a*, *gpx* and *g6pd*, known to be involved in a range of toxicological response to xenobiotics was analysed in the gills and liver using real-time PCR. Uptake of silver particles from the water into the tissues of exposed fish was low, but nevertheless occurred for current estimated environmental exposures. Of the silver particles tested, N_{10} were found to be the most highly concentrated within gill tissues and N_{10} and N_{Bulk} were the most highly concentrated in liver. There were no effects on lipid peroxidation in any of the tissues analysed for any of the silver particles tested, and this is likely due to the low uptake rates. However, exposure to N_{10} particles was found to induce expression of *cyp1a2* in the gills, suggesting a possible increase in oxidative metabolism in this tissue.

Keywords: silver; rainbow trout; lipid peroxidation; nanoparticles; nanotoxicology; gene expression.

Introduction

Current growth in the nanotechnology industry and the increasing numbers of products making use of the unusual properties of engineered nanoparticles is becoming extremely important in the global economy. Increased production and use of nano-products will inevitably lead to increased levels of discharge of nanomaterials into the environment through their intentional and accidental releases or via weathering of products that contain them. The aquatic environment is particularly vulnerable as it is likely to act as a sink for many of these particles, as it does for many chemical discharges. The fate of nanoparticles in the aquatic environment, their interactions with biotic and abiotic components, and their potential to cause harm are all still poorly understood and these uncertainties are driving concerns on the risks they may pose to human and environmental health. Silver nanoparticles are already used in a variety of consumer products, notably for their antimicrobial properties, including in washing machines (Jung et al., 2007) and fabrics (Perelshtein et al., 2008), and prospective applications also include use in wound dressings (Arora et al., 2008), water treatment filters (Li et al., 2008), catalysts (Kumar et al., 2008), sensors (Schrand et al., 2008), inks (Wang et al., 2008) and pharmaceuticals (Chen and Schluesener, 2008; Sun et al., 2008).

Though their antimicrobial properties are well known (Choi et al., 2008; Jayesh et al., 2008), silver nanoparticles have also been shown to cause toxicity in vertebrate cell lines with findings typified by the generation of reactive oxygen species (Hussain et al., 2005; Schrand et al., 2008), apoptosis (Braydich-Stolle et al., 2005; Park et al., 2007), increased lipid peroxidation (Arora et al., 2008), reduced mitochondrial function (Braydich-Stolle et al., 2005; Hussain et al., 2005; Schrand et al., 2008) and depletion of oxidative stress markers (Arora et al., 2008; Hussain et al., 2005). Furthermore, a recent study by Larese et al., (Larese

et al., 2009) demonstrated absorption of silver nanoparticles in the *stratum corneum* and the outermost surface of the epidermis in both intact and damaged human skin.

A few *in vivo* studies in fish have shown some evidence of enhanced toxicity compared with bulk counterparts (i.e. larger particulate silver material >100 nm). Concentration dependent mortality and developmental effects, including spinal deformities and cardiac arrhythmia, have been shown in zebrafish (*Danio rerio*) embryos exposed to silver nanoparticles (5-20 nm), at a threshold concentration of 50 $\mu g\ mL^{-1}$. (Asharani et a., 2008; Yeo and Kang, 2008). In adult zebrafish, exposure to 20-30 nm silver nanoparticles has shown a greater toxicity compared with dissolved silver (Ag^+ from silver nitrate), based on mass of metal (Griffitt et al., 2009). In that work, more silver was associated with the gills in exposures to nanoparticles (26.6 ± 8.8 nm; 1000 $\mu g\ L^{-1}$) compared with fish exposed to silver ions and there was a greater thickening of the gill filaments in the silver nanoparticle exposed fish compared with controls.

The mechanism of toxicity of silver nanoparticles in fish has not been determined. An enhanced toxicity of nanoparticulate silver, compared to silver ions (Ag^+), may result from their shape and/or size, the release of silver ions (silver ions are well known to be toxic to aquatic organisms) (Mayer et al., 2003; Walker et al., 2008; Wood et al., 1996a); or a combination of both. Navarro et al., (2008), examined the rate of photosynthesis in *Chlamydomonas reinhardtii* exposed to silver nanoparticles or silver ions in both the presence and absence of cysteine (which binds free silver ions), and showed that silver nanoparticles were more toxic than silver ions, based on the concentration of ions present, requiring a higher concentration of cysteine to eliminate the toxicity. These findings suggest that interactions between the algae and the nanoparticles may enhance the release of silver

ions, in turn suggesting that the nanoparticles acted as an effective delivery vehicle for silver ions.

Environmental concentrations of silver nanoparticles have not been determined, but estimates in natural waters range between 0.03 to 500 ng L^{-1} (Luoma, 2008). Sock fabrics are potentially a major source of silver nanoparticles to the aquatic environment; (Benn and Westerhoff, 2008) showed that washing of socks impregnated with nanoparticulate silver resulted in the release of up to 1300 µg silver L^{-1}, some of it nanoparticulate. Many factors will likely affect the relative toxicity of nanoparticles for exposed organisms in natural waters, through effects on aggregation behaviour and thus their bioavailability, including pH and ionic concentrations, and interactions between nanoparticles and organic material and natural colloids (Baalousha et al., 2008; Handy et al., 2008b). Solubility is an important consideration in the toxicity of silver nanoparticles too, and factors affecting their solubility, such as presence of algae (Navarro et al., 2008), are also likely to influence this. It is not known how, or indeed if, the release of the silver ions is influenced by the size of the silver particle.

Previous work examining the toxicity of silver nanoparticles has provided conflicting evidence on the influence of size of particle on toxicity. A study by (Carlson et al., 2008) exposing alveolar macrophages to silver nanoparticles found that the generation of reactive oxygen species was size-dependent. Inoue et al (Inoue et al., 2009) also found that particle size was a determining factor in the level of lung inflammation in mice exposed to latex nanoparticles. However, in a series of pulmonary instillation studies in rats with nanoscale TiO_2 rods and dots, (Warheit et al., 2006) did not find that toxicity to the pulmonary system was dependent on either particle size or surface area. Contrasting with this, however, a

further study by this research group (Warheit et al., 2007) found that the surface characteristics of quartz particles were responsible for the differences in pulmonary toxicity. There may, of course, be particle type specific effects. That is, nanoscale may increase the inherent toxicity of some materials, but not others.

The purposes of our study were two-fold. Firstly, to investigate the fate and effects of silver nanoparticles in rainbow trout exposed *via* the water column, including at concentrations with likely environmental relevance, and secondly, to investigate any differences in the fate and biological effects due to the size of the particle. We exposed juvenile rainbow trout to three nominal sizes of silver particle: 10 nm (N_{10}) , 35 nm (N_{35}) and 600-1,600 nm (N_{Bulk}) and to silver nitrate for 10 days and measured the silver concentration in the gills, liver and kidney. Thiobarbituric acid reactive substances (TBARS) were measured in the blood plasma and in gill and liver tissue homogenates to ascertain whether lipid peroxidation had occurred as a result of exposure. Further, expression of a suite of genes related to metabolism and response to xenobiotics, specifically glucose-6-phosphate dehydrogenase (*g6pd*), glutathione peroxidase (*gpx*), cytochrome P450 1A2 (*cyp1a2*), cytochrome P450 3A45 (*cyp3a45*), and heat shock protein 70a (*hsp70a*) were analysed in the gills and liver as additional biological effect measures reporting on specific mechanisms of toxicity. Previous studies have shown the expression of these genes to be upregulated in rainbow trout (Lee and Buhler 2003; Råbergh et al., 2000; Walker et al., 2007; Williams et al., 1996) and brown trout (Hansen et al., 2006; Hansen et al., 2007) in response to exposure to various metals.

Materials and Methods

Rainbow Trout Husbandry

Juvenile female rainbow trout with a mean total body weight of 19.52 ± 0.56 g (mean ± S.E.) and fork-length of 12.39 ± 0.14 g (mean ± S.E.) were obtained from Hatchlands Trout Farm, Devon, UK. Fish were maintained in the laboratory in 500 L tanks supplied *via* a flow-through system with dechlorinated tap water (pH 7.79 ± 0.01 (S.E.); conductivity 189.58 µS ± 0.49 (S.E)) on a 12 hour light/12 hour dark cycle and were fed on pelleted feed (Emerald Fingerling 30, Skretting, UK), at a rate of 1% of their body weight prior to exposure. Water temperatures were maintained between 9 and 11˚C throughout.

Materials and Particle Characterisation

All chemicals were purchased from Sigma-Aldrich, UK unless otherwise stated.

Silver particles (designated as N_{10}, N_{35} and N_{Bulk}) were purchased from Nanostructured and Amorphous Materials Inc., Houston, USA. Based on the manufacturers specifications, N_{10} (average particle size measured by transmission electron microscopy, TEM) silver particles were spherical particles with a specific surface area of 9-11 $m^2 g^{-1}$, bulk density of 2.05 g cm^{-3} and a true density of 10.5 g cm^{-3} and had a purity of 99.9% based on trace metal analysis. N_{35} silver particles of average size 35 nm (measured by TEM; max < 100 nm) had a specific surface area of 30-50 $m^2 g^{-1}$, bulk density of 0.30-0.60 g cm^{-3} and a true density of 10.5 g cm^{-3} and had a purity of 99.5% based on trace metal analysis. N_{Bulk} silver particles had an average particle size of 0.6-1.6 µm and purity of 99.95%.

Our own characterization of all the silver particles was carried out in HPLC-grade water or on powders. Particle sizes (hydrodynamic diameters), polydispersity index and zeta potential

were measured on a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd. Malvern, UK) operating with a He-Ne laser at a wavelength of 633 nm using back scattered light. Our results are the means of triplicate runs, and in each run 5 measurements were made and standard errors were determined from the replicate measurements. Particle sizes and particle numbers were also measured using a NanoSight NTA LM10 with a laser output of 30mW at 650 nm. Mean square displacements of single particles were determined by tracking the scattered light followed by analysis by the Nanosight software, and standard deviations of the mean values were calculated. All measurements were carried out at nanoparticle concentrations of 1 mg L^{-1} after sonication for 30 minutes. The adsorption method was used to prepare samples for AFM (atomic force microscopy) analysis. In this method, mica sheets were cleaved on both sides, and then immersed vertically into the sample solution (10 mg L^{-1}) for 30 min (in the case of N_{Bulk}, both 30 min and 4 hrs were tested). Following adsorption, the mica sheets were withdrawn from the solution and gently rinsed by immersion in deionised water to remove non-adsorbed sample. All AFM images were obtained using a XE-100 AFM (Park Systems). All scans were performed in air, at room temperature and AFM height measurements were recorded. Images were acquired in a true non-contact mode and recorded in topography mode with a pixel size of 256 x 256 and a scan rate of 0.5-1.0 Hz. 80 particles were counted in case of N_{10}, 40 particles were counted in case of N_{35} and 10 particles in case of N_{Bulk}, and mean particle sizes including standard errors are reported. The low counts reflected the absence of particles on the mica. X-ray diffraction (XRD) was performed using a Bruker AXS D8 Autosampler. EVA software programme was used for the assignment of reflections and analysis of the XRD patterns. Crystallite sizes were evaluated using the Scherrer equation and mean standard error values are reported. A JEOL JSM-7000F Field Emission Scanning Electron Microscope was employed to characterise particle size and back

scattered electron images were recorded. Air-drying of a small drop of suspension directly onto a Formvar/Carbon 300 mesh Ni grid was employed.

An FEI TECNAI F20 Field Emission gun (FEG) coupled with an x-ray Energy Dispersive Spectrometer (X-EDS) from Oxford Instruments (Oxfordshire, UK) was used for TEM particle size analysis. The TEM was operating at an accelerating voltage of 200 keV, a field emission gun at emission 3, gun lens 2-3 (apparent size) and extraction voltage of 3800-4400 eV and spot size 2-3. The aperture of the second condenser lens was nominally 50 µm and the objective aperture was nominally 40 µm. TEM micrographs were collected on a Gatan TV camera and Digital Micrograph software was used to measure particle size. TEM samples were prepared by ultracentrifugation of nanoparticle suspensions on a TEM grid at 30000 rpm (150000g) using a Beckman ultracentrifuge (L7-65 Ultrcentrifuge) with a swing out rotor SW40Ti as described in (Wilkinson et al., 1999).

BET (Brunauer, Emmett and Teller) surface areas (m^2 g^{-1}) were determined on a Coulter™ SA3100™ series surface area and pore size analyser. Samples were outgassed for 10 hrs at 200 ˚C prior to analysis. Results are the means of three measurements and the standard errors are reported.

Experimental Design

Eight fish were deployed into experimental glass tanks measuring 60 cm x 30 cm x 38 cm (L x W x H) with a total volume of 36 L, and aerated with 10 cm airstones. Seven treatment regimes were run in duplicate and these were as follows: 10 µg L^{-1} N_{10} silver particles (N_{10} Low), 100 µg L^{-1} N_{10} silver particles (N_{10}High), 10 µg L^{-1} N_{35}silver particles (N_{35}Low), 100 µg L^{-1} N_{35} silver particles (N_{35} High), 100 µg L^{-1} N_{Bulk} silver particles (N_{Bulk}), 0.1 µg L^{-1} silver nitrate ($AgNO_3$) and water control. Fish were exposed for 10 days and were not fed for the duration of the exposure.

Dosing stocks of the silver particles were made by suspending 360 mg of each particle in 1 L ultrapure water (Maxima ultrapure water, Elga) and sonicating for 30 minutes, and diluting as required. The silver nitrate dosing stock (360 µg L^{-1}) was also made up in ultrapure water and sonicated similarly. Experimental tanks were dosed 24 hours prior to addition of the fish, then drained and re-dosed immediately before adding the fish to minimize reduction of nominal dosing concentrations through adhesion of the particles/chemical to the glass and airstones. Water changes of 75% (27 L) and corresponding re-dosing was carried out every 48 hours. Dosing stocks were sonicated for 30 minutes prior to each dosing.

Water samples (9 mL) were taken immediately after addition of the fish, at 1, 2, 4, 8, 12 and 24 hours, before and after every water change, and at the end of the exposure for analysis of silver concentration by ICP-OES. Water pH and conductivity were monitored daily and ranged between 7.58-8.54 and 179-212 µS, respectively.

Experimental Sampling

At the end of the exposure period (10 days), fish were euthanised with an overdose of benzocaine. One mL blood was taken from each fish and centrifuged at 12,000 x g for 5 minutes at 4°C and the plasma removed and stored at -20°C for analysis of thiobarbituric reactive substances (TBARS). Both gill arches were removed and divided as follows: approximately 50 mg was taken and flash-frozen in liquid nitrogen and stored at -80°C for RNA extraction and subsequent gene expression analysis; approximately 100 mg was taken and frozen in liquid nitrogen and stored at -20°C for analysis of TBARS and the remainder was weighed, frozen in 50 mL falcon tubes and stored at -20˚C before preparation for measurement of silver content using inductively-coupled plasma optical emission

spectroscopy (ICP-OES). Additionally, from one fish in each tank, a 4 mm section of gill tissue was taken and fixed in formalin for histopathological analysis. A further 4 mm section was taken from one fish from the N_{10} High treatment and one control fish and fixed in 2% paraformaldehyde/2.5% glutaraldehyde for TEM analysis. The liver was dissected out from each fish and a 50 mg sample taken and stored at -80˚C for subsequent RNA extraction. In addition, a 100 mg section taken and stored at -20˚C for TBARS analysis, and a 4 mm^3 sample taken and fixed in formalin for histological processing and tissue effects analysis. The remaining liver tissue was weighed, frozen and stored at -20˚C prior to preparation and analysis for silver content by ICP-OES. Kidneys were dissected out, weighed, frozen and stored at -20˚C for analysis silver content via ICP-OES.

Determination of Silver Concentrations in Fish Tissues

Tissue and blood samples were defrosted and digested at room temperature with a combination of 4 mL concentrated HNO_3 (AR grade, Fisher Scientific) and 1 mL hydrogen peroxide (Laboratory reagent grade, Fisher Scientific) for 24 hours before being heated for 24 hours on a Gerhardt Kjeldatherm digester unit at 125°C. The temperature was then increased to 190°C to evaporate the nitric acid and the samples subsequently re-dissolved in 10 mL 10% HNO_3 and 200 µL of 10% Triton-X 100. The samples were then analysed on a Vista-MPX CCD Simultaneous ICP-OES. Water samples were prepared for analysis by the addition of 1 mL HNO_3 and 200 µL 10% Triton-X 100. Calibration standards containing 500 µg L^{-1} and 1000 µg L^{-1} and a quality control standard containing 400 µg L^{-1} silver were made up from a stock solution of 157.5 mg L^{-1} silver nitrate in 10% HNO_3. Internal standards of 1, 10, 100, 500 and 1000 µg L^{-1} were made using all silver particle types suspended in 10% HNO_3 dispersed with 10% Triton-X 100 and used according to the exposure regime samples being measured, i.e. N_{10} internal standards for N_{10}exposure samples. All samples and

standards were sonicated for 30 minutes prior to measurement. The detection limit of the ICP-OES for silver in the tissue and water samples was 10 μg L^{-1}.

Histological analyses

Trout liver and gill tissues were fixed in 10% neutral buffered formalin for 6 hours before being transferred to 70% industrial methylated spirits (IMS). The tissue samples were dehydrated in series in IMS (AR grade Fisher Scientific) in a Shandon Hypercenter XP Tissue Processor and embedded in paraffin wax. The samples were sectioned on a rotary microtome (Leica). Five microscope slides, with 5-6 sections per slide, were prepared for each tissue sample) and the slides were stained with haematoxylin and eosin in a Shandon automatic slide stainer (Thermo Scientific, UK). Tissues were examined and assessed for any signs of histopathological changes using a Zeiss Axioskop 40 light microscope with an Olympus DP70 Digital Microscope Camera and AnalySIS Image Processing Software and examined for evidence of gill or liver injury.

Gill tissues were fixed for Transmission Electron Microscopy (TEM) adopting an in-house method. Briefly, tissues were fixed for 2hrs in 2% paraformaldehyde/2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4), washed three times in 0.1M phosphate buffer for 5 mins and fixed in 1% osmium tetroxide in phosphate buffer for 1 hour. The tissues were then washed in deionised water for 5 x 5 minutes, before being cut into 1 mm^3 pieces and subsequently suspended in 2% uranyl acetate for 1 hour. The tissues were then dehydrated in an ethanol series: 30%, 50%, 70%, 90% and 100% (x2), in each for 10 minutes and embedded in TAAB resin. The tissues were blocked in shallow planchets and placed in a 60°C oven for 20 hours. Tissues were sectioned on an ultramicrotome (Ultracut, Reichert) and examined for structural alterations and subcellular localization of silver using a Joel TEM 1400 transmission electron microscope.

Measurement of Lipid Peroxidation

Measurement of 1,1,3,3-tetramethoxypropane (malonaldehyde; MDA) using the TBARS assay is widely used as an indicator of lipid peroxidation. The protocol adopted was similar to that described previously (Conner *et al.*, 2006). Briefly, 8 standards of MDA at concentrations ranging from 0.625 μM to 100 μM were prepared from a 500 μM stock solution of MDA in 2% ethanol. Tissue samples were homogenized with a hand-held tissue homogenizer with 250 μL 100 mM HEPES. The samples were then centrifuged at 1600 x g for 10 minutes at 4°C and the supernatant reserved. 100μL of tissue homogenate/plasma and standards were mixed with 500 μL of 0.4% thiobarbituric acid (40 mg in 10 mL) in 10% acetic acid, pH 5.0.. The tubes were heated to 90°C for 1 hour and then cooled to room temperature under tap water. 600 μL of butanol was added and the mixture was then vortexed and then centrifuged at 3,100 x g for 10 min. The butanol phase was removed and 150 μL placed in duplicate in a flat-bottomed, 96-well microtiter plate and the absorbance measured at 532 nm on a Molecular Devices SpectraMax 340pc microtiter plate reader. A standard curve was prepared and MDA concentrations were determined accordingly.

Gene expression analyses

Total RNA was extracted from each tissue sample using Tri Reagent (Sigma) following the manufacturer's instructions. Total RNA concentrations were determined by measuring the absorbance at 260 nm using a Nanodrop ND-1000 spectrophotometer (NanoDrop

Technologies, USA). The RNA was treated with DNase (Cat. No. M6101, Promega) and subsequently reverse-transcribed to cDNA using M-MLV Reverse transcriptase (Cat. No. M1701, Promega) and random hexamer primers as previously described (Filby and Tyler, 2005).

Real-time PCR assays were developed for the quantification of a selection of genes involved in the response to xenobiotics and their metabolism in rainbow trout. Primers specific for rainbow trout glucose-6-phosphate dehydrogenase (*g6pd*; Genbank Accession no. EF551311: 5′-CGGTTGTCTGTGTTCTTC-3′/5′-GGTGCTTGATGTTCTTGG-3′), glutathione peroxidase (*gpx*; Genbank Accession no. AF281338: 5′-GCTCCATTCGCAGTATTC-3′/5′-TCCTTCCCATTCACATCC-3′), cytochrome P450 1A2 (*cyp1a2*; Genbank Accession no. U62797: 5′-CTTCCGCCATATTGTCGTATC-3′/5′-CCACCACCTGCCCAAAC-3′), cytochrome P450 3A45 (*cyp3a45*; Genbank Accession no. AF267126: 5′-GTCCTCTCACCTTCCTTTACC-3′/5′-TCTGCCTGCTTCTTCATTCC-3′), heat shock protein 70a (*hsp70a*; Genbank Accession no. NM_001124228: 5′-CTGCTGCTGCTGGATGTG-3′/5′-GCTGGTTGTCGGAGTAAGTG-3′) were designed for use in real-time PCR with Beacon Designer 7.2 software (Premier Biosoft International, Palo Alto, CA, USA) according to manufacturer's guidelines and purchased from MWG Biotech. Assays were optimized and validated for real-time PCR using SYBR Green chemistry as described previously (Filby and Tyler, 2005).

Assays had detection ranges of at least three orders of magnitude. Specificity of primer sets throughout this range of detection was confirmed by the observation of single amplification products of the expected size and melting temperature. All assays were quantitative with standard curve (mean threshold cycle [Ct] vs. log cDNA dilution) slopes of -2.754 (*g6pd*), -3.367 (*gpx*), -2.901 (*cyp1a2*), -2.722 (*cyp3a45*), -3.538 (*hsp70a*), translating to high PCR

efficiencies (E) of 2.31 (*g6pd*), 1.98 (*gpx*), 2.21 (*cyp1a2*), 2.33 (*cyp3a45*), 1.92 (*hsp70a*). Over the detection range, the linear correlation (R^2) between the mean Ct and the logarithm of the cDNA dilution was >0.98 in each case.

Gene expression was measured for each gene in liver and gill samples using SYBR Green chemistry with the iCycler iQ Real-time Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) according to the protocols described previously (Filby and Tyler, 2005).

For each gene, samples were analyzed in triplicate. Relative expression levels were calculated using an efficiency-corrected version of the arithmetic comparative $2^{-\Delta\Delta Ct}$ method, as described previously (Filby and Tyler, 2005). The housekeeping gene ribosomal protein L8 (*rpl8*) was also measured in each sample and was used for relative quantification because its expression did not change for the tissues analyzed following any of the treatments.

Statistical analyses

Data are expressed as mean values ± standard error of the mean (S.E.) and statistical analyses were performed using SPSS version 16.0, with the significance value set at 0.05. All data were checked for conformity with the assumptions of normality (normality of error and homogeneity of variance). When these assumptions were not met, data were either transformed to meet these assumptions or equivalent non-parametric tests were conducted (see relevant sections of the results for further details).

Results

Particle characterisation

Particles with zeta potentials more positive than +30mV or more negative than -30mV are normally considered stable (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948) in the absence of steric stabilisation. If the particles have a higher density than the solution they will eventually sediment. Zeta potential measurements of all silver particle types in HPLC-grade water suggested that the particles had a high propensity for aggregation (Table 1.) which was confirmed by hydrodynamic diameter measurements of the particles. The N_{10}silver particles had the most negative zeta potential value (-12.5 mV), on average formed the smallest aggregate sizes in water (ca. 590 nm, measured by DLS) and had the lowest polydispersity index suggesting that aggregate sizes were more uniform compared to the two other particle types. The N_{35} silver formed the largest aggregates in water (ca. 2030 nm) and had a high polydispersity index suggesting that both larger and smaller aggregate sizes were present. N_{Bulk} silver had an average aggregate size of 940 nm and a polydispersity index of 0.69 suggesting aggregates were of a more uniform size than for the N_{35}silver particles. Aggregation, followed by sedimentation, would have removed much of the material from the water column limiting potential uptake of the NPs. Furthermore, it might be expected that diffusive uptake into gills would be limited from this aggregation.

Particle sizes determined from Nanoparticle Tracking Analysis (NTA) are lower compared to the values obtained from DLS technique (Table 1), as previously found (Domingos et al., 2009), possibly due to the bias towards large aggregates in DLS. The N_{35} and N_{10} silver particles show similar hydrodynamic diameters from NTA analysis. However particle number concentrations differ significantly: 1.07×10^8 for the latter and 0.21×10^8 for the

former. The higher particle number concentration for the N_{10} silver partly explains the higher tendency for aggregation, as is also observed on back scattered electron images. It is also clearly seen that the number of smaller particles is higher for the N_{10}particles compared to the N_{35} giving good agreement with the TEM data (Fig. 1. A-C and Table 1.). In the case of N_{Bulk} silver, single particles between 1 and 2 μm could be identified alongside smaller particle sizes.

Ag crystallite sizes were calculated using the Scherrer equation from XRD spectra. As expected and in agreement with the Bragg peak broadening of the XRD patterns (Fig. 1. D), the N_{10} silver showed the smallest crystallite size (21 nm), indicated by the peak broadening in the XRD data, not seen for the larger sized particles. However, N_{35} silver and N_{Bulk} silver showed similar crystallite sizes (68 nm and 60 nm, respectively) suggesting that the N_{35} silver contains mainly large particles similar to the N_{Bulk} particles (also observed by TEM imaging, Fig. 1. E-G). XRD analysis confirmed cubic crystal system (face-centred lattice) in case of all the three silver samples.

Semi-quantitative AFM analysis showed the presence of small particles with approximate sizes of 46 nm in the case of N_{10} silver giving very good agreement with TEM data (Table 1). However, single large particles/aggregates between 140-190 nm were also identified in agreement with NTA data. In the case of N_{35} silver , particles with approximate sizes of 90 nm were identified together with larger particles/aggregates between 130-140 nm, giving good agreement with both TEM and NTA data. Smaller particles of approximate size 10 nm were also observed, again confirming the sample polydispersity and giving good agreement with the NTA data. In the case of N_{Bulk} silver (30 min adsorption time) only smaller particles of an approximate size of 18 nm were observed, suggesting that larger particles were not sticking to the mica slide support due to low diffusivities and low adhesion to the mica. However, the bulk particle sample clearly contained very small (nano)particles,

although in much lower concentrations than the N_{10} and N_{35} samples. In addition, the 4 hrs adsorption time employed for N_{Bulk} silver identified particles with approximate sizes of 147 nm, but very small particles (~18 nm) were also observed in this sample.

The BET method was used for determining Ag specific surface areas. The measured BET surface area values are lower than those supplied by manufacturer (Table 1), but in agreement with all the measured size data. The low values observed are consistent with both the larger particles observed and the possibility of reduced specific surface areas as NPs become densely packed and aggregated during outgassing. As expected, the smallest surface area (0.6 $m^2\ g^{-1}$) was obtained for the N_{Bulk}, while N_{10} and N_{35} showed similar specific surface areas (2.0 $m^2\ g^{-1}$ and 2.9 $m^2\ g^{-1}$, respectively). Synthesis of all data indicates that the N_{10}particles were clearly smaller than the N_{35} particles and these were smaller than the N_{Bulk} particles. Nevertheless, there was considerable sample polydispersity.

TEM analysis suggests that the N_{10} and N_{35} particles are larger than reported by the manufacturer with an average diameter of 49±18.5 and 114±65.3 nm, respectively, whereas the N_{Bulk} particles are smaller than reported by the manufacturer with an average diameter of 137.4±62.2 nm (Table 1). The N_{10} sample contained only particles < 100 nm, whereas the N_{35} and N_{Bulk} samples contained a high proportion of large particles with 47% and 75.6%, respectively, being larger than 100 nm. Additionally, the N_{10} sample contained a high proportion (51%) of particles < 50 nm, whereas the N_{35} and N_{Bulk} samples contained a very small fraction (11.6% and 1.4% respectively) of these particles. TEM analyses indicate that the N_{10} sample was different from the other two samples, which are very similar in terms of their size as determined by TEM .

Exposure Media and Tissue Silver Content

Trace metal analysis of water samples taken from the exposure tanks by ICP-OES showed the measured silver concentrations in the water were considerably lower for all exposure conditions except for the N_{35} Low concentration treatment. Silver concentrations in the N_{10} Low and $AgNO_3$ tanks were below the detection limit of the ICP-OES machine for all samples. For the N_{10} High, N_{35} Low, N_{35} High and N_{Bulk} treatments, the mean silver concentrations in the exposure media were 35.5 µg L^{-1} ± 2.44 (S.E.), 9.4 µg L^{-1} ± 4.62 (S.E.), 35.3 µg L^{-1} ± 2.64 (S.E.) and 46.6 µg L^{-1} ± 4.73 (S.E.) respectively.

Trace metal analysis of the trout tissues determined by ICP-OES showed that there was significantly enhanced level of silver in/on the gills of fish in all treatments compared with controls, except for the $AgNO_3$ exposure, where the $AgNO_3$ dose to the tanks was between 100 and 1000 fold lower than the ENP concentrations (Fig. 2.). The highest concentration of silver was observed in the gills of fish in the N_{10} High treatment group with an average of 0.61 ± 0.07 µg g^{-1} tissue (mean ± S.E.) and this was significantly higher (Mann-Whitney with Holm's Sequential Bonferroni Adjustment $U(4) = 37.00$, $Z = -3.43$, $p < 0.001$) compared with in the gills of fish from the N_{10} Low treatment, where the concentrations were approximately 50% lower. Similar mean silver concentrations were observed in the gills of fish in the N_{35} Low (0.26 µg g^{-1} tissue ± 0.06 µg g^{-1}), N_{35} High (0.25 µg g^{-1} tissue ± 0.08 µg g^{-1}) and the N_{Bulk} treatments (0.32 µg g^{-1} tissue ± 0.21 µg g^{-1}).

Analysis of the liver revealed silver uptake in all four treatment groups: N_{10} High, N_{35} Low, N_{35} High and N_{Bulk}. The highest uptake occurred in the liver of fish exposed to N_{Bulk} silver particles at 1.63 ± 0.18 µg/g tissue, compared with 1.50 ± 0.30 µg/g tissue in the N_{10} High, and 0.92 ± 0.16 µg/g tissue in the N_{35} High treatment groups. The uptake of N_{Bulk} particles into the liver was significantly higher than the uptake of the N_{35} High treatment group (Mann-Whitney with Holm's Sequential Bonferroni Adjustment $U(3) = 64.00$, $Z = -2.414$, $p =$

0.015). There was a small amount of hepatic uptake of N_{35} particles in the low exposure treatment, but this occurred in one fish only. No uptake was detected in the liver of fish exposed to $AgNO_3$ or the N_{10} Low treatment groups. Where uptake of silver into the liver occurred, the overall silver content was more than double the levels in the gill tissue in those fish. Levels of silver in the kidneys of exposed fish were below the detection limit for all treatments.

Lipid Peroxidation

Analysis of TBARS showed no evidence of lipid peroxidation in the gills of fish from any of the treatments, except for the N_{10} High treatment (that contained the highest gill silver content), with significantly lower levels of measured MDA compared with controls (ANOVA with Tukey's post-hoc test; df=6,104; F=2.629; p=0.021) (Fig. 3.). In the liver tissue homogenates, similarly there was no evidence of lipid peroxidation, except for MDA concentrations in the livers of the fish from the silver nitrate treatment which were significantly lower compared with those in the controls (ANOVA with Tukey's post-hoc test; df=6,105; F=3.993; p=0.001). Examination of TBARS in the blood plasma showed no evidence of an elevation in MDA concentration for any of the particle treatment groups. There was, however, evidence of lipid peroxidation in the plasma of fish from the silver nitrate treatment, where MDA concentrations were significantly higher than controls, and the N_{10} Low and N_{35} Low treatment groups (ANOVA with Tukey's post-hoc test; df=6,90; F=3.991; p=0.001).

Histology

Histological examination of gill tissue showed no evidence of physical damage or changes to gill filaments in treatments to silver for any of the particle sizes. Gills of fish exposed to $AgNO_3$, however, showed slight damage with deformation of the secondary lamellae observed (Fig. 4.). There was no evidence of tissue damage in the liver for any of the silver treatments compared with controls (not shown).

TEM Analysis

Micrographs of gill tissue from N_{10}High treatment show electron dense areas, which correspond to particles associated with the gill tissue. The particles are highly aggregated in clusters of approximately 600 nm, corresponding well with the hydrodynamic diameter size as measured by dynamic light scattering (ca. 590 nm) and appear to be associating with the pavement cells at the gill surface (Fig. 5.). There was no confirmation of the presence of silver in the gill tissue by EDX analysis (data not shown). However, Ag content of the tissue must be ~0.1% or greater in order to be detected using this method.

Quantitative Real-Time PCR

Analysis of the expression of five genes, that have been shown previously to be induced in rainbow trout gills and liver as a result of exposure to various metal ions, showed that their expression was unchanged as a result of exposure to silver particles, with the exception for *cyp1a2*, where there was a 3-fold induction in the gills of fish exposed to the high concentration of N_{10} particles (ANOVA: df=6,48; F=2.691; p=0.025) (Fig. 6.).

Discussion

Very few studies to date have investigated the effects of exposure to silver nanoparticles in fish and these have been focused on zebrafish embryos where mortality, notochord and heart abnormalities have all been described (Asharani et al., 2008; Lee et al., 2007; Yeo and Kang, 2008). Similarly, there is little in the literature regarding the uptake and biodistribution of silver nanoparticles into internal organs or the potential toxicity of silver nanoparticle in intact free swimming fish. Furthermore, there have been no studies in fish examining the differential uptake and toxicity of nano-sized versus bulk-sized silver particles. The main aims of this study were, therefore, to identify the target organs for silver particles of different sizes as a result of *in vivo* exposure and uptake from the water in fish, using rainbow trout as a study species, and to compare the patterns of biodistribution and toxicological effects seen for two different sized silver nanoparticles (nominally 10 nm and 35 nm) and bulk silver particles (nominally 600-1600 nm).

There was clearly a substantial difference between the manufacturer's information on material properties and those seen by our own investigations. This discrepancy has been observed by many workers and leads us to the strong recommendation that, as a minimum, commercially obtained NPs need to be fully characterised before any toxicology or other experiments are performed. The reasons for the differences are most likely to be a) incorrect manufacturer's information, possibly due to limited and/or inappropriate measurement or batch to batch variation or b) changes in material properties between synthesis and initial characterisation and use. The characterisation performed here in the silver particles is internally consistent, showing all three particles are polydisperse, with the NPs generally larger than their nominal values and existing, at least partially, as nanostructured aggregates.

The larger particles were generally better characterised by the manufacturer, but it is noticeable that there were also some small particles present in those samples. Thus, we would expect that all samples would have low (mass) bioavailability and not to behave as well dispersed 10 or 35 nm particles. Nevertheless, the NPs are likely to be representative of commercially available NPs in current commercial products. For all the measurements of silver concentration in the exposure media, except for in the N_{35} Low treatment group, the actual measurements were as much as 65% lower than the nominal dosing concentrations, even immediately after dosing. The silver concentration in the tank water were highest, as expected after the (re-)dosing of the tanks during the water changes, and then they gradually and progressively decreased over time until the next (re-)dosing event (data not shown). These data suggest that aggregation and sedimentation of the particles in the exposure media resulted in a lower silver NP concentration in the water column (and to which the fish were being exposed) compared with the nominal dosing concentration. The level of silver in the gills of rainbow trout exposed to the high dose of N_{10} silver particles was significantly higher than the uptake for all other particle sizes. Characterization of the silver particles by TEM and XRD, show that the N_{35} and N_{Bulk} silver particles were similar in terms of particle size measurements, whereas the N_{10} silver particles were markedly smaller suggesting that smaller particles show a greater propensity to associate with/enter into gill tissue than larger particles. TEM images on gill tissue from trout exposed to N_{10} particles showed the presence of silver particles. However, from the imaging alone, we cannot be definitive as to whether the particles detected were on the surface of the pavement cells or were incorporated into them. EDX analysis did not detect silver in the gills, likely because concentrations were not sufficiently high to be able to do so, but their presence was supported by the ICP-OES measurements (<1 $\mu g\ g^{-1}$). The fact that the liver content of silver was twice that of the gills would suggest transport across the gills (see also later discussion). Assuming that there is 1%

solubility of Ag in the exposure media (Fabrega et al., 2009; Navarro et al., 2008), uptake of silver in gills of fish exposed to the low dose of Ag NPs, at least, cannot be explained by NP dissolution and uptake of dissolved Ag, further indicating that some uptake of Ag NPs must have occurred.

Despite the association/uptake of silver with/into the gill tissue, there was no obvious damage to the gill filaments as a result of the exposures to silver particles, although some low level damage was noted in the gills of fish exposed to $AgNO_3$. Griffitt et al. (Griffitt et al., 2009) similarly found that more silver associated with the gills of zebrafish exposed to silver nanoparticles compared with fish exposed to soluble silver ($AgNO_3$). In that study, significant thickening of the gill lamellae was observed in fish exposed to $AgNO_3$ whereas concentrations of 1000 µg 27 nm silver particles L^{-1} did not elicit any changes in gill lamellar thickness (for a 48h exposure). The effective doses of silver particles for inducing gill lamellar disruptions in the study by Griffitt et al. were, however, between 10 - and 100-fold higher than in our study, and this likely explains the differences in the effects observed.

The levels of silver present in the liver of fish exposed to the high concentrations of all the particle sizes were approximately twice the concentrations in the gills per gram of tissue, suggesting that transportation of silver occurs within the blood from the sites of uptake. Contrasting with findings in the gills (where the amount of silver measured was highest for the 10 nm high exposure regime), both N_{10} silver and N_{Bulk} silver particles showed the highest accumulation in the liver tissue. It is difficult to equate the tissue levels of N_{10} silver or N_{Bulk} silver in the liver with those in the gills, indicating a possible alternative uptake route. Indeed, it may be the case that uptake of N_{Bulk} silver (and nano silver also) was not principally *via* the gills, but rather via the gut as a function of drinking and/or feeding on aggregated material (the fish were not fed for the exposure period). The feeding on aggregated materials that

accumulate on the bottom of the tank has reported previously for aqueous exposures to other nanomaterials (Johnston et al., 2010).

Although there was a two-fold difference in uptake of N_{10} Ag particles in the gills between the low and the high dose exposures, in none of the gill samples where uptake occurred, was the concentration of Ag in the gill 10 times higher for the high dose than for the low dose. This might be explained by lowered bioavailability of Ag ENPs in the water column as a result of increased propensity for particle aggregation at higher nanoparticle concentrations as has shown in previous studies (Johnston et al., 2010). The difference in uptake of N_{35} Ag in the liver of fish between the high and low dose exposures is close to 10-fold however, further suggesting differences in route of uptake of Ag NPs between gill and liver tissue, and that uptake into the liver as a result of fish feeding on Ag NP aggregates from the tank floor may be an important consideration.

The mechanism by which electrolytes, metal ions and organic molecules are taken up across epithelial cell layers is well described (Handy and Eddy, 2004). *In vivo*, both the gill and gut surfaces are surrounded by aqueous media which contributes to an unstirred aqueous layer above the epithelium. This layer can exchange these molecules with the mucus layer covering the epithelium, which in turn can present the molecules to the apical surface of the epithelium where uptake may occur. It is not known, however, how well this model can be applied to nanoparticles and their uptake or how the physicochemical characteristics of the particles such as size, shape and surface charge and the interaction of particles with the aqueous media might affect uptake. Furthermore, it is not known how this may influence release of silver ions from the particles. Our results suggest, however, that distinct differences may exist between the interaction of different sized silver particles with epithelial membranes and also with the interaction of silver particles with different epithelial surfaces, i.e. the gut and gill.

As Navarro et al. (2008) showed in their work with *C. reinhardtii* interaction with particles with biological membranes enhanced the release of Ag^+ from the particles, however whether or not this phenomena is also observed in interactions between silver particles and epithelial membranes in fish is not yet known.

Levels of MDA were shown to be significantly increased only in the plasma samples of fish exposed to $AgNO_3$, suggesting that some ionic silver was entering the blood stream via the gills and/or the gut epithelium and causing lipid peroxidation. Levels of MDA in the livers of these fish, however, were significantly lower than in control fish, and this was in accordance with a lack of evidence of silver uptake into the livers for the $AgNO_3$ exposure. The apparent reduction of lipid peroxidation in the gills of fish exposed to the high concentration of N_{10} silver, despite a large accumulation of silver in the gills, is a surprising finding and is in marked contrast with several studies which demonstrate the potential for silver nanoparticles to generate reactive oxygen species (ROS) and cause oxidative stress. Rahman et al., (2009) found 25 nm silver particles induced the expression of oxidative stress-related genes in the mouse brain after intravenous injection 100 mg kg^{-1}, 500 mg kg^{-1} and 1000 mg kg^{-1} and *in vitro* studies have shown that silver nanoparticles have the capacity to generate ROS (Carlson et al., 2008; Hsin et al., 2008) and cause increased lipid peroxidation (Arora et al., 2008). Both liver and gill tissues have the ability to upregulate survival genes and DNA repair mechanisms (Diehl, 2000; Hansen et al., 1996) when an organism is exposed to environmental stressors which may also explain the reduced lipid peroxidation seen in response to $AgNO_3$ in the liver and to the high concentration of 10 nm Ag particles in the gills. However, if the solubility of Ag in water is taken to be 1% (Fabrega et al., 2009; Navarro et al., 2008) we would expect to see similar responses in the blood plasma of fish exposed to low concentrations of Ag NPs as in the exposure to $AgNO_3$. The absence of such

a response may suggest that differences in dissolution rates exist between different preparations of Ag NPs.

It has been suggested that the mucus layer surrounding the gill epithelia may act as a barrier preventing nanoparticle uptake by the gills (Handy et al., 2008a) and a study by Smith et al. (Smith et al., 2007) demonstrated that carbon nanotubes readily associate and became trapped within mucus on the gill surface. If the silver nanoparticles are similarly associated with the mucus on the gills for aqueous exposures, reducing /preventing penetration into the gill epithelia, this would explain the lack of any lipid peroxidation in our exposures. Interestingly, Derksen et al. (Derksen et al., 1998) demonstrated under normal oxygen-level conditions (dissolved oxygen = 9.5 mg L^{-1}), that particulate matter associated with the gills was effectively cleared from the gills of rainbow trout within 40 hours, however both elevated and reduced oxygen levels (25 mg L^{-1} and 4.5 mg L^{-1} respectively) caused significantly reduced clearance of particulate matter, in resulting altered behaviour normally associated with respiratory stress. It is quite possible therefore, that the length of time that nanoparticles are associated with the gill via the mucus before being cleared might influence vesicular uptake or oxidative stress in the gill tissue.

The suite of five genes analysed by rtPCR were chosen as genes representing a range of toxicity mechanisms (principally for heavy metals) in living cells. The Cytochrome P450 monooxygenase system plays an important role in the detoxification of both endogenous and exogenous chemicals in animals and in fish they have often been used as biomarkers for environmental contamination (Råbergh et al., 2000). Both *cyp1a2* and *cyp3a45* have been shown to have roles in the oxidative metabolism of exogenous compounds in rainbow trout (Lee and Buhler, 2003; Råbergh et al., 2000). The expression of heat shock proteins is

associated with a general shock response which is universally conserved throughout the animal kingdom. Measurement of heat shock protein induction, in particular *hsp70*, has been proposed as a useful technique in toxicological screening and environmental monitoring as a variety of stressors including, heavy metals, teratogens, anoxia and heat have been shown to induce synthesis. Williams et al. (Williams et al., 1996) showed that accumulation of HSP70 in the gills of juvenile rainbow trout occurred as a result of exposure to cadmium, copper, lead and zinc *via* the water and *via* the diet. Both glucose-6-phosphate dehydrogenase (G6PD) and the glutathione peroxidase (GPX) enzyme family play significant roles in protecting cells from oxidative damage and measurement of their activity is commonly used as markers for oxidative stress. Both genes have multiple metal response elements in their 5' flanking region which has been proposed as the reason for their responsiveness to metals (Walker et al., 2007). Exposure of brown trout (*Salmo trutta*) to both cadmium and copper *via* the water have been shown to induce expression of *gpx* mRNA (Hansen et al., 2007; Hansen et al., 2006) and both *g6pd* and *gpx* have been shown to be responsive to zinc in the gills of rainbow trout (Walker et al., 2007). Thus, the genes selected for study represented a wide range of toxicological effect pathways. Interestingly, only the expression of *cyp1a2* was found to be significantly altered and this only occurred in the gills of fish exposed to the high concentration of 10 nm silver particles, correlating with the highest level of accumulation of silver in the gills in this treatment group. (Griffitt et al., 2009), adopting a transcriptomic approach, found none of our targeted genes were differentially expressed in the gills of zebrafish exposed to silver nanoparticles (26 nm, 1000 $\mu g\ L^{-1}$). A study by (Rahman et al., 2009) found that exposure of rats to silver nanoparticles caused significant down-regulation of *Gpx2* in the brain frontal cortex and up-regulation of *Gpx3* in the caudate nucleus. The doses of silver nanoparticles eliciting these responses, however, were between 500 and 1000

mg kg^{-1} and are approximately 1000-fold higher than the maximum concentration of nanoparticles adopted in our experiment.

Silver ions have been shown to be toxic to fish, inhibiting carbonic anhydrase activity leading to a net loss of Na^+ and Cl^- across the gills and inhibiting Na^+/K^+ATPase activity (Morgan et al., 1997). The toxic effects of silver ions are due to their interaction at the gill surface and not as a result of internal silver accumulation (Wood et al., 1996b). We, therefore, might have expected exposure to $AgNO_3$ to alter the expression of some of these genes in the gills. ICP-OES employed in this study, cannot distinguish between different valency states of metals. Although previous studies have established that Ag solubility in water is ~1% (Fabrega et al., 2009; Navarro et al., 2008) in our experiment, it is not known what proportion (if any) of the silver present in the Ag NP exposure media was in the form of Ag^+. Furthermore, it is not known in what form silver was associated with the gills or transported to the liver. The lack of any effect, however, is likely explained by the low exposure concentrations adopted (chosen to reflect a 10-fold lower concentration than the accepted 96h LC50 value for rainbow trout) and effective repair mechanisms in these tissues.

The known toxicity of silver ions has led to the proposal in a number of studies that release of silver ions (Ag^+) from silver nanoparticles could be in part responsible for toxic responses seen in exposures to silver nanoparticles (eg Navarro et al., 2008). This idea however, conflicts with the known stability of zero-valent silver in water. (Griffitt et al., 2009) conducted concurrent exposures of zebrafish to silver nanoparticles and Ag^+ at concentrations that matched the amount of silver ions released by the nanoparticles. Dissolved silver from nanoparticles was measured and found to be 0.07% of the silver nanoparticle mass. The method used however, does not specifically elucidate whether the silver present was ionic or very small zero-valent silver nanoparticles. This may explain why gill damage was observed

in Ag^+ exposures but not in silver nanoparticle exposures. Also the gill global gene expression patterns between these exposures differed markedly from each other, suggesting toxicity mechanisms differed between treatments.

Conclusion

Our results show that smaller nanoparticle sizes have a greater propensity to associate with the gills of rainbow trout, but that the mucus layer on the gills may be an effective barrier to entry of the nanoparticles into the gill cells. However, induction of *cyp1a2* in the gills of fish exposed to high concentrations of N_{10} silver particles may be indicative of oxidative metabolism in response to the exposure. At this time, it is difficult to speculate on the mechanism of action of *cyp1a2* in the gills as it is unknown whether the effects seen are as a result of exposure to the silver nanoparticles, silver ions or a combination of both. We show evidence of lipid peroxidation in the plasma of fish exposed to $AgNO_3$ however, there was an apparent decreased lipid peroxidation in the gills of fish exposed to high concentrations of N_{10} silver particles. Uptake of silver particles was demonstrated in the liver but with no differences in uptake relative to particle size. Liver burden of silver was approximately twice that seen in gills suggesting that the gut epithelium may be an important route of exposure for silver particles to fish. These results suggest that both the size of the nanoparticle and the type of epithelium where nanoparticles are presented affect their uptake. The importance of Ag^+ as the mechanism of silver nanoparticle toxicity needs further investigation and accurate measurements of the proportions of zero-valent silver and Ag^+ (and Ag^+ sorbed to NP surfaces) for the different sized nanoparticles would be needed to do this. Care should also be taken when defining dissolved silver. Ag^+ in solution and stable suspensions of zero-valent silver nanoparticles are distinctly different, but have both been described in the literature as

solutions. Our findings also revealed that for all three particle types, size and surface area measurements differed considerably to the information given by the manufacturers, emphasising the need for rigorous characterization of particles to ascertain the nature of the particles to which the test organisms are exposed. Our results show that exposure of silver nanoparticles to rainbow trout at concentrations close to current estimations of environmental levels can result in accumulation of silver in the gills and liver of fish and can affect likely oxidative metabolism in the gills.

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List of Figures

Table 1. Size and charge characterization data for silver particles suspended in HPLC-grade water.

Figure 1. Characterization of silver particles: back-scattered electron images obtained from (A) N_{10}, (B) $N_{35}$and (C) N_{Bulk} silver particles, (D) X-Ray Diffraction pattern for silver particles, TEM images obtained from (E) N_{10}, (F) N_{35} and (G) N_{Bulk} silver particles.

Figure 2. Concentrations of silver in gills and liver of rainbow trout after waterborne exposure to N_{10}, N_{35} and N_{Bulk} silver particles and silver nitrate. Data are means ±S.E. n=8 per treatment.

Liver # - Significantly lower than N_{10} High, N_{35} High and N_{Bulk} (Mann-Whitney $p<0.001$) * - Significantly higher than N_{35} High (Mann-Whitney $p<0.001$). Gills # - Significantly lower than N_{10} Low (Mann-Whitney $p<0.001$) * - Significantly higher than N_{35} Low, N_{35} High and N_{Bulk} (Mann-Whitney $p=0.001$).

Figure 3. Thiobarbituric acid reactive substances (TBARS) in liver and gill tissue homogenates and plasma of rainbow trout after waterborne exposure to N_{10}, N_{35} and N_{Bulk} silver particles and silver nitrate. Data are means ±S.E. n=8 per treatment .

Liver # - Significantly lower than Control, N_{10} Low, N_{35} Low and N_{35} High (Tukey's $p=0.001$). Gills # - Significantly lower than Control and N_{10} Low (Tukey's $p=0.021$). Plasma * - Significantly higher than Control, N_{10} Low and N_{35} Low (Tukey's $p=0.001$)

Figure 4. Gill morphology as visualised by light microscopy in rainbow trout after 10 day waterborne exposure to silver particles and silver nitrate. (A) Control, (B) 0.1 µg L^{-1} $AgNO_3$ with close-up inset of damage, (C) 100 µg L^{-1} N_{10} silver particles, (D) 100 µg L^{-1} N_{35} silver particles, (E) 100 µg L^{-1} N_{Bulk} silver particles. Arrows indicate damage to secondary lamellae.

Figure 5. TEM images of gill tissue dissected from rainbow trout after waterborne exposure to 100 µg L^{-1} N_{10} silver particles for 10 days. Images B and C are higher magnification images of aggregates in image A.

Figure 6. Expression of *cyp1a2*, *cyp3a45*, *hsp70a*, *g6pd* and *gpx* in gills and liver as determined by real-time PCR. Results are represented as means ± S.E. expressed as fold-increase in relative mRNA expression (gene of interest: *rpl8*). Experimental groups consisted of eight fish and each fish was analyzed in triplicate. Statistically significant differences in the expression levels (compared to controls) are denoted by * (ANOVA $p<0.025$).

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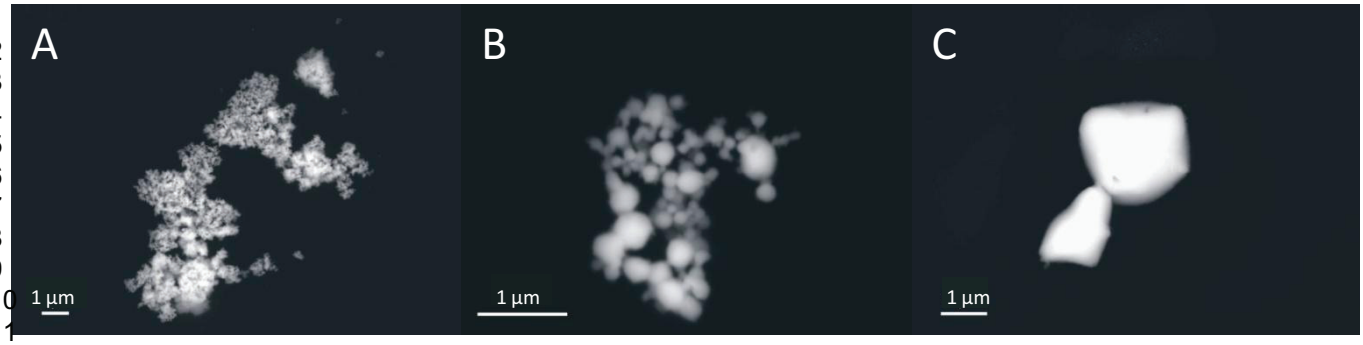
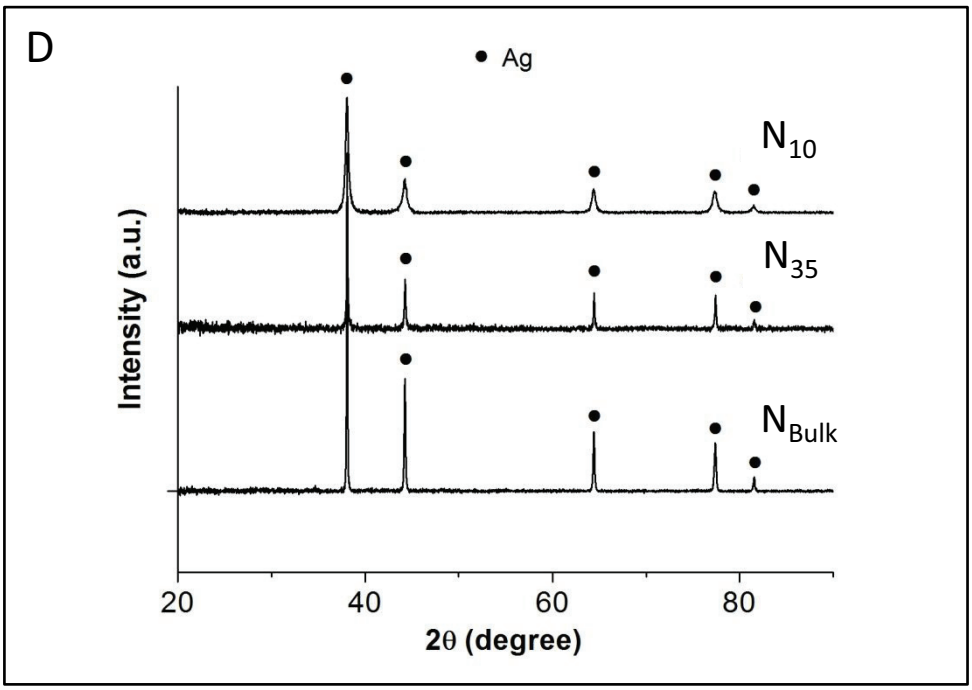
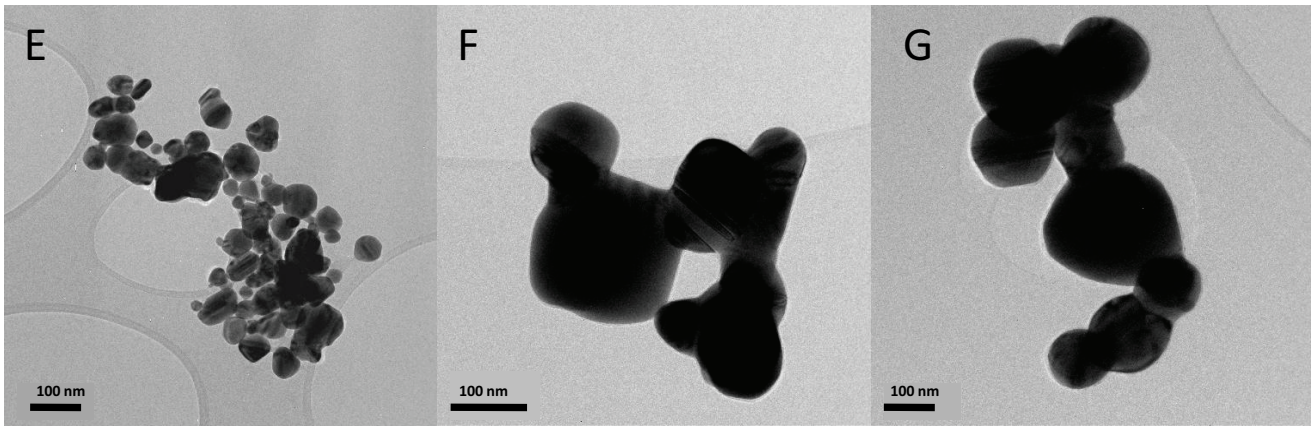
	N_{10} Silver	N_{10} Silver Manufacturers Specifications	N_{35} Silver	N_{35} Silver Manufacturers Specifications	N_{Bulk} Silver	N_{Bulk} Silver Manufacturers Specifications
Zeta potential (mV)	-12.52 ± 2.7	-	-6.50 ± 1.8	-	-2.8 ± 0.6	-
pH	7.11	-	7.34	-	6.40	-
Hydrodynamic diameter (nm) (DLS)	589 ± 101	-	2029 ± 524	-	938 ± 230	-
Polydispersity Index	0.54	-	0.93	-	0.69	-
Hydrodynamic diameter (nm) (NTA technique)	158 ± 76	-	166 ± 72	-	217 ± 130	-
Particle number mL^{-1} (NTA technique)	1.07×10^8	-	0.21×10^8	-	0.27×10^8	-
Mean primary particle size (nm) (TEM)	49 ± 18.5	10	114 ± 65.3	35 (max <100 nm)	137 ± 62.0	600-1,600
Mean particle size (nm) (AFM)	46.3 ± 10.7	-	90.0 ± 15.6	-	147.5 ± 82.3	-
Crystallite size (nm) (XRD technique)	21.2 ± 0.5	-	68.0 ± 2.0	-	60.0 ± 4.6	-
Surface Area ($m^2 g^{-1}$) (BET)	2.0 ± 0.2	9-11	2.9 ± 0.2	30-50	0.6 ± 0.1	-
Bulk Density ($g cm^{-3}$)	-	2.05	-	0.3-0.6	-	-
True Density ($g cm^{-3}$)	-	10.5	-	10.5	-	-
Purity (trace metal analysis)	-	99.9%	-	99.5%	-	99.95%

Table 1 Scown et al.







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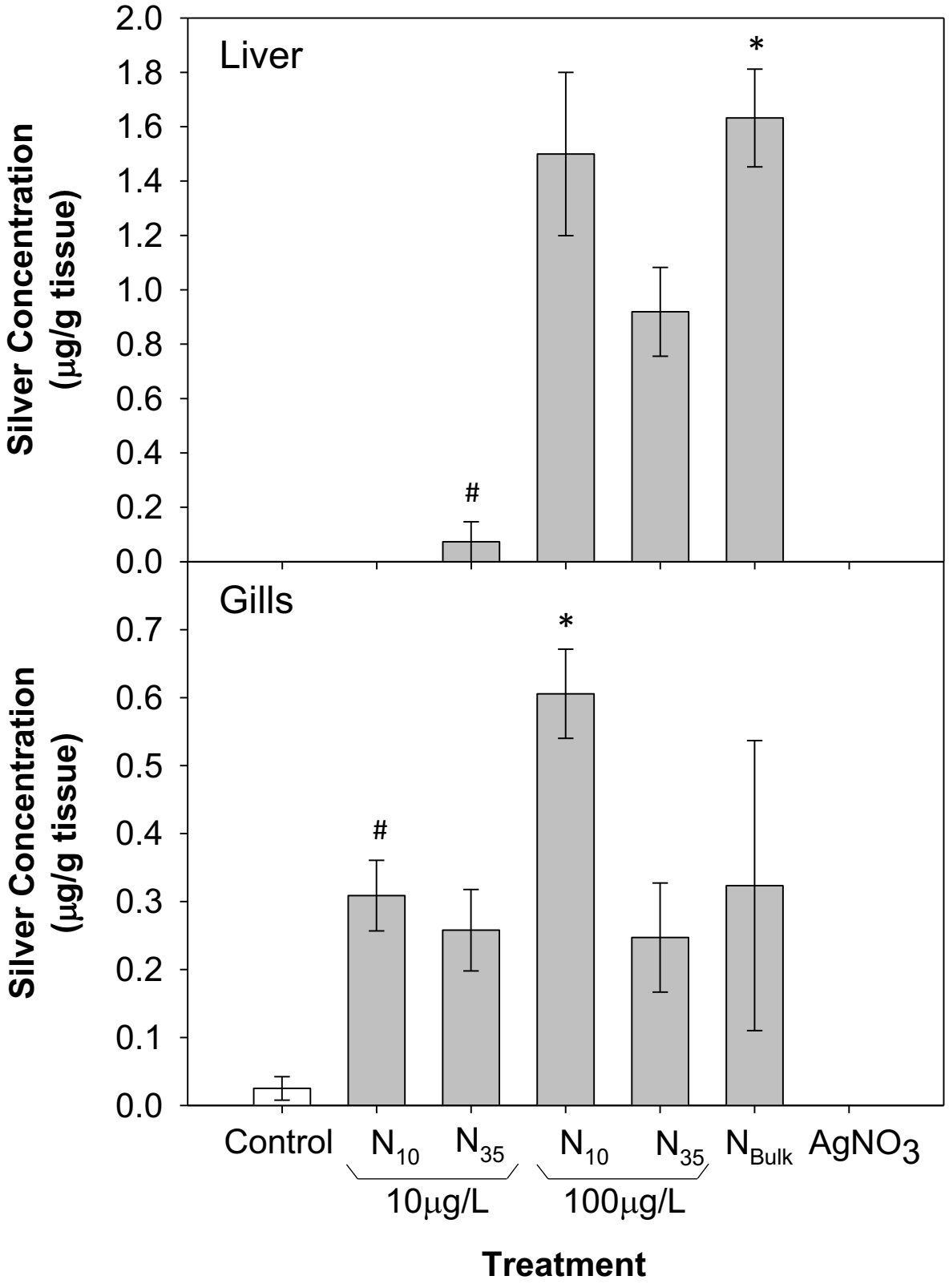

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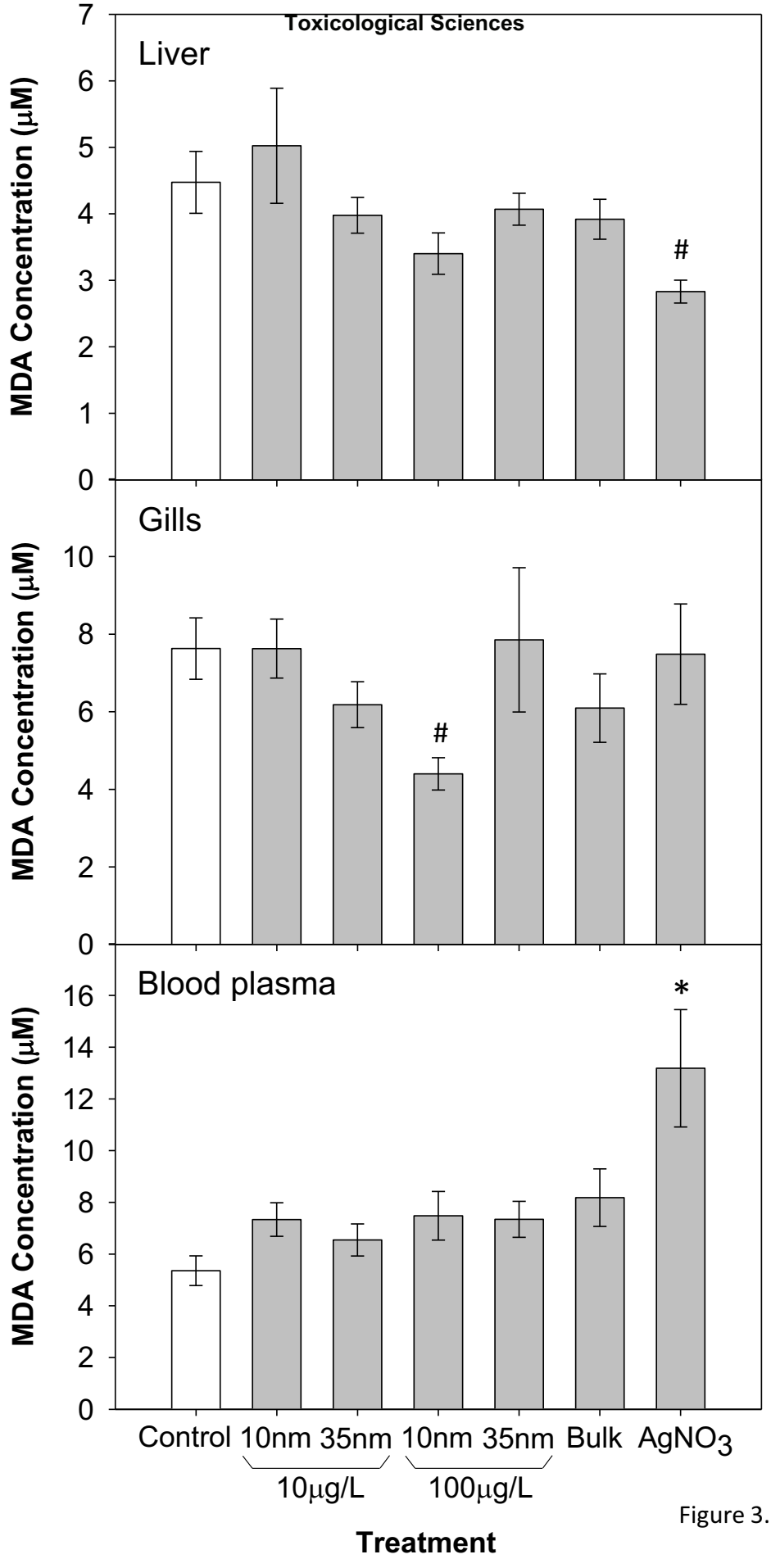

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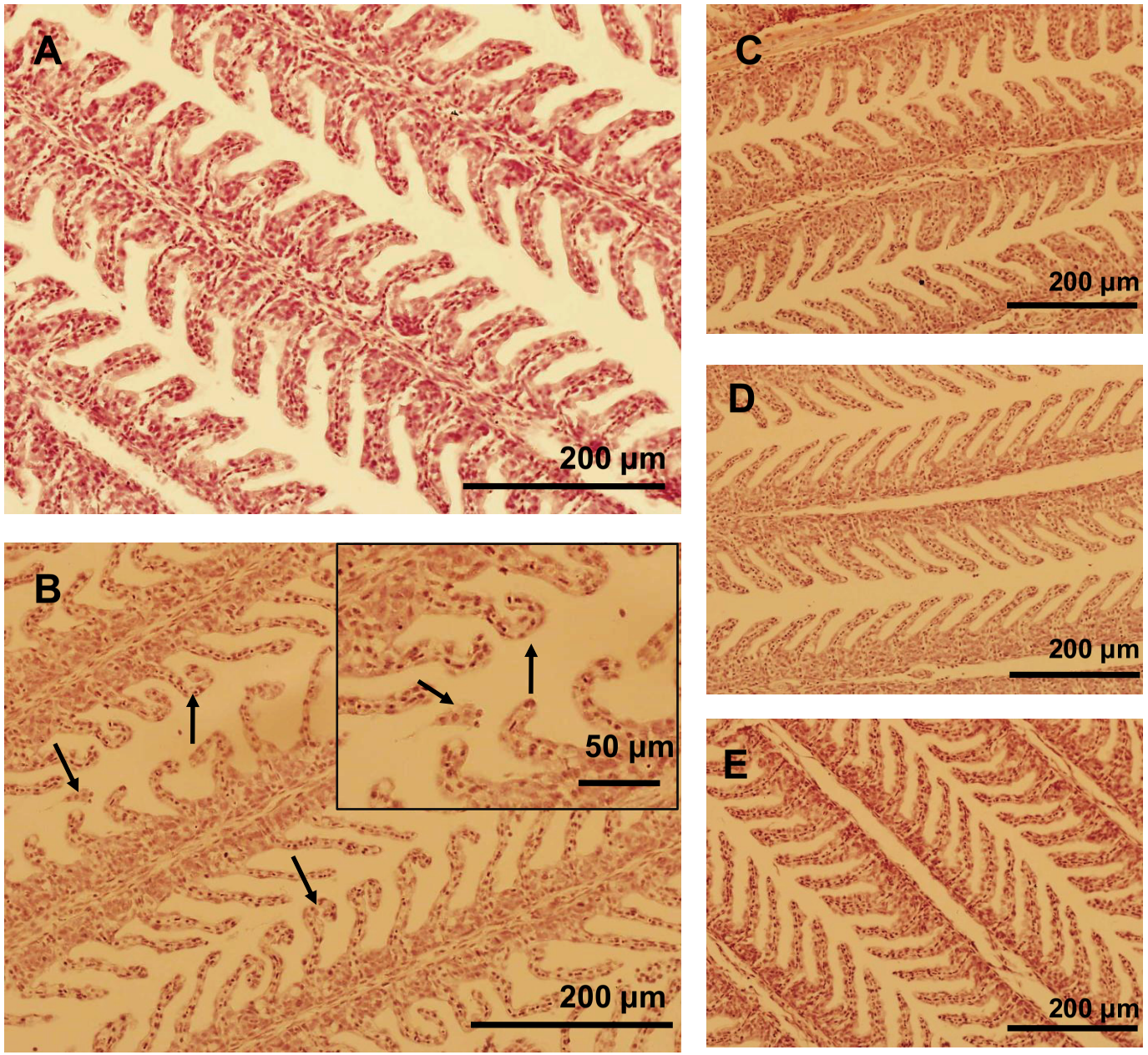

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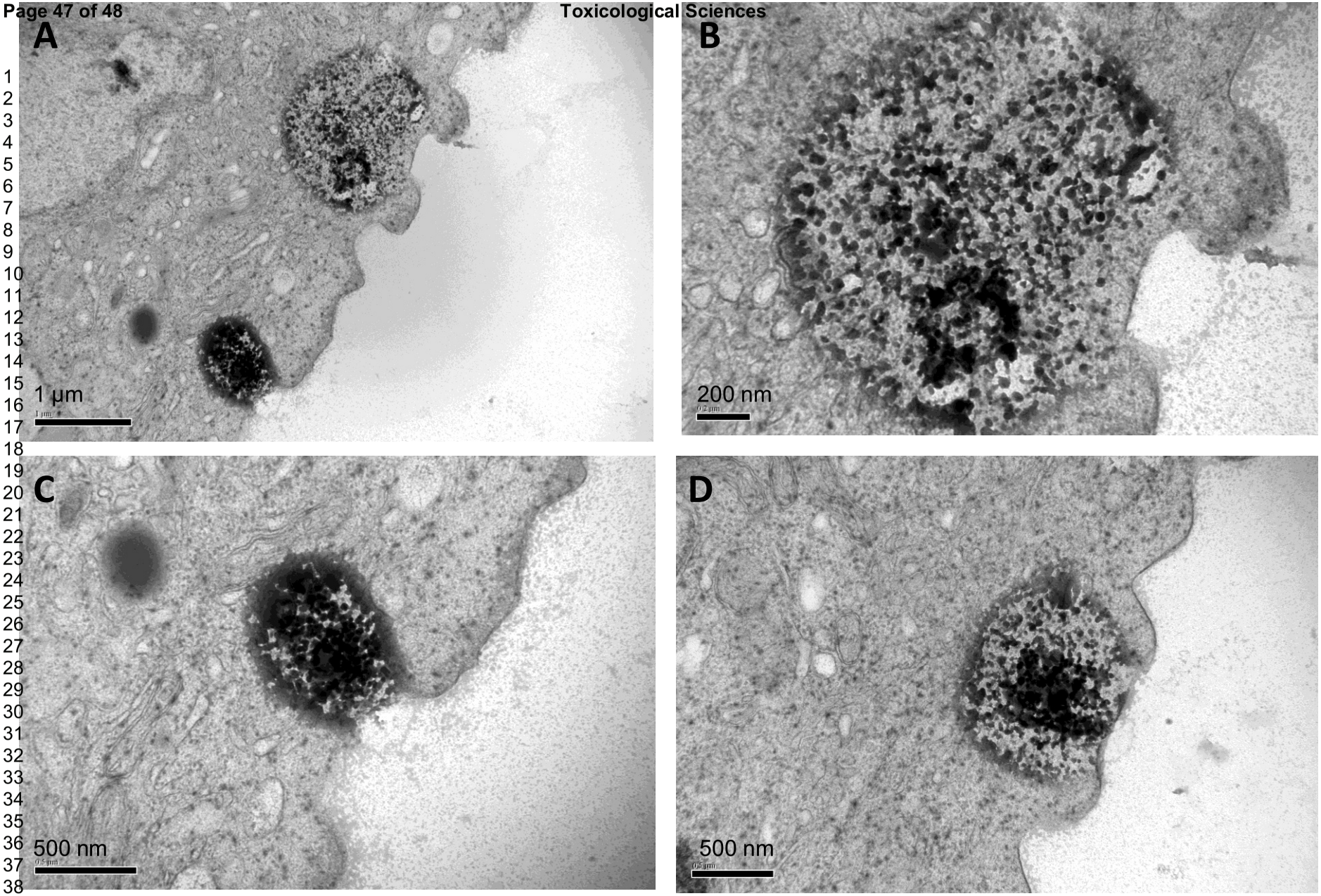

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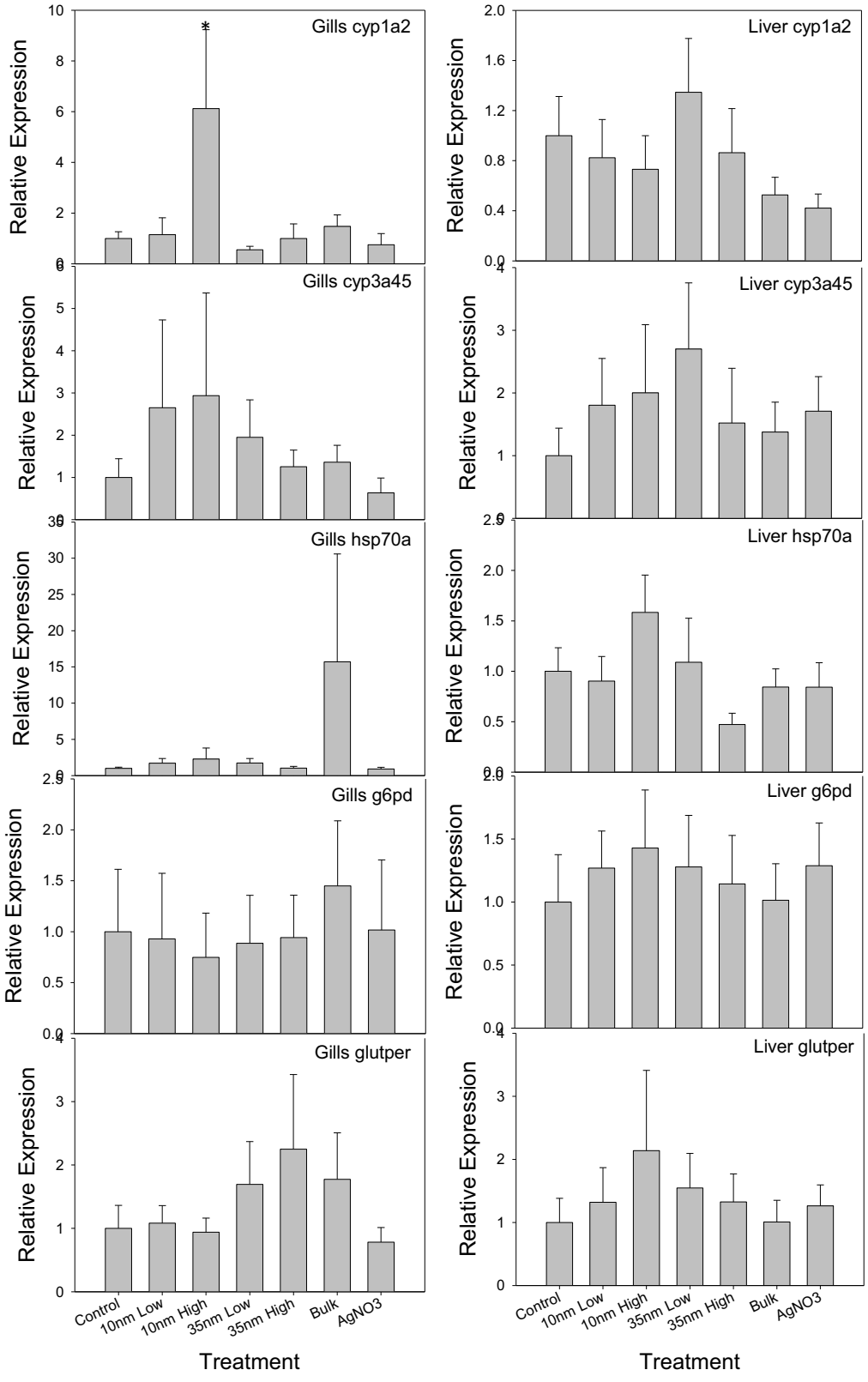

Scown et al. Figure 6

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Assessment of cultured fish hepatocytes for studying cellular uptake and (eco)toxicity of nanoparticles

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Environmental context. The production and application of engineered nanoparticles is rapidly increasing, and development of suitable models for screening nanoparticles for possible toxic effects is essential to protect aquatic organisms and support the sustainable development of the nanotechnology industry. Here, the suitability of isolated rainbow trout hepatocytes was assessed for high through-put toxicity screening of nanoparticles and for studying uptake of nanoparticles into cells.

Abstract. Relatively little is known regarding the fate and possible toxic effects of engineered nanoparticles (ENPs) in the aquatic environment. We assessed the suitability of isolated trout hepatocytes for high throughput toxicity screening of ENPs, exposing them to a variety of metal and metal oxide nanoparticles and their bulk counterparts. We found no effects of the ENPs on cell viability, or on lipid peroxidation, with the exception of exposure to ZnO nanoparticles, or on glutathione-S-transferase (GST) levels, for exposure concentrations up to 500 $\mu g\,mL^{-1}$. All ENPs, however, were internalised in the cultured hepatocytes, as shown by coherent anti-Stokes Raman scattering (CARS) as an imaging technique. Our findings suggest that fish hepatocyte cultures are suitable for studies investigating the cellular uptake of ENPs, but they do not appear to be sensitive to ENP exposure and thus not a good in vitro model for nanoparticle toxicity screening.

Additional keywords: coherent anti-Stokes Raman scattering, in vitro, metal oxides, rainbow trout, silver.

Introduction

Nanotechnology exploits the unusual chemical and quantum properties of materials manufactured at or below the nanoscale. Over the past decade, the industry has expanded rapidly and engineered nanoparticles (ENPs) now have wide industrial applications and are incorporated into many consumer products.[1–3] As the number of nanoparticle types and applications increase, so does the likelihood that they will be released into the environment in significant quantities. The aquatic environment acts as a sink for most environmental pollutants and will thus likely receive ENPs from waste water and accidental release from factories and through degradation and wear of products containing ENPs. To date, however, there is a lack of data on the environmental fate, bioavailability and biological effects of ENPs in aquatic organisms.

There is a need to develop pragmatic and appropriate experimental approaches and techniques for the testing and screening ENPs for possible biological effects to assess potential risks. Given their very diverse nature (i.e. large number of types, shapes, forms and different functionalisations) this ideally requires high throughput screening methods.

Most previous nanoparticles toxicity studies to date have been concerned with identifying the risks associated with inhalation of metal oxide nanoparticles and absorption through the skin. Many of the in vitro systems used for screening nanoparticle toxicity therefore have made use of terrestrial vertebrate cell lines such as alveolar macrophages,[4] bronchial epithelial cells[5] and pneumocytes and dermal fibroblast cell lines.[6] Uptake of nanoparticles by cells has been demonstrated[7,8] as well as a range of cellular responses such as inflammation,[9] generation of reactive oxygen species,[4] lipid peroxidation[5] and alterations to membrane integrity.[6]

Until recently little attention has been paid to potential risks associated with exposure of aquatic organisms to ENPs and the corresponding need for relevant in vitro models. Primary cultured fish hepatocytes have an established history for screening environmental contaminants, including endocrine disrupting chemicals, pharmaceuticals and heavy metals for their potential

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impact on fish and other aquatic organisms[10,11] and potentially offer a suitable method for screening ENPs (eco)toxicity.

There are only a very few techniques for accurately imaging or characterising nanoparticles, especially when in biological samples and ideally a multi-method approach is preferred.[12] In terms of assessing uptake into organisms or cells there are fewer standard imaging methods, although transmission electron microscopy (TEM) is sometimes used.[13] Gaining insight (through imaging) of the cellular localisation of particles can help to identify likely processes targeted and thus possible biological effects.

High resolution TEM offers the resolution capacity to identify crystalline structures of nanoparticles.[14] This technique, however, requires a dry sample and images are acquired under high vacuum conditions, consequently prohibiting live imaging. The characteristics of ENPs under such conditions are therefore not necessarily representative of their characteristics in situ. Furthermore, TEM is generally only able to visualise ENPs that are electron dense excluding visualisation of non-metal coatings or shells.[15] When considering uptake of ENPs into cells or whole organisms several techniques have been applied. Confocal microscopy has been used to determine the biodistribution of multi-walled carbon nanotubes (MWCNTs) in zebrafish embryos[16]; however, non-metallic ENPs must be fluorescently labelled which has ramifications when considering the toxicity potential of a novel material.

Dark-field microscopy has also been used to visualise uptake of silver nanoparticles into both zebrafish embryos and nematodes[17,18] but the technique offers minimal penetration. Samples must also be extremely pure and the high levels of illumination required for visualisation introduces the possibility of photodamage. Coherent anti-Stokes Raman scattering (CARS) microscopy is emerging as a powerful tool for biological imaging, offering several advantages over conventional microscope techniques, including label-free contrast, increased depth penetration, reduced phototoxicity and, as shown by Moger et al.,[19] exceptional capabilities for locating ENPs within biological samples with 3-D sub-cellular resolution. CARS microscopy exploits the non-linear optical response of a sample to generate image contrast derived from vibrations of molecular bonds. By simultaneously illuminating the sample with two excitation wavelengths, with the frequency difference chosen to match the vibrational frequency of chemical bonds within the desired sample component, a strong 'anti-Stokes' signal is generated which is used as image contrast. In the same way that nanoscale materials have unique chemical properties, ENPs also exhibit extraordinary optical properties which give rise to enhanced non-linear optical responses, and hence large CARS signals that are independent of the vibrational frequency being probed.[19] This nanoscale enhancement allows detection of particles far smaller than expected. The label-free nature of the technique eliminates the chemical perturbation seen when using fluorescent labelling of either ENPs or counter staining of surrounding tissues, both of which can modify the transport kinetics, cellular uptake and cytotoxicity of ENPs.

The aim of this study was to assess a rainbow trout (*Oncorhynchus mykiss*) hepatocyte primary cell culture, used routinely for toxicity assessments of conventional xenobiotics, for screening nanoparticle cellular uptake and toxicity. We exposed rainbow trout hepatocytes to three metal oxide ENPs that were well characterised: titanium dioxide (TiO_2), zinc oxide (ZnO), cerium oxide (CeO_2) and two sizes of the metallic nanoparticle silver (Ag) and their corresponding bulk counterparts, chosen because of their relatively high-volume industrial use. We evaluated lactate dehydrogenase (LDH) release, as a measure of overt toxicity (cell membrane integrity), and lipid peroxidation and glutathione-S-transferase (GST) activity as endpoints for processes shown to be affected by some of these ENPs in other biological systems. Glutathione-dependent enzymes such as GST are involved in detoxification of a range of xenobiotic compounds, including metals in cells, and an increase in this enzyme activity is often suggestive of cellular defence against oxidative stress.[20] We further applied CARS to investigate the uptake capability of the selected ENPs into the cultured cells.

Results

Particle characterisation

Particles dispersed in aquatic media with zeta potential values that are more positive than 30 mV or more negative than −30 mV are generally considered to be stable.[21,22] Zeta potential measurements for all particle types in both HPLC-grade water and M199 cell culture media were within these values suggesting that all particles will show a propensity to aggregate when in suspension (Table 1). This is confirmed by large and variable hydrodynamic diameter measurements made by DLS and when compared with average particle size. Although DLS results must be treated with caution when measured in polydisperse and aggregated samples, trends suggest that significant aggregation has occurred in water and, especially in exposure media. DLS-based sizes are uniformly higher in media than in water, suggesting greater aggregation and this is confirmed by the near zero zeta potentials of almost all particles. Nevertheless, TEM images (a selection are shown in Fig. 1) suggest that none of the ENP types are present as uniformly distributed particles, and there is extensive polydispersity of the particles and aggregation of particles into larger aggregates in the nanoparticulate forms. We, therefore, expect the ENPs to behave as particles much larger than the suggested manufacturer's size, which had limited accuracy.

ENP toxicity and biological effects measures in cultured hepatocytes

Triplicate trout hepatocyte cultures were conducted to assess the toxicity of the metal and metal oxide ENPs, Ag, TiO_2, ZnO, CeO_2 and their bulk counterparts.

Examination of the trout hepatocytes at 24 h and 48 h revealed the cells were viable and confluent in the controls, ENP and bulk particle exposures and showed no abnormalities in their physical appearance. Thus measurements of molecular endpoints should not be unduly affected by any low overall level of cell mortality. There was no evidence for any overt cytotoxicity as determined by the measurement of membrane integrity (LDH leakage), for any treatment compared with the controls (Fig. 2). There was also no significant difference in LDH release between bulk particles and their corresponding nano-sized counterparts.

Investigations into the ability of nanoparticles to induce intracellular oxidant production (using thiobarbituric acid reactive substances, TBARS, as a reporter of malondialdehyde, MDA) found significant lipid peroxidation only upon exposure to zinc oxide ENPs, where MDA concentrations were approximately three times higher than levels in the controls (ANOVA with Tukey's post-hoc test; d.f. = 11,51; $F = 2.945$; $P = 0.006$) (Fig. 3). Cells treated with 500 $\mu g\,mL^{-1}$ H_2O_2 as a positive control, however, failed to elicit a response significantly different

Table 1. Manufacturers specifications and TEM images, size and charge characterisation data for nano-sized and bulk particles suspended in HPLC-grade water and M199 cell culture medium

	Manufacturers specifications			Characterisation measurements				
Dispersion media	Particle type	Purity (based on ICP trace metal analysis)	Average particle size (nm)	Specific surface area ($m^2\ g^{-1}$)	Hydrodynamic diameter – z-average (nm)	Polydispersity Index	Zeta-potential (mV)	pH
HPLC grade water								
Nanopowder	TiO_2	>99.9%	23.2	46.3	189	0.27	+16.38	6.44
	ZnO	–	71	15	1207	0.72	−17.49	7.20
	CeO_2	–	10.6	76.0	338	0.40	+13.52	7.10
	Ag 10 nm	99.9%	10	9–11	589	0.54	−12.52	7.11
	Ag 35 nm	99.5%	35	30–50	2029	0.93	−6.50	7.34
Bulk powder	TiO_2	99.4%	–	–	801	0.67	+19.96	6.99
	ZnO	>99.9%	480	–	1335	0.38	−28.50	6.71
	CeO_2	>99.9%	750	–	14 724	0.90	−0.31	7.35
	Ag	99.95%	600–1600	–	938	0.69	−2.77	6.40
M199 cell culture media								
Nanopowder	TiO_2	>99.9%	23.2	46.3	1393	0.62	−9.54	7.08
	ZnO	–	71	15	5697	1.00	−4.41	6.97
	CeO_2	–	10.6	76.0	2974	0.91	−7.78	7.34
	Ag 10 nm	99.9%	10	9–11	9866	0.95	−2.60	7.35
	Ag 35 nm	99.5%	35	30–50	9088	0.52	−0.27	7.58
Bulk powder	TiO_2	99.4%	–	–	1533	0.77	−7.23	7.31
	ZnO	>99.9%	480	–	1303	0.87	−0.23	7.21
	CeO_2	>99.9%	750	–	18 852	0.69	−0.24	6.99
	Ag	99.95%	600–1600	–	1602	0.69	−4.79	6.93

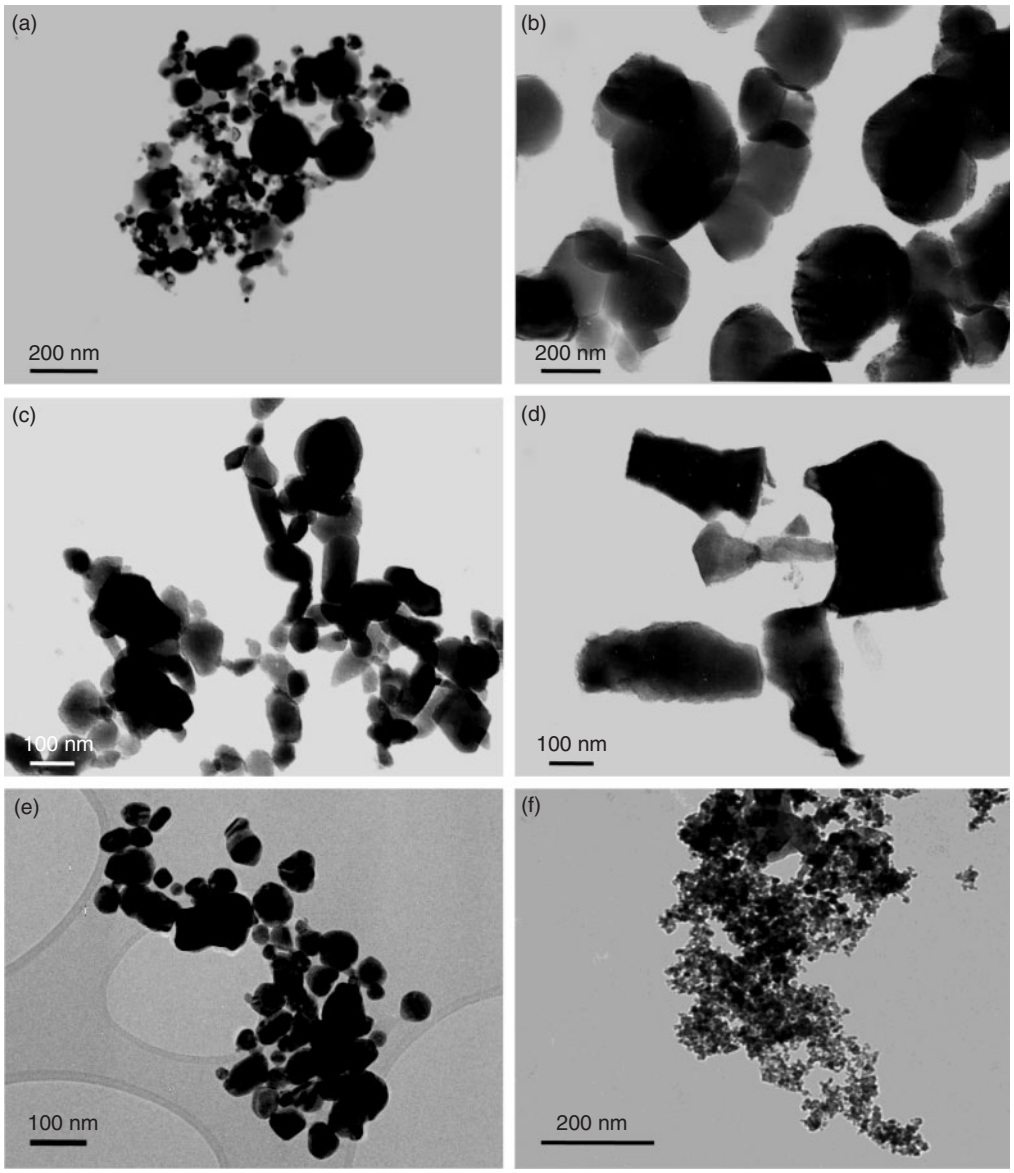

Fig. 1. Transmission electron microscopy (TEM) images of nano- and bulk particles in dry powder form. (a) TiO_2 nanoparticles; (b) TiO_2 bulk particles; (c) ZnO nanoparticles; (d) ZnO bulk particles; (e) Ag 10 nm nanoparticles; and (f) CeO_2 nanoparticles.

from the control cells in culture. The MDA concentration in an equivalent sample of untreated liver tissue homogenate was over 4-fold higher (4.47 μM) than the MDA concentration in the isolated hepatocyte controls (0.68 μM) suggesting a diminished capability of the hepatocytes to respond to cellular stress from xenobiotic compounds.

A significant increase in GST activity was only measured in cells exposed to zinc oxide bulk particles at an exposure concentration of 500 $\mu g\,mL^{-1}$ (ANOVA with Tukey's post-hoc test; d.f. = 13,59; $F = 5.276$; $P < 0.001$) (Fig. 4). Treatment of cells with 500 $\mu g\,mL^{-1}$ H_2O_2, 10 $ng\,mL^{-1}$ and 500 $ng\,mL^{-1}$ $AgNO_3$ did not cause a significant increase in GST activity compared with control cells in culture. GST activity in untreated liver tissue homogenate was two orders of magnitude higher than enzyme activity in the cultured control hepatocytes again suggesting a reduced capacity for responses to cellular stress from xenobiotics for trout hepatocytes in culture.

Imaging uptake of ENPs into cultured trout hepatocytes using CARS

The incorporation of ENPs to cultured cells was assessed visually for various metal and metal oxide ENPs (Fig. 5). Here we show signals from CARS images derived from primary hepatocytes dosed with CeO_2, TiO_2 and Ag nanoparticles. Green image contrast corresponds to the F-CARS signal from the cellular structure and red to the E-CARS ENP signal. The origin of the signal from the ENPs appears to be on the same plane as the nucleus (Fig. 5), suggesting uptake of ENPs into the cell. To confirm this, a series of 2-dimensional images were taken in the *x*–*y* plane, each separated in the *z*-direction by an increment of 0.25 μm, creating a 'z-stack' and producing a 3-dimensional interpretation of the image. This clearly placed the ENPs at an intracellular location within the hepatocytes (Fig. 6). No quantitative information on uptake, however, can be derived from these images.

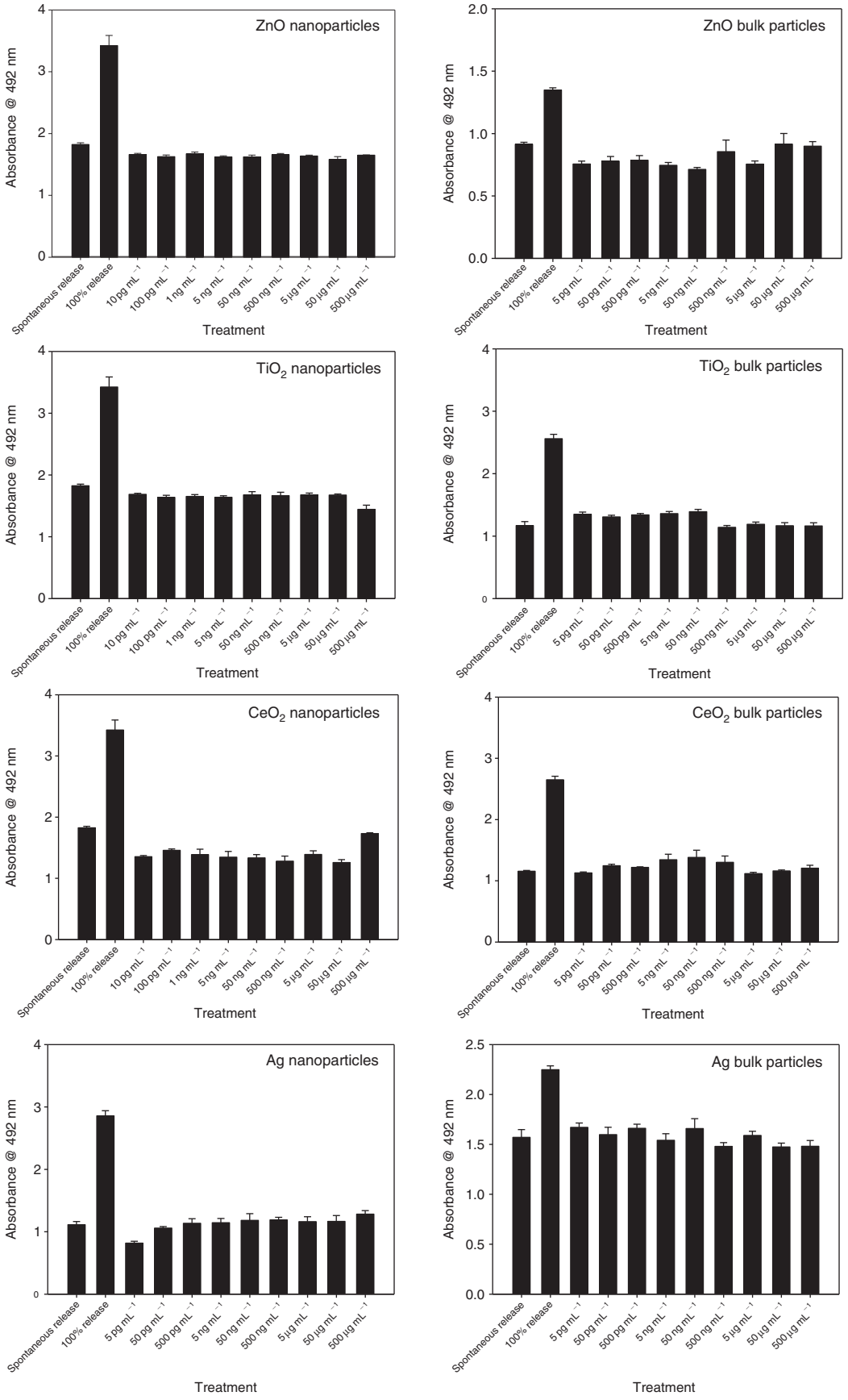

Fig. 2. Lactate dehydrogenase (LDH) release from primary trout hepatocytes into the culture medium after 24 h treatment with nanoparticles. Results are given as the mean and each carried out in triplicate. Data are means ± s.e.

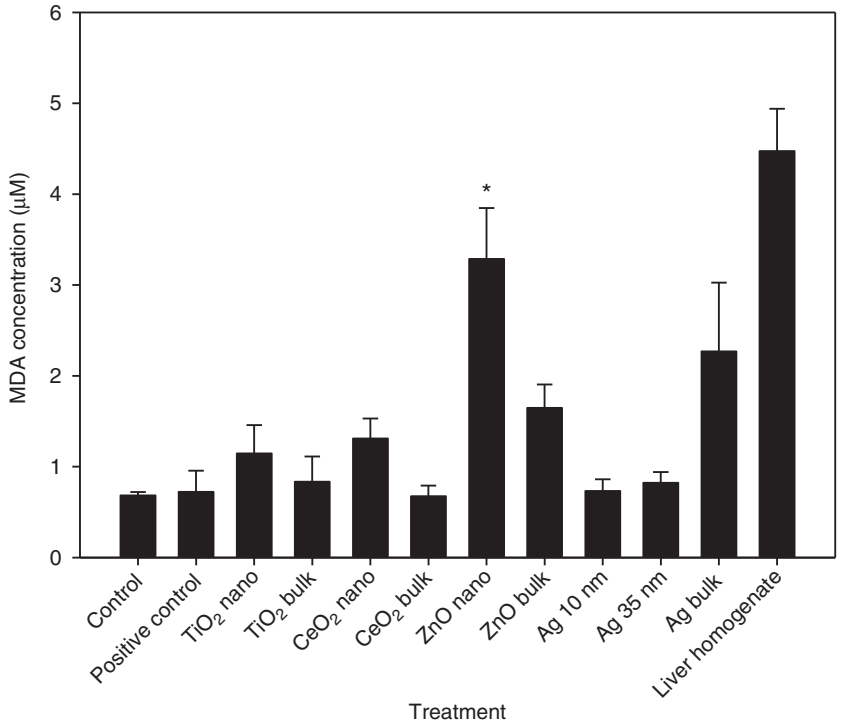

Fig. 3. Thiobarbituric acid reactive substances (TBARS) in cultured hepatocytes exposed to various nano-sized and bulk particles. Data are means ± s.e. *, significantly higher than control, positive control, Ag 10 nm, TiO_2 bulk and CeO_2 bulk (Tukey's $P = 0.006$).

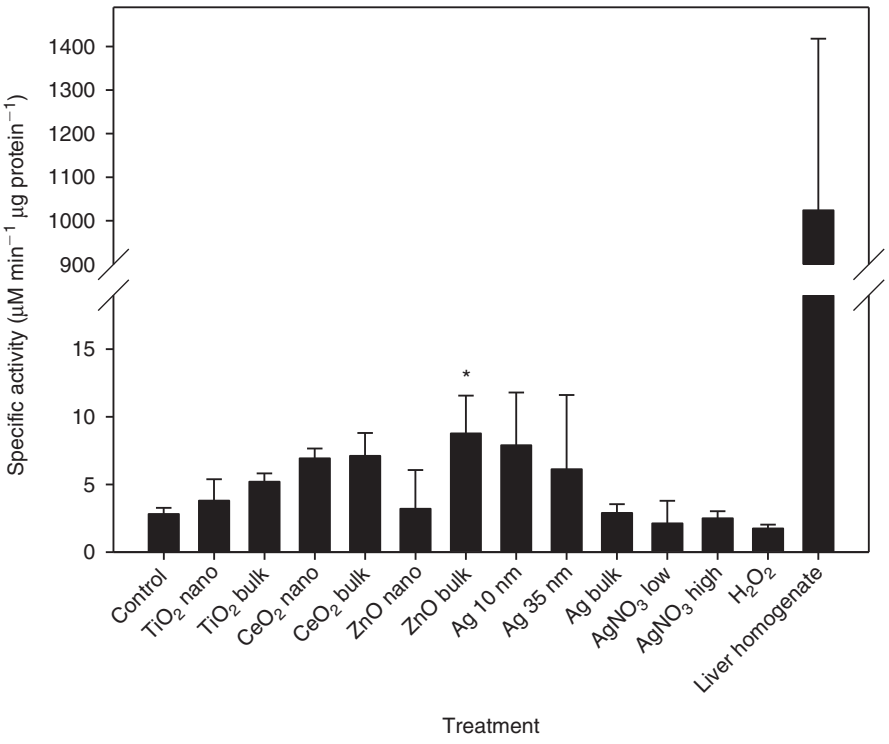

Fig. 4. Glutathione-S-transferase (GST) activity in rainbow trout hepatocytes after exposure to nano-sized and bulk particles of TiO_2, ZnO, CeO_2 and Ag. Data are means ± s.e. *, ANOVA, $P < 0.001$

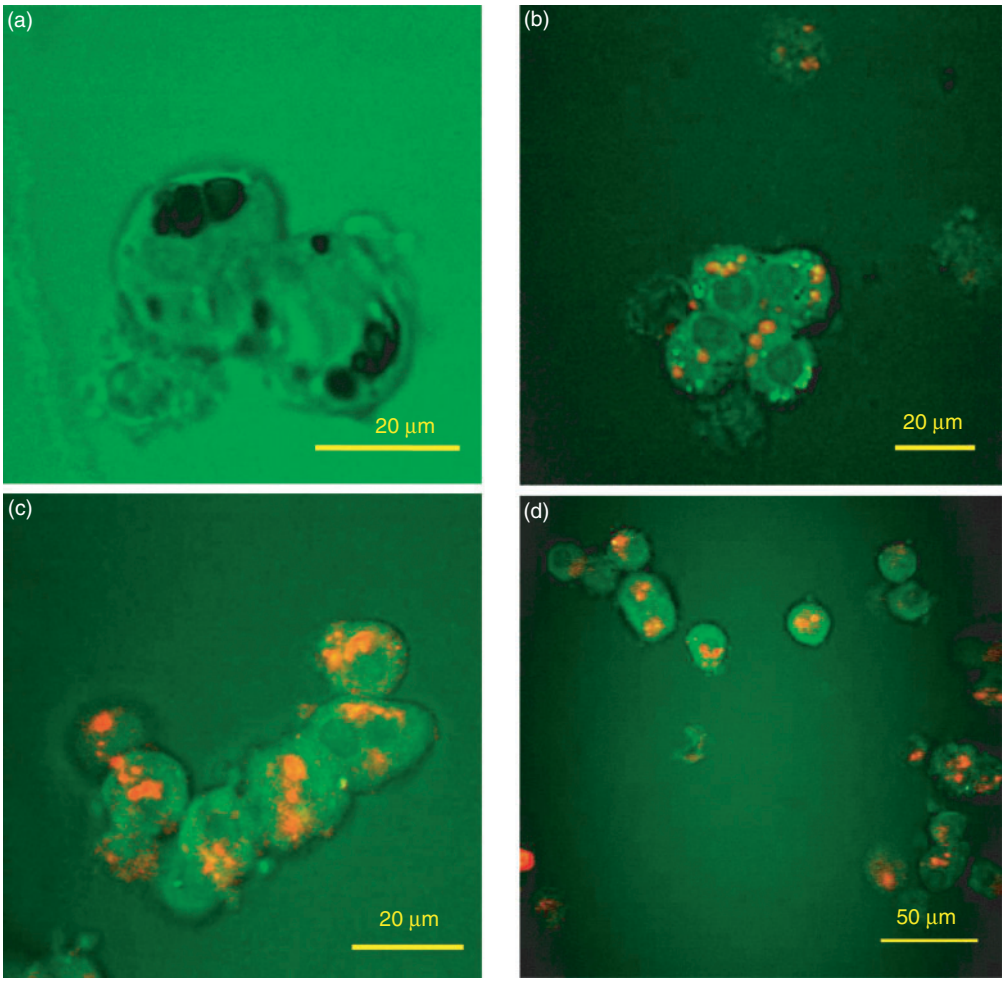

Fig. 5. Combined F- and E-CARS images of primary hepatocytes (a) control, and dosed with (b) CeO_2, (c) TiO_2 and (d) Ag nanoparticles. The F-CARS image (green) provides the cell structure and outline and the E-CARS (red) displays the very strong ENP signal. With the nucleus in focus these images are interpreted as cross-section through the middle of the cell.

Discussion

Isolated hepatocytes have been used for many years for studying liver uptake, excretion, biotransformation and toxicity of drugs and other xenobiotics and they have provided an effective system for screening chemicals for toxicological effects, including in fish.[10,20,23] Furthermore, hepatocytes are relatively easy to culture, their basic cell functions are well characterised and they have enzyme systems that are representative of many other cell types.[24] Cultured human and rat hepatocytes have been used to investigate the toxicology of various ENPs[25,26]; however, despite the fact that the aquatic environment will likely receive ENPs from a variety of sources in the near future, very few studies have used hepatocyte cultures derived from aquatic organisms to investigate the effects of ENPs. At the time of writing, the authors are aware of only one other study using fish hepatocytes to assess the toxicity of cadmium telluride quantum dots.[27]

Nanoparticle particokinetics within cells in culture are influenced by a range of factors including pH, ionic strength and viscosity of the culture media as well as particle size, shape and charge density.[28] All of these factors influence the agglomeration and sedimentation rate of the particles within the media, which in turn determines the bioavailability of particles to the cells and resultant toxic effects.

TEM imaging of the ENPs showed aggregates and fused particles were present when the particles were in dry powder form; however, characterisations on our ENPs established zeta potential values close to zero point charge when the ENPs were suspended in HPLC-grade water. This meant there was a propensity for the particles to aggregate and when suspended in M199 cell culture medium, this effect was exacerbated, likely due to the high cation concentration, suggesting that cells were likely exposed more to large aggregates rather than to discrete particles.

Limbach et al.[29] found that although smaller sized cerium oxide nanoparticles (25–50 nm) were able to penetrate human lung fibroblasts by diffusion, they underwent rapid agglomeration in the exposure media leading to sedimentation which limited their transport into cells. Larger sized particles (250–500 nm), however, remained more discrete in the exposure media and were taken up more readily. Teeguarden et al.[28] found that smaller nanoparticle agglomerates remaining in suspension in the exposure media did not necessarily come in contact with cells on the bottom of culture vessels, emphasising that measurement of the relative toxicities of nanoparticle types must take into account bioavailability and uptake into cells (effective dose), as well as the nominal dose. These studies highlight the need not only for thorough characterisation of nanoparticles in

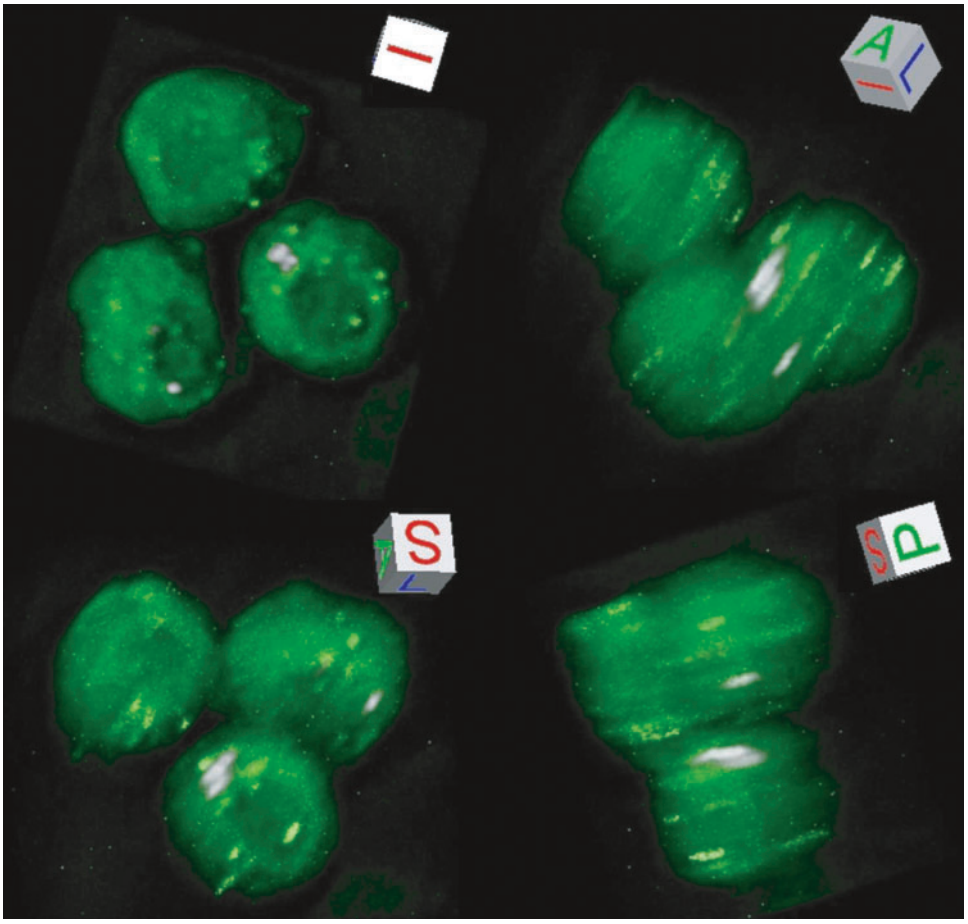

Fig. 6. Captured still frames depicting the 3-dimensional reconstruction from z-stacks showing a cluster of hepatocytes dosed with TiO_2 ENPs at 1000 ng L^{-1}. Frames are representative images of a 360° rotation, illustrating the source of the ENP signal as within the cell membrane in all three dimensions.

the exposure media, but also examination of their agglomeration, sedimentation and uptake behaviour within the culture system to gain any meaningful understanding on the uptake dynamics of nanoparticles to cells.

Membrane integrity as measured by LDH release was not shown to be affected by any nanoparticle or bulk particle type at any of the exposure concentrations, showing a distinct lack of evidence for any overt toxicity. These findings are in contrast with several other studies which have shown an increase in LDH leakage in cells exposed to metal and metal oxide nanoparticles at concentrations comparable to those used in our exposures. ZnO nanoparticles have been shown to cause LDH leakage in mouse embryo fibroblast and neuroblastoma cells at concentrations of between 10 and 100 $\mu g\,mL^{-1}$,[30,31] TiO_2 nanoparticles caused LDH leakage in mouse neuroblastoma cells and BRL 3A rat liver cells at concentrations of between 100 and 250 $\mu g\,mL^{-1}$[26,30] and Ag nanoparticles were found to cause LDH leakage in BRL 3A cells at concentrations of between 10 and 50 $\mu g\,mL^{-1}$.[26] In theory, this disparity could be due to a low level of responsiveness of the trout hepatocytes to ENPs or because levels of uptake of ENPs to the cells were too low to elicit a response.

Despite the lack of overt toxicity, CARS imaging showed that the selected ENPs were taken up into the cultured trout liver cells. All types of metal oxide and metal ENPs generated a significantly large CARS signals to be detected in both epi- and forward-directions and at both vibrational frequencies (~2855 cm^{-1} and ~2300 cm^{-1}). This is because metal oxides are wide band gap semi-conductors and thus can be visualised across a broad wavelength spectrum. When imaging away from naturally occurring vibration resonances (~2300 cm^{-1}), C–H bonds that would otherwise fluoresce are negatively contrasted and appear as dark areas (Fig. 5a). A strong signal at both wavelengths was a requirement for positive identification of the ENPs against signals produced by the presence of lipid rich structures.

The CARS z-stacks (3-day images) of cells provided strong evidence to suggest that TiO_2, CeO_2 and Ag nanoparticles were all taken up from the culture medium into the liver cells. This implies that nanoparticles enter the cells either by active endocytotic mechanisms or passively by diffusion through the membrane without compromising membrane integrity. The cultured hepatocytes contained aggregates of particles of various sizes and although some studies indicate passive diffusion of ENPs as an important means of uptake,[32] a recent review[33] has suggested that diffusion of nanoparticles through cell membranes is improbable due to the propensity of nanoparticles to aggregate in aqueous media and proposed that uptake through vesicular transport was more likely. It is not possible to determine the size of the smallest aggregate present within the cells, as the ENPs are smaller than the diffraction limit of the CARS system where the spatial resolution in the EX plane is 200 nm; therefore individual nanoparticles cannot be resolved.[34] However,

in terms of uptake this is not critical as the ENPs appear as aggregates within the cell, far larger than the spatial resolution limit. This is best illustrated in Fig. 5c, where particles appearing to be of similar size have uneven brightness, indicating the presence of different sized aggregates. The effect is further exaggerated by the nonlinear concentration dependence of Raman scatterers on the CARS signal. We were unable to identify the sub-cellular localisation of the ENPs or their aggregates using CARS because the resolution of the system is limited compared with other techniques such as electron microscopy; however, other studies have shown that ENPs are compartmentalised in lysosomes once inside the cells.[35]

In our studies, significant lipid peroxidation was only found only as a result of exposure to ZnO nanoparticles. Other studies have demonstrated ZnO nanoparticle exposure caused lipid peroxidation-mediated genotoxicity in human epidermal cells at concentrations of 0.08–0.8 $\mu g\,mL^{-1}$[36] and ROS-mediated toxicity in exposures to zebrafish embryos at concentrations of 1–10 $mg\,L^{-1}$.[37] It has been suggested that observed toxicity as a result of exposure to some metal or metal oxide nanoparticles, including ZnO and Ag, may be a result of both nanoparticle exposure and release of metal ions from the nanoparticles into the exposure media.[37–39] It was surprising therefore to find that neither silver nanoparticles or any of the other ENPs induced a detectable induction of lipid peroxidation, as oxidative stress is a common finding as a result of metal oxide and metal nanoparticle exposure.[40–42] Silver nanoparticles have previously been shown to cause lipid peroxidation in human carcinoma and sarcoma cells at a concentration of 6.25 $\mu g\,mL^{-1}$[43] and toxicity and apoptosis mediated by generation of ROS in alveolar macrophages and mouse embryonic fibroblasts at a concentration of 50 $\mu g\,mL^{-1}$.[44,45] Both TiO_2 and CeO_2 nanoparticles have also been found to induce ROS in human bronchial epithelial cells at concentrations of 5–40 $\mu g\,mL^{-1}$.[42,46] In goldfish skin cells, oxidative DNA damage as a result of hydroxyl radical formation was observed at TiO_2 concentrations of 1–100 $\mu g\,mL^{-1}$.[47] However, in a study using the rainbow trout gonadal cell line RTG-2 oxidative damage as a result of TiO_2 nanoparticles was not observed at concentrations up to 50 $\mu g\,mL^{-1}$.[41]

Interestingly, in our studies, exposure of isolated trout hepatocytes to 500 $\mu g\,mL^{-1}$ hydrogen peroxide failed to elicit an oxidative response as has been demonstrated in mammalian cell culture exposures,[48] suggesting a low responsiveness for oxidative damage.

The glutathione-S-transferase enzyme family play an important role in the defence of cells against xenobiotics.[49] They are involved in detoxification both through catalysing the conjugation of reduced glutathione, via a sulfhydryl group to electrophilic centres on a wide variety of substrates, and through direct binding of compounds including free radicals and peroxides.[20,50] Few studies have so far investigated the effect of nanoparticle exposure on GST activity. Park et al.,[42] however, found that expression of the GST gene was induced in a human bronchial epithelial cell line as a result of exposure to TiO_2 nanoparticles. In contrast, in vivo exposures of the terrestrial isopod *Porcellio scaber* to TiO_2 nanoparticles via the diet have shown conflicting results. In one study, a dose-dependent increase in GST was observed at doses of 0.5–3000 μg TiO_2 per gram of food,[51] but in the other by the same research group, no effect was seen.[52] Increased GST activity has been associated with metal exposure in several fish species.[53–55] In our study, significant increases in GST activity were only observed in exposure to ZnO bulk particles. As for our assessments on lipid peroxidation, however, there was no increase in GST activity on exposure to 500 $\mu g\,mL^{-1}$ H_2O_2, 10 $ng\,mL^{-1}$ and 500 $ng\,mL^{-1}$ $AgNO_3$. Furthermore, GST activity in homogenised liver tissue was two orders of magnitude higher per mg of protein compared with the hepatocyte controls, suggesting that GST activity was considerably diminished in the cultured cells. Previous published material provides an array of contrasting data on the suitability of rainbow trout hepatocytes for assessing toxicity endpoints. Some studies have demonstrated isolated fish hepatocytes to be good models for measurement of toxicological endpoints such as GST activity,[20,56] but others have shown lower activity in hepatocytes than whole liver samples[57] and both liver cell mono-oxygenase activity and conjugating activities can be rapidly lost in hepatocyte cultures.[24,58]

The TBARS assay shows that significant lipid peroxidation occurred as a result of exposure to ZnO nanoparticles. If the mechanism of ZnO nanoparticle-induced lipid peroxidation is via the dissociation of Zn^{2+} ions into the culture media, arguably, GST activity would be expected to be elevated since GST catalyses the conjugation of zinc ions to glutathione sulfhydryl groups. GST also functions in the detoxification of ROS, meaning the absence of raised GST activity as a result of ZnO nanoparticle exposure was unexpected. Only ZnO bulk particles showed significantly elevated levels of GST activity, but were not shown to cause lipid peroxidation, suggesting that the elevated activity was not due to detoxification of ROS, but as a result of conjugation of Zn^{2+} ions with glutathione. The ability of zinc ions to cause oxidative stress in the livers of exposed fish, however, is well known[59–61] suggesting either other mechanisms are acting to increase GST activity, or that ROS are being generated, but are not inducing lipid peroxidation. Dissociation of silver ions from silver nanoparticles has been shown to occur and has been implicated causing toxicity in zebrafish embryos exposed via the water[62] and in inducing oxidative stress in the mouse brain following intraperitoneal injection.[40] The lack of response in both the TBARS and GST activity assays in response to silver, therefore, was unexpected and further supports a diminished response of the hepatocytes to to the ENPs.

Despite a lowered functional capacity of the isolated trout hepatocytes, visually, there were no signs of abnormality and the cell membranes were intact as assessed by LDH release. The protocol used in these studies was identical to that developed by Bickley et al.[63] in our laboratory where the isolated fish hepatocytes are responsive to both cyp1A induction on toxicant challenge and vitellogenin mRNA induction after 24 h exposure to oestrogen. The absence of responses of the positive controls in the TBARS and GSH assays indicated that the isolated hepatocytes, however, were not suitable for signalling effects of ENP on lipid peroxidation or GST activity.

Conclusions

In conclusion, we have shown that some metal and metal oxide ENPs are bioavailable to fish hepatocytes in culture, and isolated trout hepatocytes thus offer a model for studying the cellular uptake of ENPs. None of the metal and metal oxide ENPs were found to be overtly toxic to trout hepatocytes in culture, however, and the hepatocytes were not found to be sufficiently responsive for the measures of lipid peroxidation or GST activity to be developed as an effective screening system for ENPs.

Although rainbow trout hepatocytes have previously been shown to be suitable for examining a wide range of physiological

parameters including responses to conventional xenobiotics,[64] it has also been shown that some liver functions may be lost upon culturing of the cells.[24,58] Even if a certain level of responsiveness is maintained, subtle responses may not be measureable suggesting hepatocytes may not be suitable for investigating the toxic responses that may be induced by novel compounds such as ENPs.

Experimental methods

Fish husbandry

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Hatchlands Trout Farm, Devon, UK, and were maintained in 500-L tanks supplied via a flow-through system with dechlorinated tap water on a 12 h light–12 h dark cycle. Water temperatures were maintained between 9° and 11°C throughout and trout were fed maintenance food rations (Emerald Fingerling 30, Skretting, UK), at a rate of 1% of their bodyweight. Trout were starved for 24 h before hepatocyte isolation.

Materials

All chemicals were purchased from Sigma-Aldrich (Gillingham, UK) unless otherwise stated. Nanoparticle and bulk particle preparations of TiO_2, ZnO and CeO_2 were all purchased from Sigma-Aldrich. Silver particles (10 nm, 35 nm and bulk particles) were purchased from Nanostructured and Amorphous Materials Inc., Houston, TX, USA. All nano- and bulk particle types were uncapped and unstabilised. Characterisation information provided by the manufacturer is shown in Table 1. Our own characterisation of the particles included images of all particles in dry powder form by transmission electron microscopy (Fig. 1), and measurements of hydrodynamic diameter, polydispersity index and zeta potential performed on a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd, Malvern, UK) for all particle types in HPLC-grade water and in M199 cell culture media (Sigma Aldrich). All measurements were carried out at nanoparticle concentrations of 1 mg L^{-1} after sonication for 30 min. The data from our characterisation of the particles is discussed in the results section.

Hepatocyte isolation

Hepatocyte isolations and cultures were based on the validated protocol of Bickley et al.[63] All solutions were sterile filtered and all instruments and glassware were autoclaved before use to maintain aseptic conditions. Perfusions solutions based on HEPES-buffered Hank's salts[65] were prepared as described in Bickley et al.[63] Briefly, salts were dissolved in ultrapure water (Maxima ultrapure water, Elga) and the resulting solutions adjusted to pH 7.3 and sterile filtered through a 0.2-μm filter (Millipore, USA).

Prior to liver perfusion, rainbow trout were anaesthetised by immersion in benzocaine until loss of equilibrium was achieved and injected with 200 μL heparin and re-immersed in the benzocaine until opercular movements ceased, at which time the brain was then destroyed. Under sterile conditions the body cavity was opened, taking care not to perforate the intestines, to expose the internal organs.

A 25-gauge needle was inserted into the hepatic portal vein and secured using a haemostat clamp. The liver was then perfused with 100 mL pre-perfusion solution (Hank's solution with 2.2 g L^{-1} Na_2-EDTA × $2H_2O$) and then 100 mL perfusion solution (Hank's solution containing 358 mg L^{-1} $CaCl_2 \times 2H_2O$ and 120 mg L^{-1} collagenase D) and then flushed with 100 mL Hank's solution with 880 mg L^{-1} Na_2-EDTA × $2H_2O$. The liver was then excised from the body cavity into a Petri dish, held on ice, teased apart using forceps and a razor blade in calcium and magnesium free (CMF) Hank's solution, and then the resulting suspension passed through a series of three mesh sizes: 250, 100 and 50 μm to further separate the cells. The suspension was then centrifuged at 75*g* for 5 min at 4°C and the resulting pellet re-suspended in CMF, centrifuged and washed a further two times. Finally the pellet was re-suspended in sterile M199 (Sigma Aldrich), pH 7.3, supplemented with 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin and 10 μg mL^{-1} streptomycin. Cell viability was examined by a trypan blue exclusion test and the cell number counted using a haemocytometer. Serowell flat-bottomed 96-well microtitre plates (Bibby Sterilin Ltd, UK) were seeded with 200 μL cell suspension per well at 1.5×10^6 cells mL^{-1} (optimum cell density cited by CytoTox 96® Non-Radioactive Cytotoxicity Assay kit, Promega, UK) for the lactate dehydrogenase (LDH) assay and for the TBARS assay and glutathione-S-transferase (GST) assay a 200 μL cell suspension at 2×10^7 cells mL^{-1} was used (optimum cell density cited by TBARS Assay kit, Cayman Chemical, USA). The cells were maintained for 24 h in a humidified incubator at 11°C to allow cells to become confluent before nanoparticle exposure.

Nanoparticle exposures

Trout hepatocytes were exposed to nanoparticles 24 h after isolation. For LDH determinations, nanoparticle and bulk particle suspensions of TiO_2, ZnO, CeO_2 and Ag with concentrations of between 20 pg mL^{-1} and 1 mg mL^{-1} in M199 in 10% FBS were made and were sonicated for 20 min before dosing. Cells were dosed by 100 μL media replacement with the nanoparticle suspensions, at concentrations of between 10 pg mL^{-1} and 500 μg mL^{-1} in quadruplicate wells and exposed for 24 h in a humidified chamber at 11°C. For lipid peroxidation and GST assessments dosing suspensions were prepared in a similar way with a single dosing concentration of 500 μg mL^{-1} used for both analyses.

ENP toxicity assessment – lactate dehydrogenase assay

The viability of hepatocytes 24 h after isolation, before ENP exposure, and after 48 h (post ENP exposure) was assessed visually under an inverted microscope (M40-5818, Wild Heerbrugg, Switzerland). Toxicity of the ENPs to the cultured hepatocytes was assessed using LDH release. The LDH assay was carried out using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega), following the manufacturers guidelines. Briefly, half of the control cells (4 wells) were lysed by incubating with 20 μL 9% (v/v) Triton® X-100 for 45 min at 37°C. The plates were then centrifuged at 250*g* for 4 min at room temperature. A 50-μL aliquot of cell supernatant from control and ENP treated wells was then transferred to a fresh 96-well flat bottom microtitre plate. LDH assay solution, reconstituted in phosphate buffered saline + 1% bovine serum albumin (BSA) (50 μL) was then added to each well, after which the plate was incubated in the dark at room temperature. During this time the reduction of NAD^+ to NADH is catalysed by LDH released from cells. NADH in turn reacts with a tetrazolium salt to form a red formazan product. The reaction was stopped by the addition of 50 μL 1 M acetic acid and the resulting absorbance measured at 490 nm on a Molecular Devices SpectraMax 340pc microplate reader. The absorbance readings for exposed cells were compared against readings for

spontaneous cell release and 100% LDH release from hepatoctes lysed with Triton® X-100.

Lipid peroxidation and glutathione-S-transferase activity

Thiobarbituric acid reactive substances assay

Metal-induced toxicity in cells is often mediated by the production of reaction oxygen species such as the superoxide ion, hydrogen peroxide, and hydroxyl radical.[66,67] Measurement of 1,1,3,3-tetramethoxypropane (malonaldehyde, MDA) using the TBARS assay is widely used as an indicator of lipid peroxidation. The protocol adopted was similar to that described previously.[68] Briefly, eight standards of MDA at concentrations ranging from 0.625 to 100 μM were made up for the TBARS assay using dilutions of a 500-μM stock solution of MDA in 2% ethanol prepared in M199 media. Cells were also exposed to 500 μg mL^{-1} H_2O_2 as a positive control. After exposure, cells were homogenised using a Teflon pestle and handheld homogeniser and the homogenate centrifuged at 1600*g* for 10 min at 4°C. Supernatant and standards (100 μL) were then mixed with 500 μL of 0.4% thiobarbituric acid (40 mg in 10 mL) in 10% acetic acid, pH 5.0 in 1.5-mL microcentrifuge tubes. The tubes were heated to 90°C for 1 h and then cooled to room temperature under a stream of flowing tap water. Butanol (600 μL) was added and the mixture vortexed vigorously mixed for 5 s. The mixture was then centrifuged at 3100*g* for 10 min and the 150 μL of butanol phase removed and placed in duplicate in a flat-bottomed, 96-well microtitre plate and the absorbance measured at 532 nm on a Molecular Devices SpectraMax 340pc microtitre plate reader. A standard curve was prepared and MDA concentrations were determined accordingly. The TBARS assay was conducted for each ENP treatment in three separate hepatocyte isolations.

Glutathione-S-transferase (GST) assay

GSTs play a major role in the detoxification of xenobiotic chemicals within cells and measurement of increased activity is often used as a biomarker for exposure to environmental contaminants.[56,69,70] Following exposure to nanoparticle and bulk particle types for 24 h, GST activity was assessed in the hepatocytes. Preparations of 100 mM phosphate buffered saline pH 7.3 (PBS), 10 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 10 mM reduced L-glutathione (GSH) were warmed to 25°C in a water bath. Cells and liver tissue were sonicated with a sonic probe (Sonicprep 150 MSE Scientific Instruments, UK) over ice for 20 s. The resulting homogenate was centrifuged for 20 min at 4°C at 10 000*g*. A 40 μL aliquot of the resulting supernatant was added to microtitre plates in quadruplicate over ice using PBS as a blank and 200 μL PBS and 20 μL GSH were then quickly added to all wells and the reaction followed spectrophotometrically at 340 nm at 25°C for 3 min. Cells exposed to 500 μg mL^{-1} H_2O_2 and 10 ng mL^{-1} and 500 ng mL^{-1} $AgNO_3$ were set up as positive controls due to the ability of H_2O_2 to induce oxidative stress in cell cultures in vitro[48] and the known toxicity of $AgNO_3$ in fish tissues.[54,71] In order to ascertain whether the response of primary hepatocytes differed compared with dissected whole liver tissue, 100 mg of rainbow trout liver tissue was dissected from an untreated fish was run in parallel with the treated hepatocytes. GST activity was expressed in nanomoles of conjugated reduced glutathione min^{-1} mg^{-1} protein (extinction coefficient, $\varepsilon_{340} = 9600$ L mol^{-1} cm^{-1}).

GST activity in the cells was quantified in cells and tissue relative to the total protein content of the sample. Protein concentration was determined in cell homogenates using the Bio-Rad Reagent Protein Assay (Bio-Rad, USA) following the manufacturers guidelines. Briefly, cell homogenates were diluted by 1 in 10 with ultrapure water (Maxima ultrapure water, Elga). Eight BSA standards of between 0 and 1 mg mL^{-1} were made. Diluted homogenate and standards (5 μL) were then added to a microtitre plate in duplicate. Bio-Rad Protein Assay Reagent was diluted 1 in 5 with water and 200 μL added to each well. The absorbance was measured at 595 nm on a Molecular Devices SpectraMax 340pc microtitre plate reader. A standard curve was prepared and protein concentrations were determined accordingly.

Coherent anti-Stokes Raman scattering

Coherent anti-Stokes Raman scattering (CARS) microscopy was adopted to visualise the internalisation of the ENPs into the hepatocytes. Imaging was performed using a modified commercial inverted microscope and confocal laser scanner (IX71 and FV300, Olympus UK), as described previously.[19] Laser excitation was achieved using an optical parametric oscillator (Levante Emerald, APE Berlin) pumped with a frequency doubled Nd:Vandium picoseconds oscillator (OPO LEVANTE™, High-Q Laser Production GmbH, Austria). The optical parametric oscillator (OPO) produced signal and idler beams with pulse duration of 6 ps and a repetition rate of 76 MHz. A 60X, 1.2 NA water immersion objective (UPlanS Apo, Olympus UK) was used to focus the laser excitation into the sample. Due to the directional nature of CARS, forwards (F)- and epi (E)-detected images contain complementary structural information.[72] As described previously, E-CARS provides preferential contrast of sub-wavelength particles, which when combined with the corresponding F-CARS image allows the location of nanoparticles to be co-registered with biological structures with sub-cellular spatial resolution.[19] The F-CARS signal was collected by an air condenser (NA = 0.55) and directed onto a red sensitive photomultiplier tube (R3896, Hamamatsu UK) via a mirror and collimating lenses. The E-CARS signal was collected using the objective lens and separated from the pump and Stokes beam by a long-wave pass dichroic mirror (z850rdc-xr, Chroma Technologies USA) and directed onto a second R3896 photomultiplier tube at the rear microscope port. The anti-Stoke signal was isolated at each photodetector by a single band-pass filter cantered at 750 nm (HQ750/210, Chroma Technologies USA).

Cellular structure was imaged using the CH_2 stretching vibration (2855 cm^{-1}) collected in the forwards direction. Contrast of ENPs was achieved by imaging the cell with a vibrational frequency away from naturally occurring vibration resonances (~2300 cm^{-1}) and the signal collected in the epi-direction to enhance the detection of sub-wavelength particles. The F- and E-CARS detected images were merged in false colour to provide simultaneous visualisation of the ENPs and surrounding cellular structures.

Three-dimensional data to prove cellular internalisation of the exposed ENPs were acquired by taking stacks of 2-dimensional images in the *x*–*y* plane each separated by an increment in the *z*-direction, which was achieved by alteration of the objective focus. The 3-dimensional reconstruction of the datasets was performed using Osirix open source software (OsiriX Imaging Software, see http://www.osirix-viewer.com, accessed 3 December 2009).

Statistical analyses

The data are expressed as mean values ± s.e. and were analysed using SPSS version 16.0, with α set at 0.05. All data were checked

for conformity with the assumptions of normality (homogeneity of variance and normality of error). If these assumptions were not met, data were transformed to meet these assumptions.

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Manufactured nanoparticles: their uptake and effects on fish—a mechanistic analysis

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Abstract There is an emerging literature reporting toxic effects of manufactured nanomaterials (NMs) and nanoparticles (NPs) in fish, but the mechanistic basis of both exposure and effect are poorly understood. This paper critically evaluates some of the founding assumptions in fish toxicology, and likely mechanisms of absorption, distribution, metabolism and excretion (ADME) of NPs in fish compared to other chemicals. Then, using a case study approach, the paper compares these assumptions for two different NPs; TiO_2 and C_{60} fullerenes. Adsorption of NPs onto the gill surface will involve similar processes in the gill microenvironment and mucus layer to other substances, but the uptake mechanisms for NPs by epithelial cells are more likely to occur via vesicular processes (e.g., endocytosis) than uptake on membrane transporters or by diffusion through the cell membranes. Target organs may include the gills, gut, liver and sometimes the brain. Information on metabolism and excretion of NPs in fish is limited; but hepatic excretion into the bile seems a more likely mechanism, rather than mainly by renal or branchial excretion. TiO_2 and C_{60} share some common chemical properties that appear to be associated with some similar toxic effects, but there are also differences, that highlight the notion that chemical reactivity can inform toxic effect of NPs in a fundamentally similar way to other chemicals. In this paper we identify many knowledge gaps including the lack of field observations on fish and other wildlife species for exposure and effects of manufactured NMs. Systematic studies of the abiotic factors that influence bioavailability, and investigation of the cell biology that informs on the mechanisms of metabolism and excretion of NMs, will greatly advance our understanding of the potential for adverse effects. There are also opportunities to apply existing tools and techniques to fundamental studies of fish toxicology with NPs, such as perfused organs and fish cell culture systems.

Keywords Fish · Trout · Zebrafish · C_{60} fullerenes · Titanium dioxide · Nanoparticles · Toxic mechanisms

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Introduction

The presence of manufactured nanomaterials (NMs) and nanoparticles (NPs) in the environment now seems inevitable given the rapidly growing number of products containing these materials (Roco 2003; Aitken et al. 2006; Boxall et al. 2007). Nanomaterials are used in a range of domestic appliances and household products, in the manufacture of textiles and electronics, as well as medical products and in bioremediation technology. Clearly all of these will create waste water or effluents, and there are concerns about the environmental risks of nanotechnology which need to be balanced against their undoubted benefits to human society (e.g., Royal Society 2004; Crane and Handy 2007; Owen and Handy 2007; Defra 2007). Handy and Shaw (2007) recently reviewed the risks to human health via the environment, and identified a number of exposure routes including the discharge of NPs to water and agricultural land. It seems that both aquatic and terrestrial habitats will be exposed to manufactured NPs. The

environmental risk to terrestrial wildlife has yet to be addressed, but there is an emerging literature on effects data for fish and aquatic invertebrates. Descriptions of the possible biological effects of various types of NPs and NMs have been reviewed elsewhere (e.g., Moore 2006; Crane and Handy 2007) and it is not our intention to repeat those observations, but rather, to critically evaluate our knowledge on the effects of manufactured NPs from the view point of toxic mechanism. Given the lack of data on terrestrial vertebrates, our analysis will focus on fish and the aquatic environment. We aim to identify the main toxic mechanisms involved in causing (or likely to cause) adverse effects on the health of fish. We identify the body systems, and the key concerns in fish toxicology that are critically different from biomedical studies and rodent models. Finally using a case study approach, we highlight some of the knowledge gaps and complexities in NM ecotoxicology by studying two very different groups of manufactured NMs (C_{60} fullerenes and related carbon-based NMs, compared with titanium dioxide as relatively well studied metal-based NP).

Fish habitats and the physico-chemistry of manufactured NPs

The chemistry of colloids and naturally occurring NPs has been extensively studied in the aquatic phase (Elimelech et al. 1995; Grasso et al. 2002; Buffle and van Leuween 1992; Lead and Wilkinson 2006; Stolpe and Hassellöv 2007) and we have been able to transfer some of this knowledge to manufactured NPs in the context of ecotoxicology (see recent review of NP ecotoxicology and chemistry, Handy et al. 2008a). The physico-chemistry, behaviour and measurement of manufactured NPs in the aqueous phase and the key chemistry issues for the ecotoxicologist are described elsewhere in this special issue (e.g., Christian et al. 2008; Handy et al. 2008b), including the dispersion of manufactured NPs in different natural waters, their surface chemistries and reactivity, and their aggregation and colloid chemistry. There is still much to learn about the complex behaviour of natural NPs, and even more to learn about manufactured materials. Nonetheless, there are some key differences in physico-chemistry between freshwater and marine environments, and at sediment–water interfaces compared to the water column, that have implications for fate and behaviour, and exposure of fish to NPs. First, the high ionic strength of seawater will tend to cause aggregation of NPs (in the absence of any stabilising organic matter), and some NPs can aggregate in the presence of cations such as Ca^{2+}. It is therefore likely that marine fishes, fish living in some hard waters, and benthic species at the sediment interface, will be exposed to aggregates of NPs rather than material in the liquid phase (colloid suspensions, see below). However, the presence of organic matter such as some fulvic and humic substances (Lead and Wilkinson 2006; Handy et al. 2008a) can stabilise NPs in the liquid phase. This effect is possible in some soft freshwaters where natural organic matter may be present. So, it seems likely that NP behaviour will be different in the various habitats where fish are found. It may also be altered by rainfall, or other weather events, that dramatically alter hydrology and the presence of organic matter, particulates, and/or the salinity of the water. The biological consequences of exposure to aggregates as opposed to suspensions of NPs will partly depend on the epithelial biology of the animals (discussed below), and we should not make the erroneous assumption that aggregated material will not necessarily be bioavailable. It may simply change the mode of delivery from respiratory exposure in the water column to dietary exposure via the sediment phase. Of course, the aggregation process may also change the overall size of the material presented to the organism, from the nanoscale of the primary particles to perhaps the micron scale for larger aggregates.

Routes of uptake in fish and the physico-chemistry of manufactured NPs

There are some emerging features of the physico-chemistry that are highly relevant to routes of uptake in fish. One of the founding assumptions in the ecotoxicity of chemicals is that there is likely to be close relationships between the physical or chemical form of the substance in the environment, the ability of the organism to absorb it, and any subsequent toxic effects. This fundamental assumption remains for manufactured NPs in fish toxicology, but in addition to the usual abiotic factors (temperature, dissolved oxygen, salinity, water hardness, etc.) consideration of the details of colloid and aggregation chemistry is needed. The first point to make is that manufactured NPs generally do not exist in true *aqueous* solution, and the concept of *solubility* is therefore less relevant, except in the context of the gradual dissolution of atoms or ions from the surface of the NP into the aqueous phase (Handy et al. 2008a). It is difficult to generalise about the chemistry of manufactured NPs in liquids such as natural waters, because the diversity of the manufactured materials will always generate exceptions. Nonetheless, many manufactured NPs exist as emulsions or suspensions in the liquid phase (not strictly *aqueous*, Handy et al. 2008a), and may be designed with no consideration of water at all in the manufacture. One exception to this generalisation would be NMs or NPs that have been surface coated or functionally modified in some way to make them more acceptable for biological

applications. This might include making the material miscible with physiological salines, or solvents used for drug delivery in clinical studies.

However, this general chemistry indicates that fish would usually be exposed to NP in suspension, and not directly to chemicals in aqueous solution. Clearly, NPs are so small that any suspension would easily have access to the water flowing between the secondary lamellae of the gills and would have access to the gill surface layers. In addition, any water drunk by the fish (e.g., stress induced drinking, see Smith et al. 2007) will present NPs to the gut mucosa. Thus, NPs in the liquid phase could present either a respiratory or dietary exposure risk. Both the gill epithelium and the gut epithelium are typical vertebrate epithelial layers, and the basic features of epithelial design are not that different from other vertebrate animals (e.g., rat gut or lung), so some of the concerns we raise here may also apply to other animals. The gill or gut surface of fish consists of the bulk fluid (e.g., river water, gut luminal fluid), which contributes to the formation of an unstirred layer (USL) over the epithelium (Fig. 1). This boundary layer of water can exchange with the mucus covering the epithelial cells, and the mucus layer in turn can present materials to the apical surface of the epithelia (review, Handy and Eddy 2004). The behaviour of metal ions, their diffusion and ion exchange properties in the USL, and with mucus have been well described (see Handy and Eddy 2004); and even applied to ligand models to predict toxic effects of metal exposure (e.g., biotic ligand models, BLM, Paquin et al. 2002). Similar models have been developed for organic chemicals (e.g., Erickson and McKim 1990). The central question is whether or not any of these models could be applied to NPs. Figure 1 outlines the key issues for NPs in the microenvironment of an idealised fish epithelium. The movement of NPs in the bulk water will be by Brownian motion and other diffusional processes, and while this may be slower than that of single atoms or small organic molecule, it is not necessarily fundamentally different in the context of providing material to interact with the USL. Movement of NPs from the bulk water into the USL would be governed by peri-kinetic forces (shear forces) at this interface (see Handy et al. 2008a for details of the theory) that are partly determined by the speed of flow of the bulk water compared to the USL, particle size/shape, and the viscosity of the USL. Similar ideas also apply to atoms and small molecules (Pedley and Fischbarg 1978), and so although the causes of the forces may be different for NPs compared to molecules or atoms (i.e., other chemicals), the net effect remains the same.

Substances in the boundary water of the USL may then move into the mucous component on the epithelial surface. Fish mucus is similar to the mucus of other vertebrates, consisting of about 97% water, some mucoproteins with highly conserved sialic acid, carboxylic acid and sulphated residues; and various soluble materials such as electrolytes (Handy and Eddy 2004). Mucus is therefore a viscous solution of polyanions, and is well known for its ability to

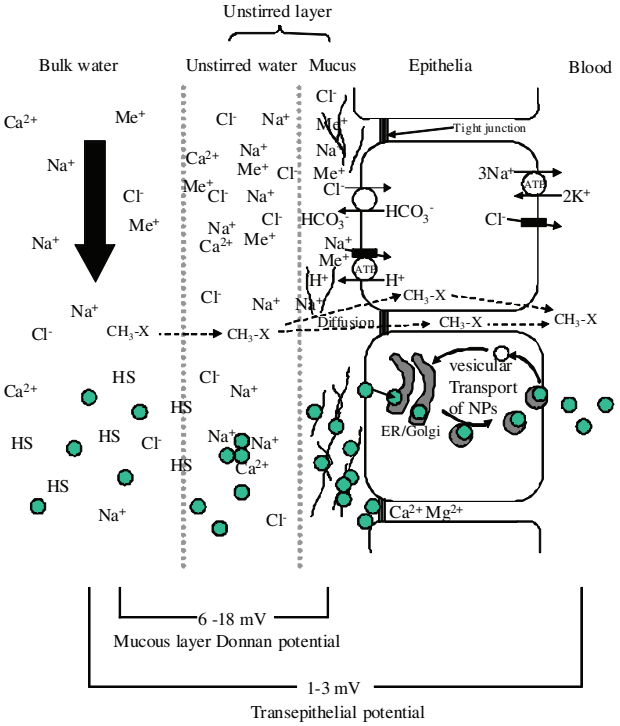

Fig. 1 An idealised diagram of the freshwater fish gill showing the mechanisms of uptake for electrolytes, toxic metal ions (Me^{+}), and small lipophilic organic chemicals (CH_3–X), compared to nanoparticles (NPs, filled circles). Modified from Handy and Eddy (2004). The substances in the bulk solution (the freshwater) must diffuse into an unstirred layer (USL) comprising of water/mucus secretions, prior to transfer across the gill epithelium. The upper portion of the diagram shows electrolytes and toxic metals ions which diffuse into the USL, and may bind to strands of mucus (mostly polyanionic) where the exclusion of free anions like Cl^{-} from the mucus layer contributes to the Donnan potential at the apical surface. Electrolytes and toxic metal ions usually move through the cell using ion transport pathways (Na^{+} transporters are illustrated here). In contrast, small lipophilic organic chemicals can diffuse into the USL and then through the cells (transcellular diffusion), or between the cells via the tight junctions (paracellular diffusion). The situation for NP uptake across the gill will be a little different. NPs will diffuse into the USL, albeit at a slower rate than smaller molecules or solutes, and may be influenced by humic substances (HS). Cationic NPs will bind to strands of mucus (electrostatic attraction, fundamentally similar to other cations), but regardless of surface charge, may also become entangled in the mucoproteins (steric hindrance) to prevent uptake by the epithelial cells. NPs are too large to be taken up by ion or other transporters on the cell membranes, and although diffusion cannot be excluded for lipophilic NPs. The Ca^{2+} and Mg^{2+} rich environment in the tight junctions suggest that NPs would aggregate rather than diffuse through the paracellular route. Diffusion of charged NPs into the USL will be affected by the Donnan and transepithelial potentials, in a similar way to other charged substances. NP uptake through vesicular transport seems likely

chelate cations, especially toxic metal ions with a high charge density and low ionic mobility in solution relative to H^+ (Handy and Eddy 1991). In essence, the surface charge of NPs and their electrostatic properties (Handy et al. 2008a) are not fundamentally different from charged ions in terms of biological effect. Arguably, NPs with a polycationic (or anionic) surface may simply present a bigger charge layer and longer distances over which electrostatic forces may interact. Thus NPs might bind more strongly, or more quickly, to the mucoproteins. Alternatively, NPs are bigger than atoms, and steric hindrance (size, shape) may slow electrostatic interactions with individual mucoproteins. However, this may not matter, as any non-spherical shape would be more likely to tangle with strands of mucus. Thus overall, one could argue that manufactured NPs might be trapped in the mucus layer, and perhaps do so more readily than other substances. This is certainly the case with single walled carbon nanotubes which readily associate with gill mucus in trout (Smith et al. 2007).

The biological implications of this USL/mucus chemistry are both beneficial and adverse. The function of the mucus layer is to protect the fish from irritants in the external environment. Trapping chemicals in the mucus, with subsequent sloughing of the contaminated mucus will protect the sensitive epithelial cells. There is no reason to suspect that this sloughing of mucus would be prevented by NPs, in fact quite the opposite, with anecdotal reports from researches of mucus precipitates appearing quickly in the fish tanks during NP exposure. However, the disadvantage is that the supply of mucus will eventually be exhausted, and this might occur more quickly with NPs compared to some other substances.

The next step in biological uptake is the transfer of material from the USL across the apical membrane into the cell and then through the cell to the blood (transcellular uptake), or alternatively, paracellular diffusion through the tight junctions between the cells into the blood. For respiratory exposure, fish have a much tighter gill epithelium (e.g., McWilliams and Potts 1978) compared to the lung epithelium of mammals and so the paracellular route is less vulnerable in fish. On a size basis alone, NPs are less likely to penetrate the tight junctions than smaller molecules or atoms. The tight junctions between gill cells (and gut cells) also contain large amounts of Mg and Ca ions, and this matrix would cause aggregation of the NPs which would further slow penetration. The permeability of tight junctions are slightly modulated by physiological status such as the effects of cortisol (Kelly and Wood 2001). So it is possible that under certain conditions that tight junction permeability to water molecules and ions will change, but this does not mean that the permeability to NPs will increase as these are much larger than single atoms or water molecules.

Moore (2006) presents a theoretical argument for vesicular trafficking of NPs through cells, and this remains a concern for fish, especially in the gut epithelium where fish are able to take up materials by endocytosis. In this regard, fish may be more vulnerable than mammals, since fish guts are able to take up much larger materials by endocytosis across the gut (a difference that has long been exploited in the oral delivery of fish vaccines). Manufactured NPs would be too large to cross the cell membrane on membrane transporters, but the diffusion of lipophilic NPs directly through the cell membrane cannot be excluded at present. Of course, all of the above arguments apply to an intact epithelium that is functioning relatively normally. All of these rules are abandoned when the gill or gut is compromised by severe inflammation or erosion. Any substance in the external environment could simply diffuse directly into the blood through the damaged tissue. Manufactured NPs do cause inflammation of the gill and injury to the gut at low mg concentrations (e.g., TiO_2 NPs Federici et al. 2007; carbon nanotubes, Smith et al. 2007), so in these situations direct uptake to the blood is theoretically possible. However, it may be less likely than some other substances, because the high ionic strength associated with body fluids leaking out of the injured tissue would tend to aggregate and precipitate the NPs. In this regard, aggregation may be beneficial in preventing NP uptake across a compromised epithelium.

The skin comprising the general body surface of the fish is often considered as a robust barrier to the external environment, and like that of mammals consists of three layers; the epidermis, dermis (scales in the case of fish), and the hypodermis. In fish and amphibians which have wet skin, the epidermis is protected by mucous secretions rather than by the keratinised epithelial cells found on the dry skin of some reptiles, birds, and mammals. So for fish at least, the skin mucus will act as a barrier to NPs as it does for other chemicals. Metal ions do not easily penetrate the skin of fish, mainly because of a perceived sparseness of metal transporters in the skin cells compared to the gills. Charged NPs may be trapped in skin mucus for the reasons outlined above. Lipophilic molecules diffuse slowly through fish skin, and it is likely that similar, but much larger, lipophilic NPs will diffuse very slowly (or not at all). There are several reports of NP uptake in mammalian skin cell cultures (see Handy and Shaw 2007 for discussion of culture studies), but these are not representative of intact vertebrate skin (e.g., no basement membrane, often only one of the layers in the culture system) and ecotoxicologists should not draw conclusions about the permeability of fish skin to NPs from such studies. We should, however, be concerned about the relative size of the lymphatic drainage under fish skin compared to that of mammals. In fish, unlike mammals, the lymphatic system has an extensive

network in close proximity to the skin and drains rapidly into the main circulation. Therefore, if the skin is compromised (by infection or inflammation) then it is likely that manufactured NPs could quickly gain access to the circulation and internal organs. Thus fish with skin infections or skin abrasions may present a higher risk of internalising manufactured NPs than healthy fish.

Other external surfaces include the buccal cavity, olfactory openings, eyes, and urinary/genital openings. The skin in these areas is of similar basic construction to the skin elsewhere. However, there are nerve endings for olfaction and for taste in the former surfaces, and these present direct external access to the nervous system. Fish are known to accumulate chemicals through the olfactory bulb (e.g., mercury, Borg-Neczak and Tjalve 1996), but the cellular mechanisms often involve uptake of the metals into the nerve cells via membrane transporters, or axonal transport of organic chemicals along the axis of the nerve fibre. It seems probable that NPs are just too large to take advantage of these uptake mechanisms. However, it does not mean that NPs will not alter olfaction by interfering with receptors in the epithelium, and could still have profound effects on fish behaviour, and therefore survival. We also cannot exclude neurological injury via secondary effects of NPs to cause oxidative stress, or changes in local blood flow in the CNS (Smith et al. 2007). In mammals, and probably in fish, the eye represents a possible route of diffuse entry of organic chemicals directly into the optic nerve and therefore the brain. The vitreous humor in the eye of vertebrates is a saline gel, and this at least would cause NP aggregation. This may prevent diffuse uptake along the optic nerve, but equally, NP precipitation could impair vision and may even cause mechanical damage to the retina.

Distribution, metabolism and excretion

Handy et al. (2008a) reviews the sub-lethal effects of manufactured NPs in fish including absorption, distribution, metabolism, and excretion (ADME). This review identifies the gills, gut, liver and brain as possible target organs for the toxic effects of some manufactured NPs in fish, as well as a range of toxic effects including oxidative stress, cellular pathologies consistent with tumour formation in the liver, some organ specific iono-regulatory disturbances, and vascular injury. However, many knowledge gaps and technical barriers remain. For example, the lack of simple, routine methods for the direct measurement of manufactured NPs in tissues is limiting our knowledge on body distribution. Electron microscopy of dissected tissues is very labour intensive, and although fluorescent polystyrene microspheres may be used to demonstrate the possibility of NP uptake into juvenile stages of fish (Kashiwada 2006), there is a need to develop suitable labels for manufactured NPs that may be of environmental relevance. This would include some validation that the label remains attached to the NP once inside the organism. These technical issues currently prevent the establishment of a definitive list of target organs for manufactured NPs and quantitative analysis of distribution (such as % of body distribution in each organ). It is possible to identifying target organs on the basis of biological effects, but as Federici et al. (2007) point out for TiO_2 NPs at the gills, surface acting toxicants can generate injury to internal organs through secondary means (e.g., diffusion or bulk transport of oxygen radicals, release of inflammatory factors into the blood, etc.). There is a concern that NPs could be potent surface acting toxicants, and toxicologists should not make the assumption that injury to an internal organ equals the presence of the NP in that organ. Of course this is an academic point from the perspective of biological effects, the fact that an organ or body system is damaged is the key issue. However, there is an environmental monitoring/regulatory implication. National monitoring programmes for fish periodically measure chemical residues in fish tissue to assess contamination in the environment, derive implications for fish health, or the safety of a fishery for human consumption. Regulators do not yet have the scientific basis (distribution and accumulation studies) on which to found such a monitoring scheme for manufactured NPs.

Studies on the metabolism and excretion of manufactured NPs are limited for similar technical reasons. However, it is worth considering whether there are any fundamental reasons why these processes would be different for manufactured NPs compared to other substances. Fish can excrete substances from the blood via the liver, kidney, gills, and also through exuded skin mucus (intracellular in origin). One assumption for excretion from the blood is that the substance should be in the aqueous phase rather than attached to the external surface of a blood cell, or bound to plasma proteins (e.g., albumins, lipoproteins). This assumption is based on the fact that cells and plasma proteins are usually retained in the blood, while the contaminant of interest appears in the urine or bile. Manufactured NPs tend to stick to the surface of cells and will aggregate in saline solutions or attach to proteins (Handy et al. 2008a), but this is not an uncommon problem with other substances. The real problem may be whether or not the manufactured NP will pass through a liver cell into the bile, or be filtered at the kidney. Hepatic excretion relies on exocytosis and vesicular trafficking to form the bile, and given these vesicles may be around 200 nm (Hampton et al. 1988), it is at least feasible for primary particle sizes smaller than this to enter the vesicles. For filtration in the vertebrate kidney, the molecular weight cut off is around 60 kDa, and it therefore seems unlikely that

NPs bigger than a few nm would pass through the glomerular filter. Of course, this route of excretion would not be available to stenohaline marine species (e.g., aglomerular kidneys), or sea water-adapted fish that are producing little or no urine in the hyperosmotic environment. In the case of the gills, the branchial route of excretion usually only becomes important when diffusion gradients for the toxic substance are reversed to the outward direction (e.g., by placing the fish in clean water). Fish are not known to be able to perform extensive vesicular trafficking from the basolateral membrane to the apical membrane of the gill, and so excretion by vesicular trafficking at the gills seems unlikely. If the tight junctions also prevent paracellular losses, then it seems probable that the gills will have a small (or even negligible) role in the excretion of manufactured NPs.

Fish are known to deposit metal granules in the liver (e.g., Lanno et al. 1987), and it would seem logical that manufactured NPs could also form hepatic deposits. It is possible that fish may have some capacity for inert storage of particles in some organs. If the liver is capable of excretion of NPs into the bile, then presumably enterohepatic circulation and re-uptake of the NP across the gut is possible. However, there is no data this process for NPs in fish and it remains unclear whether or not bacteria in the gut would modify the NP before it is re-absorbed. A final alternative to excretion or storage is the dissolution or metabolism of the NP to its molecular components. Dissolution is likely to be important for mineral-based particles. However, the detailed kinetics of these processes has not been reported in biological fluids. It may occur within a few minutes or many hours depending on the properties of the NP. There is also a concern that metal-based NPs could deliver very high free metal ion concentrations locally in/on the cell, resulting in latent metal toxicity from metal NP exposure.

The metabolism of manufactured NPs that are organic, or have organic residues on the exterior surface (i.e., an accessible component that could be metabolised), will depend on several factors. Clearly, we do not know if the substrate binding sites of enzymes involved in metabolism will be able to recognise or bind functional groups that are attached to NPs. On many enzymes the substrate binding site involves a few amino acid residues, or a spatial arrangement that allows the substrate to penetrate into the tertiary structure of the protein (substrate access channel topography, e.g., Deng et al. 2002). The distances involved will be typically tenths of a nanometer, so it seems unlikely that NPs that are 10s of nanometers wide could bind directly to active sites. Alternatively, there are plenty of examples where chemicals can disrupt protein structures and enzyme function without docking with substrate binding sites (e.g., metals binding to any accessible thiol group on a protein structure). Nonetheless, it is worth speculating on some possible issues that require data to substantiate or eliminate concerns about metabolism. These are illustrated by considering metabolism by oxidation, and the issues include:

(i) Whether or not the manufactured NP can be sterically available to bind to enzymes in the hepatic endoplasmic reticulum (ER) so that the cytochrome mono-oxygenases (CYP enzymes) are active and can breakdown the material (assuming the NP can access the liver at all).
(ii) Assuming that metabolism will work on the surface of the NP, once associated with the ER, that the enzymes present will continue to breakdown the surface residues on the manufactured NPs. This may initially involve adding functional groups (phase 1 reactions) so that the NP is larger, but eventually may be metabolised to basic components (e.g., CO_2 and water).
(iii) For surface coated NPs with multiple layers of different materials, what happens to the enzymatic-dependent metabolism of the underlying layer once the initial layer is metabolised or modified? For example, if the underlying layer requires a completely different set of reactions to be metabolised (e.g., halogenations instead of oxidations), how would the NP now be gain access to the next enzymes required?
(iv) The NPs do not interfere with the electron transfers to the various cytochromes involved in oxidation and reduction reactions. Otherwise the reactions will stop, or alternatively, run out of control (excessive electron shuttle) causing damage to the cell.

Currently ecotoxicologists do not have answers to any of these questions on NP metabolism for fish (or mammals), although attempts are being made to measure CYP activity from some species (e.g., fathead minnows, Oberdörster et al. 2006). Furthermore, complete metabolism of the NP may not be necessary, as long as the particle is made small enough to be excreted by the liver or kidney, and has a functional hydrophilic surface that enables the particles to disperse in the blood plasma, urine or bile.

Case studies

The purpose of these case studies is to highlight whether or not the manufactured NPs are meeting some of the founding assumptions for exposure and effect in fish toxicology, and also to identify any additional issues about the toxicity of NPs that are unintuitive or not predictable from knowledge of the nearest equivalent bulk chemical. The

founding assumptions are imbedded in the discussions above on exposure, and ADME. The main concerns in fish toxicology include:

(i) The physical and chemical behaviour of the toxic substance in the external medium will influence bioavailability and therefore toxicity. Some selected abiotic factors (temperature, salinity, dissolved oxygen, type of organic matter etc.) should influence bioavailability or toxicity in a predictable manner.
(ii) The chemical reactivity of the toxic substance with the molecular components of the tissue (e.g., SH-residues, lipids, nucleic acids etc.) is predictive of the types of toxic effects and mechanisms involved (e.g., an oxidising chemical is expected to cause oxidative stress).
(iii) Particle size or shape is important in toxicity. Different sizes (e.g., TiO_2 NPs versus TiO_2 bulk powder) or shapes (e.g., fullerenes compared with carbon nanotubes) of the same chemical substance should have different toxic effects.
(iv) There is some evidence or indication that the NP follows the general rules for ADME outlined above. These include uptake or effects at sites of entry into the fish (gills, gut or skin), identification of some target organs and predictions of how the material is carried in the blood, the possibilities for metabolism or storage, and likely routes of excretion.
(v) Similar to bulk chemicals, manufactured NPs with different chemistries should have different toxic effects (e.g., comparison of fullerenes with TiO_2 NPs).

Case study I—toxicity of carbon-based nanomaterials in fish

Since the first manufactured nanomaterial (C_{60}, Kroto et al. 1985) was synthesized and the era of nanotechnology initiated, manipulation of the arrangement of carbon atoms has been of intense interest because of the potential for novel materials to dramatically alter many aspects of human life. Although current production of carbon nanomaterials (e.g., fullerenes and nanotubes) is limited and environmental contamination has not substantially occurred (or at least not measured yet); projections are that these materials will be extensively produced in the future and subsequently released into the environment (Colvin 2003). The potential biological effects, bioavailability, and behaviour of each carbon NM will depend not only on the physico-chemistry of the material, but also how it reacts with other abiotic components in the environment.

Manufactured carbon NMs differ from their bulk carbon counterparts (e.g., graphite, diamond, and carbon black) by the arrangement of the carbon atoms and the specific properties conferred by this arrangement. Numerous shapes of carbon NMs are possible and are defined by carbon–carbon bonds (primarily sp^2 in character) that form a cage-like structure, which can vary from spherical and perfectly symmetrical (e.g., the C_{60} fullerene, Kroto et al. 1985) to elongated tube-like structures (e.g., single walled carbon nanotubes, SWCNTs, Iijima 1991). High bond strength of the single and double bonds of the carbon atoms and the partially delocalized π electrons define many of the unique characteristics of carbon NMs. Carbon NPs can be derivatized by addition of functional groups to thereby generate NPs with dramatically different physicochemical properties (e.g., water solubility). As new uses are developed for derivatized carbon NMs it is expected that these functionalized NPs will also ultimately present as environmental contaminants.

Un-derivatized fullerenes and carbon nanotubes (CNTs) are extremely insoluble in water (Andrievsky et al. 1999) and will likely exist on the water surface or at the water–sediment interface until physical mixing occurs within the aquatic environment. Extensive mixing in the laboratory can lead to formation of C_{60} aggregates (100 nm diameter) with charged colloidal characteristics (Andreivski et al. 1999). However, aqueous preparations of CNTs require a surfactant (e.g., sodium dodecyl sulphate) to form aggregate suspensions (Smith et al. 2007). There is evidence that C_{60} aggregates consist of hydroxylated C_{60} on the exterior of the aggregate that interact with water molecules and un-derivatized fullerenes on the interior (Fortner et al. 2005). Evidence also suggests that C_{60} can be hydroxylated during the mixing process as longer mixing duration (up to 2 months in daylight) enhances aggregate formation (Oberdörster et al. 2006). Hydroxylation of CNTs will dramatically enhance their ability to disperse in water. However, the potential for hydroxylation to occur in the environment is unknown. Factors that could increase the hydroxylation of carbon NPs include the temperature, pH, and amount of irradiance. In addition to hydroxylation, it is possible that abiotic processes cause formation of other derivatives of carbon NPs that have different physico-chemical properties. The extent to which derivatization of carbon NPs may occur in environmental media and the chemical products that may result is currently unknown.

Carbon NMs will interact with dissolved organic material (DOM) in the aquatic environment and this interaction will affect their presence in the water column. Dissolved organic material can associate with C_{60} (Lee et al. 2007), and presumably hydrophobic components of DOM protect the fullerene from the hydrophilic environment. Carbon nanotubes will likely have similar interactions with the DOM, and this association will also permit CNTs to exist in the water column. Clearly, interactions of carbon NPs with organic material and physical processes of mixing and

re-suspension of sediments will be a dynamic process that will dictate the movement of these materials between environmental compartments and towards potential routes of exposure in aquatic organisms.

The physicochemical properties above uptake via the water column, contact with sediments, or via the diet are probable, and the uptake mechanism outlined in Fig. 1 may apply. Endocytosis may be possible for single NPs or aggregates. The potential for individual carbon NPs to dissociate from aggregates and enter the lipid bilayer of cell membranes is an interesting possibility but remains unsupported (Lee et al. 2007). Behaviour of carbon NPs in fish blood has not been documented, but un-derivatized carbon NPs are extremely hydrophobic. It is possible that individual NPs may enter the circulation and be transported by lipoproteins present in the blood. If carbon NPs behave as other hydrophobic contaminants (e.g., PCBs) they may accumulate in lipids or fatty tissues (e.g., mesenteric fat around the gut, endocrine tissues, the brain). It is unknown if hydrophobic materials would constitute a concern for bioaccumulation similar to PCBs and other hydrophobic organic chemicals. Hydroxylated carbon NMs would be more miscible with water and therefore presumably accumulate in lean tissues with high blood flows (e.g., liver, kidney, gill), and perhaps behave more like water soluble chemicals such as metals. There are, as yet, no data on carbon NM accumulation patterns in fish tissues because of lack of methods for tracking and measuring these materials in tissues at relevant detection levels. Resolution of some of these technical challenges may be through use of labelled NPs thereby facilitating interpretation of the movement of the material among compartments in organisms and chemical changes to the NPs.

Although many different model organisms have been exposed to carbon NMs in laboratory experiments, little is known about metabolism of these materials. Rats given intravenous injections with radio-labelled fullerenes demonstrated that most of the nanomaterial accumulated in the liver and some was excreted through faeces suggesting biliary excretion. However, assessment of the presence of fullerene metabolites was not attempted (Rajagopalan et al. 1996). The labelled fullerenes were found associated with plasma proteins of the rats but renal excretion was not observed, likely indicating that the particles were too large or too hydrophobic to be released into urine. The issues indicated about steric hindrance and metabolism (above) would also apply to carbon NPs. To establish if metabolism of carbon NPs is occurring in organisms including fish, analytical methods to detect the formation of metabolites must be developed and applied.

Oxidative injury is one mechanism of toxicity that could be important for carbon NPs and this mechanism has been the basis of numerous toxicology investigations of these materials (e.g., Oberdörster 2004; Sayes et al. 2004, 2005). The potential for C_{60} to generate singlet delta oxygen ($^1O_2$) following absorption of energy from UV irradiation is well established (Arbogast et al. 1991), and other carbon NPs are also known to produce $^1O_2$ and reactive oxygen species (ROS). However, evidence that C_{60} generates $^1O_2$ and ROS in aqueous solution are inconsistent (Beeby et al. 1994; Yamakoshi et al. 1998; Miyata et al. 2000), and this is likely because of differences in preparation methods among studies. Recently, Lee et al. (2007) compared ROS production of C_{60} in water prepared by various methods (solvent extraction, surfactants) and concluded that irradiance absorbed by C_{60} aggregates led to generation of ROS only when C_{60} acted as an individual molecule (e.g., within a surfactant micelle), but not when C_{60} was aggregated together (i.e., solid state C_{60}–C_{60}). In the same study, ROS production was detected from water soluble hydroxylated C_{60} derivatives, which also act as individual molecules within the aqueous system. It is important to recognize that while carbon NMs can produce ROS under some conditions (e.g., C_{60} irradiated in toluene, Lee et al. 2007), these conditions may not be environmentally relevant.

Currently, the potential for carbon NMs to cause oxidative injury in fish remains controversial. An early study concluded lipid peroxidation in the brains of fish after water exposure to C_{60} (Oberdörster 2004). However, subsequent work demonstrated that toxic effects were more likely the result of degradation products of the tetrahydrofuran (THF) vehicle used to prepare the aggregates (Henry et al. 2007). Results of other investigations that have used THF as a vehicle for C_{60} in aquatic toxicity tests should be interpreted with caution, particularly as vehicle controls have not been adequately applied (e.g., Lovern and Klaper 2006; Oberdörster et al. 2006; Zhu et al. 2006, 2007). Although un-derivatized C_{60} may not cause oxidative injury, hydroxylated C_{60} fullerenes can produce ROS in aqueous environments (see above) and could explain some of the reported toxicity (i.e., oxidative injury). Carbon nanotubes can produce ROS; however, their ability to do so in environmentally relevant scenarios remains to be established. The potential for CNTs to produce ROS and cause oxidative injury in organisms needs to be clarified. Production of ROS has been documented in cultured rat lung epithelial cells exposed to single wall CNTs (SWCNTs) (Sharma et al. 2007). However, contrasting evidence indicates purified SWCNTs do not release ROS or cause toxicity in rat alveolar macrophages (Pulskamp et al. 2007). Results on oxidative injury in rainbow trout exposed to SWCNTs varied between tissues, with intestine and brain showing no effect, but elevation of glutathione in the gills and liver at some SWCNT concentrations (Smith et al. 2007).

Hypotheses on mechanisms of toxicity of carbon NPs are limited. Effects on organisms may be indirect, and

carbon NPs may not cause specific effects on unique target molecules in cells or tissues. Evidence for a lack of toxicity of C_{60} includes an experiment in which larval zebrafish (*Danio rerio*) were exposed to C_{60} aggregates (no THF or vehicles present) and assessment of changes in global gene expression was conducted (Henry et al. 2007). In that study, after a 72 h exposure, changes in global gene expression in larval zebrafish were minimal and no detoxication pathways were activated; 10 genes out of ~14,900 interrogated differed relative to unexposed controls, 8 of these genes were down-regulated, and 2 genes (unannotated) were up-regulated (~2 fold). Changes in global gene expression in fish exposed to other types of carbon NPs is an appropriate approach for developing hypotheses of toxic effects particularly when mechanisms of action are completely unknown. Indirect non-specific toxic effects of carbon NPs include physical irritation and occlusion of surface tissues (e.g., gills), which have been evidenced in some studies with aquatic organisms (e.g., daphnids, Oberdörster et al. 2006; and rainbow trout, Smith et al. 2007). Carbon NTs accumulated on gill surfaces leading to irritation and lesions leading Smith et al. (2007) to conclude that CNTs acted as a respiratory toxicant in rainbow trout.

The size and shape of carbon NPs can affect the potential for exposure and toxicity in organisms. Zebrafish embryos appear to be protected from aggregates of SWCNTs because the μm-size aggregates are larger and unable to pass through nm-size pores in the chorion Cheng et al. 2007). Direct comparisons of toxicity between carbon NPs of different sizes (e.g., C_{60} and CNTs) have not been conducted in fish. However, differences in the size and shape of the individual materials suggest different toxicities. Although the aggregates of carbon NPs of different specific types are of similar size (ranging near 100 nm) when suspended in the water column; when they interact with tissues and sites of uptake, the size and shape of individual NPs is likely to be important. The size and shape of NPs is a relevant determinant of toxicity in mammalian lung inhalation studies, with smaller particles typically leading to greater toxicity (Oberdörster 1996). Fish gill surfaces may be more susceptible to irritation by NPs of a particular size range, or specific sizes or shapes; and it may be more difficult for fish to dislodge some shapes or sizes of carbon NMs from the cell surface. Such experiments on size/shape effects remain to be conducted in fish.

Presently, there remains considerable inconsistency among investigations regarding toxicity of carbon nanomaterials. It appears that most of these inconsistencies result from different NP preparation methods, inadequate evaluation of the purity of starting materials, and likely above all, the lack of established methods to test the toxicity of NPs. In fish, establishing toxicity (e.g., LC_{50} values) of carbon NPs remains out of reach. A significant concern regarding investigations of CNTs is inadequate assessment of purity to assure that nanoparticles are free from contaminants, particularly the metal catalysts used during fabrication, which can be present in materials obtained from commercial suppliers (Kagan et al. 2006; Pulskamp et al. 2007). For example, toxicity in zebrafish embryos exposed to SWCNTs has been attributed to the presence of Co and Ni catalysts used in the preparation of the SWCNTs (Cheng et al. 2007). The effects of vehicle solvents used to generate aqueous aggregates of carbon NMs have led to potential misinterpretations of toxicity in fish (e.g., Oberdörster 2004). In order to work out toxic mechanisms due to the carbon NMs alone, toxicologists need to work with pristine NMs that are free of vehicle solvents or contaminant residue from manufacture. This may also include investigating how these pristine materials interact in the environment and subsequent effects on bioavailability and toxicity. However, the materials may not remain pristine for long. The formation of aggregates of carbon NMs in water offers the opportunity for other organic materials including toxicants to become associated with the aggregates (Yang et al. 2006) which will change bioavailability of these materials and create additional toxicological concerns. Clearly, the toxicity of carbon NMs is complex, and ecotoxicologists must learn from the experiences of using THF, and other solvents, so that results are carefully verified with a comprehensive regime of solvent controls, and physico-chemical analysis of the test medium including the presence/absence of metal contaminants. This verification is vital because some of the benefits and commercial exploitation of carbon NMs may be curtailed by incorrect data interpretation that suggest toxicity, when in fact none is present; or when the environmental relevance of a study is unclear because the physico-chemical characterisation of the carbon NPs has not been adequately reported in experiments.

Case study II—titanium dioxide toxicity to fish

The use of metal oxide NPs in industry is growing rapidly and currently these materials are used in cosmetics, sunscreens, fuel additives, as catalysts, and in numerous industrial applications (e.g., Anselmann 2001; Wakefield et al. 2005; Aitken et al. 2006). There is also a significant body of mammalian literature on the respiratory injury and inflammation associated with fine and ultrafine TiO_2 particles (e.g., Bermudez et al. 2004; Warheit et al. 2005, 2006, 2007) at least one study on the oral administration of TiO_2 NPs in mice (Wang et al. 2007), and studies on the absorption of fine ZnO and TiO_2 particles across porcine skin (Gamer et al. 2006). These product applications of TiO_2 suggest these materials are likely to be in effluents, or

released directly into the environment during use, and the known toxic effects in mammalian models raises concerns about other vertebrates including fish.

The environmental chemistry of TiO_2 NPs has been partly investigated. TiO_2 NPs can be dispersed in freshwater by sonication (Federici et al. 2007), but the primary particles tend to form aggregates of a few 100 nm dimensions, and the aggregates gradually precipitate from the water column over a few hours. TiO_2 has two major crystal structures (rutile and anastase), and the surface reactivity of the NP is closely defined by the crystal structure (Watanabe et al. 1999). Both forms are toxic (e.g., rat lung, Oberdörster et al. 1992), but in human bronchial epithelial cells (BEAS 2B cells) exposed to the different crystal structures of TiO_2 (10 or 20 nm anatase, or 200 nm rutile forms), the anatase form of TiO_2 was much more toxic inducing DNA damage, lipid peroxidation, and micronuclei formation. This toxicity is associated with the oxidising properties of TiO_2 NPs, which are capable of generating ROS. It is therefore important that ecotoxicologists give as much information about the crystal structure, or proportions of these structures in the manufactured NPs that are used for experiments. TiO_2 NPs can, in the presence of light, transfer electrons from substrates such as hydrogen peroxide (H_2O_2) or chlorinated organic compounds to rapidly generate the hydroxyl radical (Hirano et al. 2005). This chemistry therefore predicts that TiO_2 NPs should produce oxidative stress in fish, and this observation is confirmed by studies on trout where changes in tissue levels of thiobarbituric acid substances (TBARS) and total glutathione were observed (Federici et al. 2007). Thus the founding assumption that biological effect can be explained by the chemistry of the NP seems to hold true in this example.

The generation of ROS and inflammation noted in the studies on rodent lung above, and inflammatory injury to trout gills (Federici et al. 2007), raise concerns about the risk of immunotoxicity of TiO_2 NPs to fish and other aquatic organisms. Federici et al. (2007) showed no effects on total white cell counts, but the organ histology implicated a systemic inflammatory response. Unfortunately, little is known about the immunotoxic effects of Ti metal in fish, and it remains unclear if the nanoscale is adding an additional risk. However, TiO_2 NPs are known to bind lipopolysaccharides in the presence of Ca ions (Ashwood et al. 2007), and the possibility that TiO_2 could be a delivery vehicle for other immunotoxicants present in the environment cannot be excluded. Clearly more detailed investigations of the immune effects of TiO_2 NPs are needed.

Another concern about the environmental chemistry is the risk of metal toxicity from metal ions released from the NP. Bulk metal oxides are generally considered to have low solubility, and while this is also true for nano-scale TiO_2, the small particle size and large surface area:volume ratio of the particle suggests that dissolution of Ti metal or TiO_2 from the surface of the NP is possible, and should be faster than that of the ordinary bulk TiO_2 powder. This suggests the possibility of release of Ti species from the surface of the NP, but the biological consequences of this for fish are uncertain because of a lack of studies on the toxicity of Ti metal species in fish. For example, the aqueous Ti^{3+} LC_{50} for fish is not known. However, TiO_2 NPs levels up to 1 mg l^{-1} did not cause lethal toxicity in trout, suggesting low toxicity of the manufactured NP via waterborne exposure in fish (Federici et al. 2007). Similar low toxicity has also been reported for zebrafish exposed to copper NPs, with a 48 h LC_{50} of 1.5 mg l^{-1} (Griffitt et al. 2007). If free metal ion species are released, then the assumption is that the normal factors in metal toxicity will apply (hardness, pH effects etc.), but systematic investigations of abiotic factors are needed to test this assumption. Interestingly, TiO_2 is able to adsorb other toxic metals (such as Cu) and might therefore reduce the availability of other metals present in the water (Barakat 2005).

There have been no studies specifically designed to quantify the bioavailability of TiO_2 NPs to fish, but Federici et al. (2007) did show a range of biological effects without measurable increases in Ti levels in the tissues of the trout, and surface acting metal toxicity on the gills was suggested. This did not exclude bioavailability, but suggested that uptake was probably slow and that detection of new Ti due to NP exposure was hampered by a significant background level of Ti already in the tissues of the fish. However, biological effects revealed several target organs including the gill, intestine, and liver. Ti metal can be measured in all these organs and the brain (Federici et al. 2007). A more detailed analysis of the body distribution of Ti metal compared to TiO_2 NPs is needed in fish. To our knowledge, there are no measurements of Ti sequestration (storage) or excretion rates in fish. Clearly much more information on ADME of TiO_2 is needed in fish.

The effect of particle size has not been specifically tested in fish for TiO_2 NPs. However, a particle-size effect is known for toxic effects on the rat lung. Oberdörster et al. (1992) showed that the level of lung inflammation in rats was associated with the smaller ultrafine TiO_2 particle sizes which caused more adverse effects. Federici et al. (2007) point out that bulk TiO_2 powder has been used for many years as a dietary marker in fish nutrition, and this implies that the bulk form (not regarded as toxic) must be much less toxic than the nano form in fish as well. Nonetheless, a systematic investigation of the toxic effects of different particle sizes of TiO_2 is needed in fish (along with other metal NPs).

There are also some opportunities to apply a range of in vitro techniques to fill the current knowledge gaps on

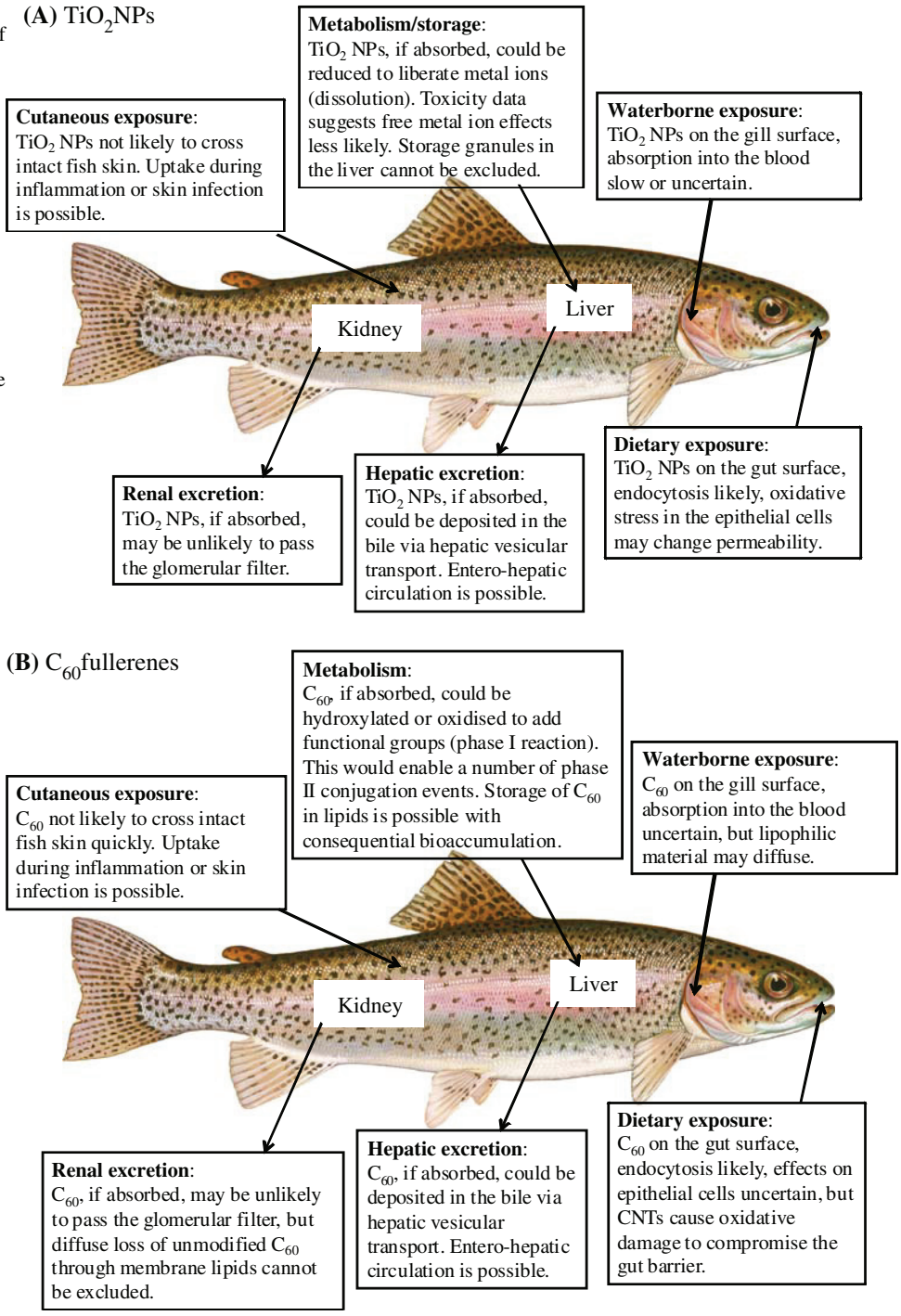

Fig. 2 Possible routes of uptake, excretion and metabolism or storage of (**a**) TiO_2 NPs compared to (**b**) C_{60} fullerenes. These materials present similar problems for uptake and excretion, but the metabolism or storage of these materials is likely to be very different, with some considerable uncertainty of how or if NPs can access enzymes involved in metabolism. Target organs are not shown for clarity, but include the routes of exposure (gill and gut especially). Once in the blood compartment the NPs would have access to all the internal organs via the cardiovascular system. Toxic effects have been noted in the liver and gills for both materials

particle size effects, body distributions, and ADME of TiO_2 NPs. Details of uptake mechanisms and bioavailability of metals have been elucidated with in vitro techniques such as the perfused gill preparation (Campbell et al. 1999), perfused gut (Handy et al. 2000) and cultured gill epithelial cells (Wood et al. 2002). These techniques should also work for manufactured NPs, provided the use of any dispersing agents do not compromise the viability of the preparations. In vitro studies using mammalian cell lines have been conducted (see Handy and Shaw 2007 for discussion), and although there are debates about the importance of dose metrics (e.g., use of mass concentration versus surface area) in some of these experiments (Oberdörster et al. 2007), the preparations remain a useful tool.

Comparison of titanium dioxide NPs with fullerenes and CNTs

The assumption that the toxicology is related to the chemical and physical properties of the NP can be assessed by comparing the TiO_2 NPs with carbon-based materials (C_{60} fullerenes or CNTs). Figure 2 summarises the key features of uptake, excretion and metabolism for TiO_2 NPs and fullerenes. Although they have very different physical structures, they do share an important chemical property—both materials are redox active and can result in the generation of ROS. From this view point they share some similar types of toxic effects (e.g., changes in tissue TBARS and total glutathione, Federici et al. 2007; Smith et al. 2007) that can be explained by the common chemistry. One might also expect some chemical differences (e.g., metal versus non-metal), but as the discussion above indicates, TiO_2 NPs do not show seem to show characteristic metal toxicity. The typically high lethal toxicity associated with waterborne exposure to free metal ions (i.e., LC_{50} values in the low $\mu g\ l^{-1}$ range) are not evident for TiO_2 NPs, and in trout at least, the major ionic disturbances and haemolytic effects typical of metals appear to be lacking for mg l^{-1} exposures to TiO_2 NPs (Federici et al. 2007). Thus TiO_2 NPs are behaving like a metal oxide with low acute toxicity, rather than like a free metal ion, and is therefore closer to the chemistry of carbon than we might expect. Hence both groups of materials (metal oxides and carbon-based NPs) show common aspects of gill pathology (also consistent with many other chemicals), both are not acutely haemolytic, but do cause oxidative stress (see discussion in Smith et al. 2007; Federici et al. 2007).

Conclusions, knowledge gaps and research needs

It is likely that manufactured NPs are in the aquatic environment and that wild fish populations are being exposed to these new materials. To date, no measurements of NP exposure or accumulation have been made in wild fish populations. Much of the available data are on freshwater fishes (in laboratory experiments), and there is a need to collect data on marine and estuarine species, and on flatfish (sediment dwelling species) where the NP physico-chemistry in these habitats may be different from freshwater. Development of techniques to measure NPs in environmental samples as well as fish tissues would be a prerequisite to such field experiments; and a monitoring programme. These would also need to differentiate between the effects of manufactured NPs and natural NPs on fish. The founding logic and assumptions used in fish toxicology can be generally applied to NPs, although there will be differences between NPs and other toxic substances. The effects of abiotic factors such as salinity, hardness, and dissolved organic matter on bioavailability of NPs to fish have not been systematically studied; but in essence the chemistry of NPs can inform of bioavailability and toxic effects in the same way as other chemicals. The uptake of NPs across epithelia might be slow, and endocytosis or related membrane trafficking systems would seem the most likely mode of entry. NPs are too large to be carried on membrane transporters at the gill or gut, but diffusion of lipophilic NPs cannot be excluded and experimental measurements of diffusion across the gill and gut are needed. Information on the distribution, metabolism and excretion of NPs in fish is lacking. The fundamental concepts and processes of metabolism or excretion could be applied to NPs, but critically, there is an urgent need to know what organelles NPs can access in each tissue. For example, for hepatic metabolism one founding assumption is that the toxic chemical has direct access to the endoplasmic reticulum. Ecotoxicologists do not yet know these details for NPs in fish. Furthermore, there are several opportunities to apply existing tools and techniques for the fundamental studies of fish toxicology. In particular, the use of perfused organs and fish cell culture systems. However, other techniques require development such as labelling methods to quantify the body distribution of different NPs.

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