Investigations into the bioavailability of manufactured nanoparticles in fish

Submitted by

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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.

...........................................................(Rhys Goodhead)
Abstract

The field of nanotoxicology has emerged as a discipline in parallel with the rapid expansion of nanotechnology and the use of nanomaterials in modern life. Assessing the potential impacts of manufactured nanoparticles (MNPs) on the environment and human health is critical to the sustainable development of the nano-industry. Current knowledge on the ecological implications of nanotoxicology has major uncertainties surrounding the fate and behaviour of nanomaterials in the exposure environment. Bioavailability, uptake and partitioning of nanomaterials to organisms are key determinates to toxicity, yet these foundations of basic data are only now just starting to emerge in any useful and coherent manner for aquatic animals. This thesis work set out to address this gap in knowledge and further our understanding of these important principles for fish.

In an attempt to develop a high through-put screening system for toxicity of MNPs, studies assessing the utility of primary isolated rainbow trout (Oncorhynchus mykiss) hepatocytes found they showed very limited responses to a range of MNPs. There was a lack of any evidence for either lipid peroxidation or xenobiotic detoxification activity. In these studies isolated trout hepatocytes were found to be unresponsive to the induction of these biological responses after exposure to positive controls. The findings demonstrated that the MNPs tested showed low toxicity generally and that fish hepatocytes do not provide a useful system for the screening of potential toxic effects of MNPs. In this cell culture work, coherent anti-Stokes Raman scattering (CARS) microscopy was applied to demonstrate that the particles supplied in the culture medium did cross the cell membrane and enter into the exposed cells.

In the second phase of the work in this thesis CARS was investigated as an experimental technique for tracing a wide range of metal and metal oxide MNPs into cells and tissues. CARS was applied to evaluate initial detection of different MNPs and investigate the imaging capability on a range of cells, tissues and organisms. Finally, CARS was applied to assess localisation ability of MNPs within biological matrices. MNPs were shown to be taken into trout hepatocytes using a 3D reconstruction to determine the origin of the MNP signal within the cell. Uptake of MNPs was established into trout gill and kidney tissue, corophium and daphnia species and were shown to have different partitioning in zebrafish embryos. In summary CARS showed great potential for tracing particle uptake and bio-distribution both in vitro and in vivo. Particular benefits include imaging MNPs in living organisms, without the need for labelling or fixing the material. Limitations of the CARS technique are also discussed.
In chapter 4, the consequences of the presence of natural organic matter (NOM) were investigated on the uptake of MNPs into fish. Carp (Cyprinus carpio) were exposed to cerium dioxide (CeO$_2$) MNPs in combination with NOM over 28 days. Elevated levels of uptake of cerium were measured in the brain, gill and kidney tissue by induction coupled plasma mass spectroscopy (ICP-MS) for fish exposed to 50 μg/l CeO$_2$ MNPs in combination with 250 μg/l of NOM. There were no such effects of the NOM enhancing uptake for the bulk CeO$_2$ particles. Detailed studies on the behaviour of the CeO$_2$ MNPs in the exposure medium demonstrated the highly complex and dynamic nature of the interactions with NOM. This study discusses some of the difficulties in the techniques, analysis and interpretation of data derived from studies of this nature. The finding that NOM may enhance MNP uptake presents a potential issue for current risk assessment criteria for MNPs that do not consider natural conditions.

The final experimental chapter considered maternal transfer as a potentially important route for exposure of embryos and early life stage animals to MNPs in live bearing animals. In this work guppies (Poecilia reticulata) were exposed to 7 nm silver citrate stabilised particles and citrate stabilised bulk sized particles, dosed via the diet for a full gestation cycle. Maternal transfer of Ag to the larvae was significantly higher for the nanoparticulate treatment compared with the bulk and control treatments and larval burden was significantly higher compared with the maternal sires. However, there was no impact of Ag on larval survival, birth weights, or on indices of body condition in the exposed adults. The enhanced uptake of nano Ag compared to bulk Ag particles into the guppy offspring emphasises the potential for exposure to sensitive early life stages of organisms, which to date has not been widely considered and suggests greater research is needed in this area.

Collectively, the studies conducted in this thesis contribute to the science base of nanotoxicology, specifically in areas where data are especially lacking and with a focus on bioavailability. These studies have identified that fish hepatocytes do not offer an effective screen for MNPs, and the data produced further suggests that the MNPs tested are not toxic in that form. Working with CARS I have helped advance the understanding on its utility for nanotoxicology studies, with regards to its application and limitation for uptake analyses. The study of MNPs in combination with NOM has identified the fundamental change that real life exposure scenarios may instigate for toxicity assessments of MNPs, with significant impact on risk assessment criteria. Finally, I’ve established that maternal transfer is an exposure route for MNPs that requires further study, with evidence of transfer to sensitive life stages in a non-mammalian system.
Acknowledgements

Ahh the acknowledgements the one place I can be a verbose as I choose!

There are so many people who have made this PhD both a fascinating and hugely enjoyable experience. The paths of numerous people have been crossed, yet despite the brevity of some, they still made lasting impression on me. I will certainly look back upon this time with great fondness.

It is traditional, of course, to thank your supervisor for their support during this period. Putting it down in such short sentences makes it seem like a rather trivial task, both to supervise and to give thanks. It could not be more different. In Prof. Charles Tyler, I have been so very fortunate to have found a supervisor whose door it is always a pleasure to walk through. Unbridled help and direction aside, the enthusiasm generated by him is one of key things that has maintained my enjoyment for science. As well as listening, with all seriousness, to my enthusiastic ideas for experiments Charles has been a brilliant mentor in guiding me to be a better scientist. It helps, of course, that many conversations end with a discussion on the latest wildlife sightings on Dartmoor. So before I start to sound sycophantic, many thanks Charles for giving me this opportunity, all the effort and time you have invested has been hugely appreciated.

A good lab group also helps the world go round, so my thanks to those who have had to deal with my singing and capers around the office and aquarium. Special mentions to Toby and his terrible taste in music, Charlie, Greg and Tamsyn for all the sport, you can never have too much and it has helped keep me sane on occasion. Marta, Okhyun, Amy, for your friendship and just generally putting up with me, Emma for bat nights and Viv for dipper days, Tess for understanding the nano problem. To those who left our shores for Australia, you have never really left. You cannot have a lab without technical support and in Jan and Mike we are very fortunate, thanks for helping me with my varied demands, often at short notice, you always came up trumps. There are of course many many others I could thank from over the four years, perhaps unknowingly you have all helped in some ways either as a support group or to share the good times with.

I have been incredibly lucky to grow up in a supporting and encouraging family, Mum, Dad, Bryn and Huw, you run throughout this work.

Of course there is often someone particular who stands out when you think about writing an acknowledgment, a person who immediately springs to mind to thank. Not, perhaps, because they have made an overt effort, but their telling contribution is that you are here, nearing the finish line. Luanne you have been my someone particular. Your generosity, encouragement and kindness have helped nurture me through to this end. I do not have the words to thank you enough.
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RESEARCH PAPERS AND AUTHOR’S DECLARATION


Statement: I, Rhys Goodhead, was involved in the following parts of the presented papers: I planned and carried out the hepatocyte isolations, exposures, LDH, TBARS and GST assays in paper 1 and co-wrote paper 1 with Tessa Scown. I was responsible for the CARS microscopy and GST work and Tessa Scown carried out the TBARS and LDH assays. Equal contribution was made towards cell isolation and exposures. Charles Tyler and Julian Moger contributed to the design of paper 2. I carried out all of the zebrafish exposure and imaging work and assisted Julian Moger with the imaging of the invertebrates for paper 2. Julian Moger imaged the gill and worm tissue and I played the lead role in writing this paper. Blair Johnstone, Jamie Lead, Mohamed Baalousha and Charles Tyler all had significant contributions towards the study design for paper 3. Blair Johnston and I carried out the preliminary experimental work for paper 3 and I was responsible for the subsequent follow up exposure. All authors contributed some part towards the writing of paper 3 however I was the predominant contributor. I prepared all of the samples for ICP-MS analysis in papers 3 and 4. I played the leading role in planning, designing, implementing and writing paper 4 with significant contribution from Charles Tyler and technical support from Victoria Jennings. Isabel Romar synthesized the silver nanoparticles for paper 4. Nanoparticle characterisation work for paper 1-4 was carried out by Mohamed Baalousha, Jamie Lead, Paula Cole and ICP-MS measurements take by Stephen Baker. All papers had large editing contributions from Charles Tyler.

The published versions of papers 1, is included in the appendix.

Other publications completed during this PhD included;


### LIST OF GENERAL ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Ag⁺</td>
<td>Silver ion</td>
</tr>
<tr>
<td>Ag</td>
<td>Silver</td>
</tr>
<tr>
<td>Al₂O₃</td>
<td>Aluminium oxide</td>
</tr>
<tr>
<td>Au</td>
<td>Gold</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C₆₀</td>
<td>Buckminsterfullerene (fullerene)</td>
</tr>
<tr>
<td>CARS</td>
<td>Coherent anti-Stokes Raman scattering</td>
</tr>
<tr>
<td>CeO₂</td>
<td>Cerium dioxide</td>
</tr>
<tr>
<td>CNT</td>
<td>Carbon nanotube</td>
</tr>
<tr>
<td>DLVO</td>
<td>Derjaguin &amp; Landau, Verwey and Overbeek Theory</td>
</tr>
<tr>
<td>DOC</td>
<td>Dissolved organic carbon</td>
</tr>
<tr>
<td>DWCNT</td>
<td>Double-walled carbon nanotube</td>
</tr>
<tr>
<td>E-CARS</td>
<td>Epi-detected CARS</td>
</tr>
<tr>
<td>ENMs</td>
<td>Engineered nanomaterials</td>
</tr>
<tr>
<td>ENPs</td>
<td>Engineered nanoparticles</td>
</tr>
<tr>
<td>FA</td>
<td>Fulvic acid</td>
</tr>
<tr>
<td>F-CARS</td>
<td>Forwards detected CARS</td>
</tr>
<tr>
<td>Fe₂O₃</td>
<td>Iron oxide</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>HA</td>
<td>Humic acid</td>
</tr>
<tr>
<td>hpf</td>
<td>Hour post fertilization</td>
</tr>
<tr>
<td>ICP-OES</td>
<td>Inductively coupled plasma – optical emission spectrometry</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma – mass spectrometry</td>
</tr>
<tr>
<td>IR</td>
<td>Infra-red</td>
</tr>
<tr>
<td>LC₅₀</td>
<td>Median lethal concentration</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>MNP</td>
<td>Manufactured nanoparticles</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MWCNT</td>
<td>Multi-walled carbon nanotube</td>
</tr>
<tr>
<td>NOEC</td>
<td>No observable effect concentration</td>
</tr>
<tr>
<td>NOM</td>
<td>Natural organic matter</td>
</tr>
<tr>
<td>NM</td>
<td>Nanomaterials</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>STM</td>
<td>Scanning tunnelling microscope</td>
</tr>
<tr>
<td>STW</td>
<td>Sewage treatment works</td>
</tr>
<tr>
<td>SWCNT</td>
<td>Single-walled carbon nanotube</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TiO₂</td>
<td>Titanium dioxide</td>
</tr>
<tr>
<td>UFP</td>
<td>Ultrafine particles</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet (also UVA and UVB)</td>
</tr>
<tr>
<td>VTG</td>
<td>Vitellogenin</td>
</tr>
<tr>
<td>WWTWs</td>
<td>Wastewater treatment works</td>
</tr>
<tr>
<td>ZnO</td>
<td>Zinc oxide</td>
</tr>
<tr>
<td>Species</td>
<td>Common Name</td>
</tr>
<tr>
<td>------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Arenicola marina</td>
<td>Lugworm</td>
</tr>
<tr>
<td>Carassius auratus</td>
<td>Goldfish</td>
</tr>
<tr>
<td>Corophium velator</td>
<td>mud shrimp</td>
</tr>
<tr>
<td>Cyprinodon variegatus</td>
<td>Sheepshead minnow</td>
</tr>
<tr>
<td>Cyprinus carpio</td>
<td>European carp</td>
</tr>
<tr>
<td>Danio rerio</td>
<td>Zebrafish</td>
</tr>
<tr>
<td>Daphnia magna</td>
<td>Water flea</td>
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<tr>
<td>Esox lucius</td>
<td>Pike</td>
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<tr>
<td>Micropterus salmoides</td>
<td>Largemouth bass</td>
</tr>
<tr>
<td>Oncorhynchus mykiss</td>
<td>Rainbow trout</td>
</tr>
<tr>
<td>Oryzias latipes</td>
<td>See-through medaka</td>
</tr>
<tr>
<td>Perca fluviatilis</td>
<td>Perch</td>
</tr>
<tr>
<td>Pimephales promelas</td>
<td>Fathead minnow</td>
</tr>
<tr>
<td>Poecilia reticulata</td>
<td>Guppy</td>
</tr>
</tbody>
</table>
Chapter 1

General Introduction
1 - GENERAL INTRODUCTION

1.1 What is nanotechnology?

Nanotechnology is the design, production and application of structures and systems in the nanoscale, which covers the size range from approximately 1 to 100 nanometres (nm). To place this in perspective, a typical atom lies in the range of 0.1 to 0.5 nm in diameter, DNA molecules are approximately 2.5 nm wide, most proteins are approximately 10 nm wide, and a typical virus 100 nm wide. A bacterium is a relative giant, measuring approximately 1000 nm.

By the end of 2011 the total government funding for nanotechnology research worldwide is estimated to be £42 billion, rising to £65 billion by 2014, with nearly a sixth of a trillion pounds invested by 2015 (Cientifica Ltd, 2011). The current total final market value of nano-enabled products worldwide is predicted to be around £200 billion and over the past decade the growth rate of the final value of nano-enabled products has averaged 25% per year (Harries and Harries Ltd, 2011). In Europe, an inventory published by the European Consumers’ Organisation (BEUC) and the European Consumer Voice in Standardisation (ANEC) of products containing nanomaterials, found 475 in 2010 compared to 151 the previous year (available from http://www.anec.org/anec.asp). However, the numbers of products and manufacturers is likely to fluctuate rather dramatically from source to source. In the UK, AZoNano.com list 276 manufacturers whereas the NanoKTN (https://ktn.innovateuk.org/web/nanoktn) has 472 companies in its directory. These listings, however, are only an estimate and may not be an actual reflection of the UK occurrence or usage of nano-enable products.

The reasons behind this immense growth that has labelled nanotechnology as the next industrial revolution (Schmidt, 2009), are the unique properties that materials can confer at the nanoscale. These properties, can potentially offer solutions to current global issues in the fields of food and sustainable energy production, medicine (where biological structures are now within a size scale that researchers are able to manipulate and control), environmental remediation, and they also facilitate development and enhancement opportunities to products and processes in almost every working sector.

However, the continually advancing use and widespread application of manufactured nanoparticles (MNPs) comes at the cost of making them increasingly likely to come into contact with humans and wildlife. Applications such as nano-enabled products within the food
industry (reviewed in Blasco and Picó, 2011), which are estimated to be worth £4.7 billion by 2014 (iRAP, 2009), are just one of the many examples by which the global population will be exposed to products, or even the particles themselves from the nano-industry. This diverse application of nanomaterials consequently expands the leaching potential and number of point sources of MNPs into the environment, from production waste to landfill, product degradation and waste water treatment works (WWTW). The term ‘ultimate sink’ has been used for the aquatic environment for many pollutants with an anthropogenic aetiology, and MNPs are likely to be no different.

It is with this at the forefront of the mind, that research into the uptake potential and effects of MNPs in aquatic organisms is undertaken. There is a considerable dearth of information on the fate and behaviour of MNPs in the environment. With altered properties from their bulk material counterparts (discussed in detail below) and a size distribution that involves quantum mechanisms and Brownian motion, their uptake pathways, bio-partitioning and effects are likely to be unlike any other conventional anthropogenic pollutant. These facts have been met with growing concerns over the safety of nanomaterials (Stern and McNeil, 2008) as their production and development runs alongside any testing for potential hazardous or detrimental effects in the real world. This leads to a rolling corollary on a ‘best current guess’ scenario as the research and understanding and commercial applications of nano materials are developed concurrently. Thus the nano-industry, if to fulfil its potential, requires scientific data, where there are gaps in our understanding, on risk assessment of MNPs to human and environmental health. This is critical to ensure its development in a sustainable manner.

This purpose of this introduction is to provide the relevant background and context to the thesis work undertaken. In the first instance an overview is presented on the most relevant and abundant nanomaterials, their unique features and how these have been exploited for commercial purposes and industrial applications. This is followed by a summary of the known nanotoxicological data including conditions affecting their fate and behaviour. Finally, this introduction explains the main objectives and rationale of the research undertaken in this thesis.

1.2 The origins of nanoparticles

Nanomaterials have numerous origins which can be roughly classified into 3 groups; natural nanomaterials, anthropogenic nanomaterials and engineered nanomaterials. Natural nanomaterials have been around for billions of years and include a wide range of materials
such as volcanic dust, soil colloids, consisting of silicate clay minerals, iron or aluminium oxides/hydroxides or natural organic matter (NOM). Some of the most common natural nanoparticles are nanocrystals of sea salt formed from evaporation of sea water sprays. Fascinatingly, some natural nanoparticles are hypothesised to potentially have had an impact on the chemical abundance required to create life on Earth! These carbon nanoparticles called C_{60} and C_{70} fullerenes have been found in meteorites and in space (Becker et al., 2000; Cami et al., 2010) and may have delivered carbon and gases to earth that allowed the development of life.

Anthropogenic or incidental nanomaterials, are largely derived from grinding, wear and combustion from human action and are thus inherently more recent. The predominant type being carbon black (soot) produced from the combustion of fossil fuels. Engineered nanomaterials are those that have been designed and manufactured specifically by man and synthesised for a specific purpose. This leads to a variety of constitutions and shapes which can be further functionalised by the addition or modification to their surface layers. The history of man and the use of nanomaterials date back to Roman times, the Lyrcurgus Cup from the 4\textsuperscript{th} C AD is made of dichroic glass which has a different colour depending on the direction light shines through it. Further investigation found the presence of gold and silver nanoparticles in that glass (Barber and Freestone, 1990). Other early examples include the presence of gold nano-sized colloids in the rose stained glass window from Notre Dame Cathedral from the 13\textsuperscript{th} Century (Haes et al., 2004). More recently, it is likely that the photography (c. 1827) utilised nano-sized silver colloids and in 1889 Lea reported the synthesis of a citrate-stabilized silver colloid (Lea, 1889) which was later measured to produce particles less than 10 nm in size (Frens and Overbeek, 1969). Industrial manufacturing of nanomaterials can also be traced back to the 19\textsuperscript{th} Century, at which time a product called “Collargol” used protein stabilised nanosilver for medical applications including gynaecological treatments of ulcers after birthing. Stemming from the desire to find a “silver solution which could be brought into the blood stream and form silver aggregates which would possess antibacterial properties and thus lead to an overall disinfection of the body” Collargol was put into production in 1897 (Boese, 1921); it had a mean particle size of under 10 nm (Bogdanchikova et al., 1992). The usage of nanosized particles described above may be purely coincidental, and not known to the manufacturer. However, patents from the mid 20\textsuperscript{th} Century specify the importance of particle size for their products these include the biocide Algaedyn, which has a high volume usage in swimming pools and was recently confirmed to contain silver particles in the nano range (Nowack et al., 2011).
As evidenced above, products and concepts on the nano scale have been around for a significant period of time, although they have more often been referred to as colloidal products. The potential of nanomaterials was recognised by an American scientist call Richard Fenyman, who gave a talk entitled “There’s Plenty of Room at the Bottom - An Invitation to Enter a New Field of Physics” (available at http://www.zyvex.com/nanotech/feynman.html) where he discussed, not just visualising, but manipulating and controlling matter on a scale of the atom. However, it wasn’t until 1974 that the term “nanotechnology” was first coined 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 (Sandhu, 2006). In 1981 the possibility of using molecular self-assembly to make functional nanoscale systems was presented by Dr K. Eric Drexler, who hypothesised working on construction of devices and materials to complex atomic specifications with the ability to manipulate biological materials (Drexler, 1981). Facilitating the progression of these ideas were the instruments capable of turning concept into practice. This was achieved with the invention of surface enhanced Raman spectroscopy (SERS) in 1977 by Van Duyne, one of the most sensitive tools used to study the chemical reactions of molecules, scanning tunnelling microscope (STM) in 1981 by Binning and Rohrer, and the development in 1986 of STM by Binning and colleagues to an atomic force microscope (AFM) (Binnig et al., 1986), allowing scientists to observe particles and molecules on a atomic scale. Around this time buckminster fullerenes were discovered (Kroto et al., 1985) as well as the purported discovery of carbon nanotubes (Iijima, 1991). However, carbon based tubes with a diameter of approximately 50 nm, were first made in 1952 and reported in the Journal of Physical Chemistry of Russia (Monthioux and Kuznetsov, 2006).

1.3 General properties of nanoparticles

Throughout history there is a trend for the progression and development of a product to make it smaller. Obvious advantages to reducing size include less material and transportation costs, space saving and often increased practicality. However nanotechnology goes beyond basic enhancements. At the nanoscale, scientists can systematically piece together materials from the bottom up, with designated materials being constructed with precise purposes in mind. Axiomatically due to their small size, nanoparticles have a very large surface area to volume ratio and therefore an increasing number of atoms are available on the surface of the material. For example, a 100 nm aggregation of atoms has 3% of the atoms present at the surface whereas for a 10nm aggregation 30% of the atoms are at the surface. Consequently with an increase in size from nanometer to micro or even millimetres, the percentage of atoms on the
surface will have a large impact on the decrease of reactivity from bulk materials compared to their nanosized counterparts.

Figure 1. Number of publications retrieved using the ISI Web of Knowledge search engine with search terms “nanoparticle” and “nano*”. Search undertaken on 14/10/11.

In the nano form the properties of materials differ to their corresponding larger or ‘bulk’ counterparts. Some of these gained attributes related to their nano size can enable the nanomaterial to function more efficiently as well as give it entirely new properties. These contribute towards novel applications such as data storage and electronics. Nano-sized induced differences include; electronic band gaps, magnetic moments, specific heat and melting points, as well as changes in material morphology and surface reactivity (Lucas et al., 2001). At the smaller end of the nanoscale (< 5nm) particles begin to take on quantum behaviour prescribing optical and electrical alterations from larger particles of the same material as the behaviour of light and matter differ (Dowling et al., 2004). All of these properties can be manipulated and exploited using the nano form of the material, with many potential applications. The level of scientific interest in nanotechnology in recent years is clearly illustrated by the rapid increase in the number of publications in the area, especially over the last 10 years (see Figure 1).
1.4 Types of nanoparticles: their specific properties and applications

1.4.1 Natural nanomaterials

Natural nanoparticles have been around for billions of years and natural events such as dust storms, volcanic eruptions, and forest fires can produce such vast quantities that they can have a global impact on air quality or air travel safety (Buzea et al., 2007). Other examples include nanoparticles found in glacial ice cores 10,000 years old (Murr et al., 2004), geological processes such as weathering which are likely to produce inorganic nanoparticles and biological mechanisms that typically produce organic nanomolecules (Wigginton et al., 2007). Furthermore, humic and fulvic acid components of natural organic matter (NOM), present in most natural water systems and soil, can be found in the nano-size range (Lead and Wilkinson, 2006).

Natural nanomaterials can be manipulated in order to serve useful functions. Current medical imaging techniques often require radioactive or heavy metals which leads to difficulties in biocompatibility within the patient and numerous natural nanomaterials have been employed as coatings to reduce toxicity, opsonisation and increase circulation of these contrast agents. Lipoproteins, viruses and ferritin are natural nanoparticles that have been harnessed as delivery vehicles for contrast-generating materials. These provide an alternative to synthetic nanoparticle systems where evasion of the immune system, biocompatibility and biodegradability are fundamentally inherent characteristics (Cormode et al., 2010).

1.4.2 Man made carbon based nanomaterials

Fullerenes are spheres, tubes, ellipsoids or sheets formed entirely from carbon rings. Buckminsterfullerenes (C60), discovered in 1985 by Kroto and colleagues (Kroto et al., 1985) were produced by design in 1990 (Kratschmer et al., 1990). Chemically and thermally stable, fullerenes can be dissolved in solvents, purified, functionalized, sublimed, polymerized, used as an electron acceptor, an efficient radical scavenger, they have unique optical properties, and unique cage structures (Murayama et al., 2005). These properties enable a plethora of applications (Figure 2), from 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) to their use in fuel cells (Hinokuma and Ata, 2001). Commonly made using an arc discharge method or by combustion of precursors such as benzene in a flame producing soot (Murayama...
et al., 2005) the versatility of fullerenes is due to their ability to have a modified surface that can enable their functionalisation for many different applications.

![Diagram of potential applications of fullerenes](image)

**Figure 2.** Potential applications of fullerenes (reproduced from Murayama et al., 2005)

Carbon nanotubes (CNTs) are amongst the most researched materials of the 21st century. Structurally, CNTs can be described as a sheet of graphene rolled into a tube. Different types of CNTs, described by the indices of their chiral vector, \( n \) and \( m \), can be obtained (Schnorr and Swager, 2010). Additionally, CNTs can vary by the number of carbon-layers in their sidewalls. Single-walled CNTs (SWCNTs), double-walled CNTs (DWCNTs), and multiwalled CNTs (MWCNTs) have been synthesized and are commercially available. Being lightweight and having a high tensile strength means that CNTs are often employed in mechanical applications, such as lightweight composite materials for bicycle frames, rackets and car body work. Further, unique electrical properties of CNTs have led to their use as transparent electrodes for organic light-emitting diodes (OLEDs), lithium-ion batteries, supercapacitors, and CNT-based electronic components (reviewed in Schnorr and Swager, 2010). Additionally, CNTs have been utilised as catalysts, in sensors as well as filters and biomedical applications (Schnorr and Swager, 2010). As mentioned previously, the modification potential of CNTs allows for this diverse specialisation of their properties for particular applications.

Graphene is termed the parent of all graphitic forms (Rao et al., 2009) and has stimulated much scientific interest in recent years. Constituting a single-atom layer of six-membered carbon rings, thus making it 2-dimensional, it is distinctly different from CNTs and other
fullerenes and possesses its own set of unique properties. These include a quantum Hall effect at room temperature (Zhang et al., 2005; Novoselov et al., 2007) ballistic conduction (Novoselov et al., 2004) high elasticity and a tunable band gap (Novoselov et al., 2004; Han et al., 2007). These properties allow applications such as transistors, hydrogen storage, conductive plastics, plasma displays, gas sensors and solar cells as well as graphene having characteristics that allow efficient doping (reviewed in Geim and Novoselov, 2007; Rao et al., 2009). Additionally, grapheme exhibits strong mechanical properties, and as such has potential application in composites similar to CNTs.

1.4.3 Quantum dots

![Design of QD core](image)

- Size/distribution
- Geometry
- Chemical composition
- Surface architecture
- Optimization of photo-physical properties

![Design of organic shell](image)

- Stability in buffer
- Compact size
- Preserved photo-physical properties
- Anchor groups
- Improved biocompatibility

![Design of surface ligands](image)

- Preserved ligand activity
- QD-ligand stability
- Controlled stoichiometry
- Controlled ligand orientation
- Pre-designed conjugation anchors
- Bio-functionality

**Figure 3.** Schematic of basic steps and design stages when engineering QD for biological applications (from Zrazhevskiy et al., 2010)
Colloidal semiconductor quantum dots (QDs) are single crystals a few nanometers in diameter. Their size and shape can be controlled precisely by the ligands, temperature and duration of their synthesis (Alivisatos, 1996). Usually made from hundreds to thousands of atoms from group II and VI elements or group III and V elements (Zrazhevskiy et al., 2010), this refined composition enables engineered size- and material-dependent absorption and emission of photons is one of their key characteristics for applications (Figure 3). The size-tunable optical properties of these nanoparticles, which are not obtained in bulk material, as well as efficient light absorption over a broad excitation spectrum, long fluorescence lifetime and resistance to the effects of photobleaching has led to QDs being championed over many dyes and conventional fluorophores for many biological imaging applications (see Alivisatos, 2004; Klostranec and Chan, 2006; Jamieson et al., 2007 for in depth reviews). Biological labelling with QDs was first used in 1998 (Bruchez et al., 1998; Chan and Nie, 1998) and can be tracked using confocal microscopy (Lacoste et al., 2000) total internal reflection microscopy (Michalet et al., 2001) or wide-field epifluorescence microscopy (Dahan et al., 2003). As well as the optical properties that QDs possess, their relatively small size assists in the engineering of biologically functional materials. For instance, fluorescent QDs can be conjugated with biomolecules such as antibodies, to target specific biological events, cellular structures and are used in a variety of bioanalytical processes as well as in drug delivery vehicles (reviewed in Bailey et al., 2004; Jamieson et al., 2007; Zrazhevskiy et al., 2010). Other applications include security tagging, anti-counterfeit pigments (Chang et al., 2004) and displays containing light emitting diodes (Kang, 2008).

### 1.4.4 Dendrimers

Dendrimers are branched synthetic nano-scale polymers with a layered architecture resembling biomolecules (Figure 4). The term dendrimer arises from the Greek words ‘dendron’ meaning ‘tree or branch’, and ‘meros’ meaning part. More specifically, dendrimers can be defined as globular, monodisperse macromolecules with novel three-dimensional polymeric architectures in which all bonds emerge radially from a central focal point or core with repeat units that each contributing a branching point in the dendrimer (Fréchet, 2002). Research into core structures with branches began in the late 1970s (Buhleier et al., 1978) and dendrimer synthesis can now be manipulated to alter molecular weight and chemical composition. Specific tuning of these properties has excited scientists with their ability to alter biocompatibility and biokinetics with much research being undertaken on their role as therapeutic transporters (e.g. Lee et al., 2005a; Wijagkanalan et al., 2011). Dendrimers are promising drug and gene delivery vehicles and with their branched structure are able to carry...
many forms of anticancer, anti-viral, anti-bacterial agents. Further medical applications include tissue repair scaffolds and optical oxygen sensors (Bosman et al., 1999; Duncan and Izzo, 2005; Lee et al., 2005a; Jain et al., 2010). Their suitability is related, not only to their three-dimensional architecture and high functionality, but also to their low polydispersity and ability to enhance solubility, bioavailability and release site sensitivity.

![Dendrimer architecture](image)

**Figure 4.** Classic dendrimer architecture (from Lee et al., 2005a)

### 1.4.5 Metal and metal oxide nanoparticles

All of the work undertaken in this thesis has been on metallic and metal oxide nanoparticles. Here I broadly introduce metal and metal oxide nanoparticles and their current usage.

The unique characteristics of metal oxides make them an extremely diverse class of materials and their properties cover many aspects of materials science and solid-state physics. The crystal chemistry of metal oxides, representing not only the crystal structure, but also the nature of bonding, ranges from simple rock salt to highly complex structures and their bonding varies from highly ionic to covalent or metallic. Associated with such changes in structure and bonding, oxidic materials can exhibit specific electronic and magnetic properties. All these properties make metal oxides a vital constituent in many technological applications.

Further to their diverse application, nanoparticulate metal and metal oxides are relatively easy to synthesise chemically with most laboratories able to undertake such processes *e.g.* (Gu and Soucek, 2007). Metal nanoparticles are usually produced *via* reduction or co-reduction of
metal salts, whereas engineered metal oxide nanoparticles, such as titanium dioxide (TiO$_2$) and zinc oxide (ZnO), are commonly made by hydrolysis of the transition metal ions (Masala and Seshadri, 2004).

Metal oxides, such as TiO$_2$ (reviewed in Chen and Mao, 2007) and ZnO, are widely exploited for their photolytic properties, and have been used as photocatalysts in solar cells, industrial catalysts, paints and coatings. They have also been used extensively in sunscreens, cosmetics, and bottle coatings due to their ultraviolet-blocking ability and the visible transparency of nanoparticulate forms (Brezová et al., 2005; Bertelli and Selli, 2006; Quintana et al., 2006). The Woodrow Wilson Database (www.nanotechproject.org) lists 59 titanium containing products, over half of which are personal care products (PCPs). Twenty nine out of 30 zinc incorporated consumer products are also PCPs. Another metal oxide of current global importance is cerium dioxide (CeO$_2$). Its nanoform is used in a variety of commercial and industrial applications including mischmetal (commonly used in the ‘flint’ ignition device of many lighters), computer chip manufacturing, solar cells, polishing agents, precision optics, gas sensors and oxygen pumps, a UV blocking agent for cosmetics and as an additive in cigarettes (Masui et al., 2000; Cassee et al., 2011). CeO$_2$ MNPs are also likely to be increasingly incorporated into commercial applications requiring high oxygen storage capacity, low redox and UV absorbing potential. However its primary function, for which is has stimulated much research, is its usage as both a catalyst and fuel additive in the automotive industry (Fall et al., 2007; Park et al., 2007; Park et al., 2008b).

Of the metallic nanoparticles, silver (Ag) is the most widely used and incorporated into everyday products. The scale of Ag MNPs applications compared to other metal MNPs is shown in Figure 5 (derived from the Woodrow Wilson Database (http://www.nanotechproject.org)) and where 313 general products globally are known currently to contain nano silver (October 2011). Properties of Ag MNPs that make this material so sought after include high electrical and thermal conductivity, non-linear optical behaviour and chemical stability. These characteristics have led to the use of Ag NPs in microelectronics (Wu et al., 2006), inks (Lee et al., 2005b; Perelaer et al., 2009) and medical imaging (Jain et al., 2008). However, the predominant property of Ag that has seen its increased addition into many products is its antimicrobial activity (Luoma, 2008). This activity is often attributed to the dissolved cation as opposed to the physical parameters of the particle itself and this has lead to many debates as to the potential toxicity of Ag MNPS, its speciation and how it should be regulated. Ag MNPs have, as previously noted, been synthesized and available for decades (Nowack et al., 2011) although the recent increase in market presence of Ag MNPs has seen
them incorporated into plastics, soaps, wound dressings, socks, and other textiles; air filters; toothpaste, baby products, vacuum cleaners and washing machines (http://www.nanotechproject.org).

Another anti-microbial metallic nanoparticle, copper (Cu), has been used in bioactive antifouling coatings, inhibiting the growth of microorganisms (Cioffi et al., 2005) as well as being incorporated into textiles (Gabbay et al., 2006). Cu nanoparticles are also used in nano inks because of its lower cost compared with the noble nanoparticles such as gold and silver. Copper (I) oxide (Cu$_2$O, also called cuprous oxide) is a $p$-type metal oxide semiconductor with promising applications of its nano-form in solar energy conversion and catalysis as well as exhibiting unique optical properties (Yin et al., 2005). Cu MNPS are now available commercially for use in applications such as additives in lubricants and acts as an effective repair and friction reducing agent (Liu et al., 2004), facial spray, shampoo’s, anti oxidants (www.nanotechproject.org) and anode materials for lithium ion batteries (Guo et al., 2002).

![Major Materials](http://www.nanotechproject.org)

**Figure 5.** MNP containing products as listed by the Woodrow Wilson database (http://www.nanotechproject.org).
“Gold will be slave or master” wrote Horace, and if current trends in gold (Au) nanoparticle applications continue it is likely that Au as MNPs will have a large influence on many areas of research. Au is one of the most stable metal nanoparticles and possesses a number of interesting properties which makes it attractive for industrial exploitation, especially within the medical field. Nanoparticles with fewer than 300 Au atoms display the typical change in size-related electronic, magnetic and optical properties of nano-scale particles. Their properties are dependent upon the particle size, interparticle distance and shape of the nanoparticles (Brust and Kiely, 2002). Physicists predicted a quantum size effect for particles between 1 and 10 nm in diameter where the particles behave in a manner relevant to quantum-mechanical rules. Free electrons in Au are trapped in such quantum particles and show a characteristic collective oscillation frequency of the plasma resonance, giving rise to a plasmon resonance band observed near 530 nm in the 5-20-nm-diameter range (reviewed in Daniel and Astruc, 2004). The deep-red colour of Au MNPs in water reflects this surface plasmon band where a broad absorption in this visible region of around 530 nm. This has been extensively studied and the optical properties of Au MNPs are its key characteristics in applications.

Like silver, one of the conventional methods of Au MNP synthesis is by reduction of Au(III) derivatives, using citrate reduction of HAuCl₄ in water, a method that was introduced by Turkevitch (1951). Other synthesis methods include microemulsions with surfactants, seeing-growth techniques and other physical methods (reviewed in Daniel and Astruc, 2004). Applications of Au MNPs are wide reaching and include immunolabeling, imaging of cells and use in biodevices for diagnostics. Au can conjugate with antibodies, peptides and drugs facilitating detection and regulation of DNA and drug delivery, sciatic nerve repair and cancer diagnosis (reviewed in Ghosh et al., 2008; Sperling et al., 2008; Sardar et al., 2009; Lim et al., 2011; Rippel and Seifalian, 2011; Stone et al., 2011; Zeng et al., 2011) Conjugates of Au MNPs and oligonucleotides potentially allow use of the programmability of DNA base-pairing to organize nano materials (Mirkin et al., 1996).

Au MNPs can provide non-toxic carriers for drug and gene delivery applications (Figure 6). Using these structures, the gold core imparts stability to the assemblage, whilst the outer layer facilitates functionalisation and tuning of surface properties (Ghosh et al., 2008). Additionally, release of the therapeutic agent can be triggered internally by thiols (Hong et al., 2006) or pH (Polizzi et al., 2007), or externally by light stimuli (Han et al., 2006).
Aluminium nanoparticles are another widely used MNP, often in the form of Al₂O₃ which has potential electronic applications as a component of high-performance polymer composites (Goyal et al., 2007), as a fuel additive to increase ignition probability (Tyagi et al., 2008) and in nanoscale metal-based energetic materials called superthermites. These have a number of military applications as well as use in thermal batteries (Valliappan et al., 2005).

Zero-valent metals are typically made by the reduction of solutions of metal salts in which alteration of the conditions and reductant in turn can alter their physical properties. For zero-valent iron, the most common of synthesized zero-valent MNPs, there are many routes and methods. These include sol–gel techniques, chemical vapour deposition, hydrothermal synthesis, pyrolysis, milling and microbial synthesis (reviewed in Abhilash et al., 2011). Nanoparticulate zero-valent iron (Figure 7) is of particular environmental relevance due to its usage in the remediation of waters, sediments and soils (reviewed in Li et al., 2006). The extent of applying MNPs to combat problems associated with organic pollutants is such that US Environmental protection agency (EPA) funds programmes that are worth over £500 million (Karn et al., 2009; Sánchez et al., 2011). Pollutants targeted for in situ remediation with nanoscale zero-valent iron include; nitrates, chlorinated organic solvents, organochlorine pesticides and polychlorinated biphenyls (PCBs). This is achieved by injection into the subsurface for groundwater remediation, often as a slurry (Zhang, 2003b). Other commercial applications of iron nanoparticles take advantage of their magnetic properties. Magnetite (Fe₂O₄) exhibits special electric and magnetic properties and below a certain size (generally 10–20 nm), exhibits a unique form of magnetism called superparamagnetism (Huber, 2005).
for magnetic nanoparticles include recording media, labelling and magnetic separation of biological materials, directed drug delivery, MRI contrast enhancement and hyperthermia treatment (Gupta and Gupta, 2005; Huber, 2005; Corot et al., 2006; Thorek et al., 2006).

**Figure 7.** The core-shell model of zero-valent iron nanoparticles. The core consists of mainly zero-valent iron and provides the reducing power for reactions with environmental contaminants. The shell provides sites for chemical complex formation (e.g., chemosorption). Here, the mechanics of arsenic (As) remediation are being schematized. (Reproduced from, Ramos et al., 2009)

### 1.4.6 Nanocomposites

In addition to the wide range of individual MNPs currently available, there is a large body of materials that are nanocomposite or nanointermediates. These are materials or matrices into which raw nanoparticles are incorporated or have been synthesized with nanoscale features to improve a particular property of the material. An analysis of the products listed on the Woodrow Wilson Database (http://www.nanotechproject.org), shows approximately 1320 consumer products, some of which have been mentioned previously in this chapter. In general they span a vast range of goods from antibacterial kitchen and tableware with nano-silver coating made by Nano Care Technology Ltd to a jet-ski hull made by Yamaha. A basic analysis of the products run in March 2011 is shown in Figure 8, with health and fitness products the
most represented in the market. Although this database is not comprehensive, the trend in nano-product applications is an indicative representation of the state of commercialised nanotechnology.

![Figure 8](image)

**Figure 8.** Number of commercial products defined by categories that contain or utilise MNPs. Analysis of the Woodrow Wilson database (http://www.nanotechproject.org).

1.5 **Nanomaterials and the environment – applications, fate and behaviour**

To set the context of environmental exposure to nanoparticles it should be noted that airborne atmospheric dust has an estimated global production of one billion metric tons per year (Kellogg and Griffin, 2006) and if a fraction of this was in the nanoscale region it would equate to millions of tons of natural nanoparticles. When compared to the production estimates for MNPs, which are a few thousand tons per year for each major type of material (Borm *et al.*, 2006), it is a relatively small amount. Thus, hypothetically, organisms have been exposed to nanoparticles for millions of years without catastrophic consequences. The reason for the intense research into exposure to MNPs is that, even though natural nanoparticles can be toxic (Lee and Richards, 2004), they tend to be ephemeral in the environment, whereas some MNPs are designed to persist, remain viable or be stabilised against natural aggregation or
dissolution processes. As a consequence, this functionalisation of MNPs can incorporate elements toxic to the environment, which are not transient and thus they can become novel pollutants of an area.

There are relatively few studies predicting total environmental concentrations of MNPs in the air, soil and aquatic compartments of the environment, although more are available for single event scenarios. Top-down perspective models consider only the MNP release from a small number of pertinent MNP containing products (e.g. Boxall et al., 2007; Blaser et al., 2008; Park et al., 2008a; O'Brien and Cummins, 2010). In contrast to the bottom-up approaches, where calculations on the whole spectrum of products and possible applications of MNPs (e.g. Mueller and Nowack, 2008; Gottschalk et al., 2009). For example, the model by Boxall and colleagues used relative importance of exposure routes to all environmental compartments as well as the proportion of available consumer products containing MNPs and concentrations of MNPs in those products as inputs, to estimate the likely environmental concentrations of MNPs in the United Kingdom (Boxall et al., 2007). Gottschalk used a ‘probabilistic material flow analysis approach from a life-cycle perspective of MNP-containing products’ and predicted that there may be risks currently for nano-Ag, TiO$_2$ and ZnO in sewage treatment effluents as well as for nano-Ag in surface waters (Gottschalk et al., 2009). Modelling of MNP presence in the environment has recently been reviewed (Gottschalk and Nowack, 2011) however, in all cases the models suffer from a paucity of input data. Empirical information is required on industrial MNP production volume, release sources during nano-product life stages as well as the form, behaviour and fate of MNPs released. Without these data, predicted environmental concentrations (PECs) are highly variable between studies and conclusions are difficult to draw.

However MNPs are inexorably leaching into the environment from an ever increasing number of point and runoff sources, be it from degradation or disposal of commercially available nanocomposites or direct application for remediation purposes. The end result, therefore, is that the burden on the environment will continue to increase. Despite this there is relatively little known about the fate and behaviour of MNPs in the environment, their interactions within real life ecosystems and the relevant potential toxic effects they may cause to the biotic components of a system. Coupled with the above modelling studies, the paucity of data on MNP levels within the various ecological compartments highlights the necessity of the further research into environmental concentrations.
1.5.1 Nanomaterials in the air

As previously mentioned, nanoparticles of natural origin, such as those produced by volcanic eruptions and forest fires, have been present in our atmosphere for millions of years (Buzea et al., 2007). In recent history anthropogenic sources of airborne particles have been produced from cooking fires (Buzea et al., 2007) and in the 20th Century from diesel vehicle exhaust fumes (Zhiqiang et al., 2000). Predominantly in response to evidence generated in the 90’s, suggesting a size dependant respiratory health concern from atmospheric particles (Peters et al., 1997; Sioutas et al., 2005), airborne exposure to nanoparticles or ultrafine particles (UFPs) has had some of the most extensive research focus. Road vehicle traffic and stationary combustion have been indicated as major sources for particles, with nanoparticles (3–7 nm) accounting for more than 36–44% of total particles measured in a study in Birmingham, UK (Shi et al., 2001). Nanometer sized particles, <100 nm, have also been documented in cites on every inhabited continent (Li et al., 1993; Trier, 1997; Hughes et al., 1998; Morawska et al., 1998; Buzorius et al., 1999; Maenhaut et al., 2002). The small size of airborne nanoparticles, enable a long retention time in the atmosphere where theoretically they can be transported considerable distances. Oberdörster (2001) also noted that adsorption of pollutants to the surface of these particles can occur, facilitating co-transport of these compounds. These findings have clear implications in the areas of nanoparticle manufacturing and nanotechnology usage, with regards to safety and potential spread of airborne particles. It must be noted however, that research has mainly focused upon ultra fine particles occurring as emission by-products of combustion and not from MNP production or usage. However, the recent addition of CeO$_2$ MNPs to diesel fuel has lead to concern about airborne exposure via exhaust fumes (Costantini et al., 2001; Park et al., 2008a). Furthermore, with the increased research investigating nanomaterials, laboratory exposure is also a concern. This has been demonstrated by a study which found that working with Ag and Al nanoparticles inside laboratory fume hoods could result in a release of >13,000 particles/cm$^3$ of airborne nanoparticles into the laboratory environment (Tsai et al., 2009).

1.5.2 Nanomaterials in the soil

Soils are complex mixtures of solids ranging from millimeters to nanometers in size, and which contain many kinds of inorganic and organic particles. Well-known examples are clay minerals, metal (hydr)oxides, and humic substances. Only a small proportion of nanoparticles in soil occur as discrete entities as, for example, organic colloids are largely associated with their inorganic counterparts or form coatings over mineral surfaces. For this reason, individual
nanoparticles are difficult to separate and collect from the bulk soil, and extraction yields are generally low. Although most anthropogenic pollutants end up in the aquatic environment, many have to pass through soil systems. Such pollutants include MNPs and predominant entry points to soils include run-off from applied nano-products, atmospheric deposition, direct application (as remediation or via sewage as a fertilizer), accidental release from factories and leaching of nanoparticles to the soil through landfill. An additional difficulty is the lack of analytical methods sensitive enough to detect such trace concentrations of MNPs. Behaviour of MNPs in the soil will depend predominantly on the soil characteristics and their influence on MNP characteristics. Naturally occurring nanoparticles in soils are known as colloids, and their behaviour is relatively well researched. Colloids are usually defined as material with one dimension between 1 nm and 1 µm (Lead and Wilkinson, 2006) and generically constitute a complex mixture including viruses and bacteria, natural organic matter (NOM) such as humic and fulvic acids, protein and polysaccharide exudates from microbes and inorganic matter such as oxides of iron, manganese, aluminium and silicon (Lead and Wilkinson, 2006; Klaine et al., 2008). Subsequently, the behaviour of MNPs entering the soil will be influenced by these colloids and the chemical and physical factors in play can lead to a multiplicity of contrary behaviours such as; aggregation, deposition, transport sorption, stabilisation and dissolution.

1.5.2.i Groundwater remediation

As previously mentioned, for some MNPs an important route of entry into the environment is via direct application for environmental remediation purposes. This is one nanotechnology application that has already progressed to commercial use (Tratnyek and Johnson, 2006) (Johnson et al., 2009). Zerovalent iron nanoparticles are highly redox active (Figure 7) and are thus employed to clean up soils contaminated with heavy metal ions, polychlorinated hydrocarbons, pesticides and radionuclides (Zhang, 2003a). These particles are applied in the form of a highly concentrated (ppt) slurry (Zhang, 2003a) which is injected into the soil forming a colloidal reactive barrier (Giasuddin et al., 2007). Another application method is by placing a solid permeable membrane coated with zero-valent iron into the soil, across the flow of groundwater. Whilst this technology is potentially very beneficial to the environment, particularly in the clean-up of large scale problematically located spills, there are also potential hazards. The effectiveness of zero-valent iron as a reducing agent for many toxic chemicals also means that its radox potential can cause oxidative stress in cells (Phenrat et al., 2009). This means that residual zero-valent iron particles after the treatment of soils may become an unwanted pollutant. Similar problems may arise with the use of static permeable membranes, where leaching of iron to the soil can reach levels of up to 70%. This means that subsequent
iron removal treatments are required if the groundwater is a source of drinking water (Nikolaidis et al., 2003). The production of toxic by-products or intermediates is also a consideration for the degradation process, and can create complex mixtures of contaminants (Barcelona, 2005). Additionally the widespread presence of humic substances (see previous section) has also been found to reduce the effectiveness of the process due to competitive binding (Giasuddin et al., 2007).

1.5.3 Nanomaterials in aquatic systems

The aquatic environment is often termed the ‘ultimate sink’ for pollutants from both the atmosphere and terrestrial environment, as well as those that enter directly (e.g. sewage treatment works). As a consequence, organisms in aquatic environments are likely to receive the greatest exposures to MNPs. Similarly to soil systems, the aquatic environment contains natural nanoparticulate matter in the form of various colloids and these will behave with the same fundamental principles and conditions as the natural colloids in soils. Thus there is tendency for nanoparticles to aggregate and fall out of solution causing sedimentation and reduced transportation within the water column. The modelling approach by Mueller and Nowack (2008) addressing the presence of MNPs within environmental compartments does not take into account the concatenated nature of the environment despite the linkage between surface waters and groundwater reservoirs (Schaller and Fan, 2009) and the documented transfer of pollutants between the two (Caruso and Dawson, 2009).

With waste water treatment works (WWTW) anticipated to receive a significant anthropogenic MNP load, this will form a predominant route of entry for MNPs into the aquatic environment. Occurrence of Ag and Au MNPs has been modelled for WWTWs (Benn and Westerhoff, 2008; Kaegi et al., 2011), but were not predicted to present a problem for WWTW processing. A further study on WWTW by Limbach et al. (2008) used a model wastewater treatment plant to investigate CeO₂ MNPs with up to 6 % (weight) of the particles found in the effluent. Additionally this study reported that stabilizing agents routinely used in nano-products caused a significant increase in the levels of cerium oxide found in the treated wastewater. Similarly a coating dependant behaviour was recorded for silica (SiO₂) MNPs (Jarvie et al., 2009). Studying presence, distribution and behaviour of MNPS at such sites of human and ecological interaction are well founded, and some of the very limited data for MNPs existence in the environment has been recorded for C₆₀ and C₇₀ fullerenes at WWTWs, with predominant concentrations being in the parts per billion range (Farré et al., 2010). Direct evidence of MNPs released into the environment, not from sewage discharge, is extremely limited. However,
data has been presented for TiO$_2$ particles originating from paint run-off from buildings (Kaegi et al., 2008) where particles in the range of 20 to 300 nm were measured at a concentration of 8 μg/l in local urban run-off.

From the few studies available and the evidence presented it seems appropriate to consider MNPs potentially as a considerable environmental pollutant of the future. Their usage in a multitude of technologies and consumer products will mean extensive release into all environmental compartments and thus exposure for essentially all organisms. Assessment for the risk associate with exposure must draw not only upon the particle characteristics themselves but also their potential concentration and interaction within the environment. To do this the analytical tools must keep apace with the science that develops and designs more specific MNPs for more specialised jobs, to enable detection, identification and characterisation of MNPs in an array of environmental matrices. The understanding of MNP behaviour in real world scenarios is critical to understanding their risk to both human health and their ecological impact.

1.5.4 Nanomaterials and natural organic matter (NOM)

Natural organic matter (NOM) is a ubiquitous component of our ecosystems, predominantly made up from a composition of humic acids and fulvic acids with a hydrophilic fraction. The effects of organic matter on nanoparticles has been reviewed both by Lead and Wilkinson, (2006); and Wilson et al., (2008). The importance of colloidal size in pollutant binding, and subsequent fate and behaviour of trace pollutants, is well known (Baalousha and Lead, 2007). With aggregation and sedimentation known to have a large influence on the fate and transport of particulate matter, including MNPs (Baalousha, 2009), it is hypothesized that NOM and its ability to chelate metals will affect the colloidal stability and subsequent bioavailability of MNPs to organisms. This is due to its influence on the surface speciation and charge of MNPs by sorbing onto the surface via a range of interactions including electrostatic and hydrogen bonding as well as hydrophobic interactions (Hug and Sulzberger, 1994; Ojamae et al., 2006). This predominantly results in a modification to the surface charge of the MNP due to their high surface charge density (Santschi, 2005). This is critical to particle behaviour as dispersed particles will only become destabilised and thus aggregated when their surface charge is near to neutral and thus removing the electrostatic or steric repulsion (Chen and Elimelech, 2007). This aggregation state is, as previously stated, an important consideration for the impact on MNP reactivity (Tseng et al., 2006) and potential toxicity (Grassian et al., 2007; Sager et al., 2007). NOM size is reported by many to be in the nanoscale with hydrodynamic diameters
reported as low as 1 nm (Schimpf and Petteys, 1997) and its geometry ranges from spheres to sheets (Namjesnik-Dejanovic and Maurice, 1997). NOM, just like MNPs is greatly influenced by pH, ionic strength of the surrounding medium and concentration (Pranzas et al., 2003; Baalousha et al., 2006), however, various studies give evidence of increased stability (von der Kammer et al., 2010; Yang et al., 2009; Zhang et al., 2009; Quik et al., 2010) and decreasing aggregation (Domingos et al., 2009; Keller et al., 2010) of MNP suspensions upon addition of NOM. Von der Kammer and colleagues (2010) suggested the availability of organic molecules adhering to the metal oxide particles can provide a barrier to aggregation. Other studies also suggest NOM adsorption can also decrease the zeta potential of nano-oxide suspensions and increase the electrostatic repulsion between particles by adsorption to the surface area, indicating that NOM-coated nano-oxides could be more easily dispersed and suspended (Harbour et al., 2007; Yang et al., 2009). Therefore interactions between MNPs and NOM will be a considerable deciding factor in the fate and bioavailability of MNPs. Whereby aggregate formation will be favoured by high molecular weight NOM compounds and therefore subsequent flocculation and removal into sediments, compared to solubilisation favoured by low molecular weight NOM compounds acting as natural surfactants and thus increasing their dispersion potential and transport capabilities (Figure 9). Most laboratory experiments assessing the toxicity of MNPs in aquatic environments have not considered the presence of NOM in their testing regimes, which could have fundamental implications for effects assessments.

Alongside NOMs ability to effect the behaviour and fate of MNPs, these natural nanoparticles have a potential role in binding trace elements and acting as co-transporters of other pollutants (Lead et al., 2005). This is evidenced in a number of studies where coatings or presence of NOM enhanced sorption of organic chemicals and polycyclic aromatic hydrocarbons to MNPs (Hu et al., 2008; Wang et al., 2008; Yang and Xing, 2009) and is an important consideration when considering that the natural environment is a mixture of a wide range of xenobiotic and pollutants.
Figure 9. Potential nanoparticle (NP) interactions with toxicants (Tox A and B), salt ions (SI), and natural organic matter (OM) such as humic acids or compounds released by plants, fungi, bacteria, and algae. Some compounds present in environmental matrices might increase the NPs’ stability (OM) and thus bioavailability (represented as solid arrows entering organisms), whereas others (salt ions) might foster the aggregation of NPs, thus reducing their bioavailability (represented as dotted arrows not entering organisms), or physically restraining NP-organism interactions. (Reproduced from, Navarro et al., 2008)

1.6 Nanotoxicology

The nanoscale properties that have been manipulated and exploited for commercial applications may also give rise to their potential to induce adverse effects for unintended exposures to MNPs. Displaying quantum behaviours and enhanced reactivity, MNPs could produce unexpected interactions with exposed organisms especially considering that their small size potentially facilitates bypassing of key biological barriers that prohibit standard xenobiotic entry.

As with most toxicants and pollutants established or hypothesised to enter the environment, most research has focused upon human health effects and nanotoxicity in mammalian systems. Recently, however, studies of a more environmental nature have been undertaken. Further areas such as potential routes of uptake and toxicodynamic studies of MNPs have been explored with both fate and toxicity studies taking place in vitro and in vivo on cell and
tissue types that are considered most likely to be challenged. The next section introduces the current understanding of the mechanisms of MNP toxicity and gives an overview of toxicological data for the most prominent MNP types focusing on the studies involving fish.

1.6.1 **What makes nanomaterials toxic?**

1.6.1.i **Size and surface area**

The fundamental property of a nanoparticle is, of course, its nano dimension. Nanomaterials have different properties from their ‘bulk’ counterparts based entirely on their size characteristic alone. This difference is often due to the simple physics of having a greater surface area to volume ratio but it is predicted that MNPs in the diameter range 1–10 nm will follow quantum-mechanical rules (Alivisatos, 1996). In terms of toxicity a size related increased surface area can lead to an enhanced dissolution of materials (e.g. for some metals) and thus lead to the release of potentially toxic ions, or an increase in reactive sites (Oberdörster, 2000). Additionally due to their small size, MNPs have been shown to be retained in many cells and organs to a greater extent compared with larger particles (Nativo *et al*., 2008). There are many studies that show a size dependant increase in toxicity as particles size decreases (Renwick *et al*., 2004; Karlsson *et al*., 2009; Van Hoecke *et al*., 2009; Merrifield *et al*., 2010; Pelletier *et al*., 2010) as well as a more specific size dependent generation of reactive oxygen species (Choi and Hu, 2008) and an even more detailed observation where the mechanism of toxicity varied for particles 0.2 nm different in size (Pan *et al*., 2007). However, there are also a number of studies that do not show size related effects in relation to uptake or toxicity (Limbach *et al*., 2005; Grassian *et al*., 2007; Hardas *et al*., 2010). As with many studies, toxicological results are usually multifaceted, although size is an obvious initial determinant of nanoparticle toxicity, many other factors have important contributions to make.

1.6.1.ii **Charge and aggregation**

As size determines a large part of uptake and toxicity for MNPs it is self-evident that processes effecting particle size will also play a role in toxicity assessments. Stability and aggregation behaviour of MNPs within various environmental matrices, as previously mentioned, is determined both by the physicochemical properties of the surrounding media and the properties of the MNPs themselves. Aggregation forms clumps of particles that can fall out of the nano size range and thus alter the properties more akin to the ‘bulk’ material. The degree of aggregation of MNPs has been demonstrated to affect the organism exposure levels and the
nature of the exposed particles (Römer et al., 2011) and aggregated MNPs have also shown to be less cytotoxic (Wick et al., 2007). However, there are also results that show MNP aggregates demonstrating toxicity in their own right (Zhu et al., 2009; Wong et al., 2010).

Charge in itself has also been implicated as an influencing factor in MNP transport and toxicity, with different surface charges resulting in different uptake and toxic effects (Lockman et al., 2004; Mayer et al., 2009). As previously stated various environmental parameters govern MNP dynamics and physical state. Thus, the exposure conditions can also modify the toxicity of a particle as the pH, composition and concentration of ions in the surrounding media or environment will be likely to affect the charge on a particle and therefore its aggregation behaviour and further interaction with any organisms.

1.6.1.iii Natural organic matter

As NOM presence has a direct effect on aggregation states it is hypothesised that it should increase bioavailability to organisms, however, no studies to date have expounded on this. NOM effects on toxicity however, have shown to be fairly consistent with the majority of studies reporting a protective effect of NOM. These include studies showing reduced toxicity and bioavailability to invertebrates, algae and bacteria (Li et al., 2010; Lee et al., 2011; Van Hoecke et al., 2011) as well as a reduced toxicity of metals to fish (Richards et al., 2001; Hoang et al., 2004) all in the presence of NOM. A number of studies also indicate the decrease in toxicity in the presence of NOM as a consequence of binding of the ionic forms of the metal by NOM, thus restricting its uptake (Karen et al., 1999; Zhou et al., 2005). Nevertheless there are a few studies indicating NOM can have enhancing toxicity effect for nanomaterials and other contaminants (Meinelt et al., 2003; Wiench et al., 2009).

1.6.1.iv Shape

The shape of MNPs can play a crucial role in a determining a toxicological response (Albanese et al., 2010) as exemplified by needle-shaped carbon nanotubes, which impale entire cells (Liu et al., 2009). Another study showed dendritic nickel nanoclusters to be more toxic to zebrafish (Danio rerio) embryos than nickel nanoparticles (Ispas et al., 2009). As further demonstration, the asbestos type toxicity noted from carbon nanotubes (Poland et al., 2008), shown both in vitro (Hirano et al., 2008) and in vivo (Warheit et al., 2004) is likely to be due to physical similarities (Soto et al., 2005). However, care must be taken when interpreting studies...
assessing similarities between nanotube and asbestos type toxicity to ensure that the measured parameters are accurate and/or realistic (Donaldson et al., 2008)

1.6.1.v Particle chemistry

Chemical composition is another determinate of MNP effects on biological systems. MNPs of different chemical compositions have been demonstrated to have differing biological effects (Renwick et al., 2004; Griffitt et al., 2008) other chemical factors such as the ability of a nanoparticle to generate reactive species (Sayes et al., 2006; Xia et al., 2006) and the oxidation state of the nanoparticle (Wörle-Knirsch et al., 2007) are also key determinants of toxicity.

1.6.1.vi Nanoparticle solubility, ion or particle toxicity

Metal speciation models are equilibrium models, which are in contrast to the current description of MNPs behaviour which is a dynamic process where the system is dependent on the amount of energy added to the MNP dispersion and the physico-chemical properties of the particles (called Derjaguin, Landau, Verwey and Overbeek (DLVO) theory, Derjaguin and Landau; Verwey and Overbeek, 1948), see Handy et al (2008c) for discussion with relevance to MNPs within the environment. The solubility of the nanoparticle is a significant factor in determining toxicity (Brunner et al., 2006; Gagné et al., 2008; reviewed in Shaw and Handy, 2011). There has been much early ambiguity in many nanoparticle studies with regards to teasing apart the effects of MNPs and their dissolved ions (Lubick, 2008). Most studies have ionic controls implemented into their designs but the results can still be conflicting (Kim et al., 2011; Navarro et al., 2008). Figure 9 demonstrates the variable route of uptake in a fish gill between MNPs and ion metal forms. This figure implies that MNPs are too big to use ion transporters, or paracellular diffusion pathways, and that the most likely route of uptake is via a cytosis pathway. However, for MNPs less than 20 nm that have a hydrophilic surface or achieve no net charge subsequent to biological coating (such as mucus, Handy and Maunder, 2009), it is possible for these MNPs to diffuse through the cell membrane. This is likely to be a property that is selectively engineerable and when comparing MNP and ionic transfer the properties of size and hydrophobicity should be considered. A study comparing the effects of three MNPs and their associated dissolved salts (Griffitt et al., 2009) found that both ionic and nanoparticulate forms increased metal levels in gill tissue after 48 h however one of the MNPs (Ag) showed a greater uptake compared to the dissolved treatment, although actual internalisation of the particle was not measured and the result could be a function of association. Dissolution of MNPs is still of concern as the MNPs can act as vehicles for the
metal ions, and association with tissues could impart either a chronic release of metal ions or a regional acute concentration. To discern between particulate and ionicly driven toxicity is a challenge that faces nanotoxicologists and although correction can be added for dissolution rates of various metals there are many environmental scenarios that can affect these parameters. Therefore establishing uptake or at least effective proximity of MNPs within an experimental system is critical to assessment.

Figure 9. An idealised diagram of the freshwater fish gill showing the mechanisms of uptake for electrolytes, metal ions (Me+), and electroneutral diffusion of some small organo-metals (CH$_3$-X), compared to nanoparticles (filled circles). Figure and legend modified from Handy et al., (2008b), and Handy and Eddy (2004). The substances in the bulk solution must diffuse into an unstirred layer (USL) comprising of water/mucus secretions, prior to transfer across the gill epithelium. Electrolytes and metal ions usually move through the cell using ion transport pathways (here Na+ transporters are illustrated). In contrast, small electroneutral organo-metals may diffuse into the USL and then through the cells (transcellular diffusion), or between the cells via the tight junctions (paracellular diffusion). Nanoparticles will diffuse into the USL...
and may be influenced by humic substances (HS). Cationic MNPs will bind to strands of mucus via electrostatic attraction but, regardless of surface charge, may also become trapped in the mucus preventing uptake. MNPs are theoretically too large to be taken up by ion or other transporters on the cell membranes, although diffusion cannot be excluded for some MNPs that are lipophilic or if tissue is damaged in any way. In conclusion, the current data suggests that under ideal circumstances MNP uptake will predominantly involve vesicular transport, this may in turn vary in its relevance depending on other physic-chemical properties of the MNP in question.

1.6.1.vii Surface chemistry

The surface chemistry of MNPs impacts such factors as their catalytic activity, ability to generate reactive species and general cytotoxicity and is a dominant factor in determining toxicity that size nor surface area can account for (Warheit et al., 2007). This has been demonstrated for different crystal structures of nano-TiO₂ (Warheit et al., 2006; Sayes et al., 2006). The addition of functional groups to the surface of MNPs is one way in which their surface chemistry can be manipulated. This has been shown to both reduce (Sayes et al., 2004) and increase their toxicity (Magrez et al., 2006) as well as alter the mechanisms of toxicity (Isakovic et al., 2006; Pan et al., 2007; Gu et al., 2009), depending on their functionalisation type. These examples highlight the effect that surface chemistry can have on particles and the engineering potency of MNPs. Specific coatings can be applied to MNPs, either to enhance their biological compatibility, or to act as stabilisers for suspension and monodispersion in aquatic matrices. The function of the coatings can therefore affect their toxicity either indirectly by altering the MNP’s aggregation tendencies, and thus their size, or directly by altering the surface activity (Limbach et al., 2007). Natural coatings can also occur and oxidation of a nanosized zerovalent iron was found to decrease its toxicity (Phenrat et al., 2009).

In summary, a variety of parameters relating to the physical and chemical characteristics of MNPs have been shown to influence their toxicity. These parameters however are still poorly understood with many studies producing contradictory results, making predicting effects difficult. In a real world scenario it will be the combination of many characteristics both environmental and particulate that will affect the behaviour, fate and toxicity of MNPs. Thus exposure media, dose metrics and route of exposure must all be considered for assessment of an MNP and then reconsidered for each different MNP. Understanding of these characteristics
is vital to attaining a more uniform understanding and comparison of observed effects (Powers et al., 2007; Warheit et al., 2008).

1.6.2 Nanotoxicology and fish

Since the establishment of a link between mesothelioma and asbestos exposure in the 1960’s and 70’s (Elmes et al., 1965), research has been conducted to elucidate the effects of similarly structured ultrafine particulate matter on human respiratory health (Peters et al., 1997; Sioutas et al., 2005). Much research was carried out on rodent models and showed that these ultrafine particles, or nanoparticles, are capable of inducing adverse effects in the lungs (Donaldson et al., 1990; Driscoll et al., 1991; Driscoll et al., 1995; Lam et al., 2006). These early investigations initiated a whole raft of toxicity studies on MNPs and this has set a precedent for toxicity studies using MNPs generally. Consequently, the majority of early nanotoxicology assessments have been conducted on mammalian systems and most commonly for inhalation-based exposures. All of the work done in this PhD was on fish and thus all of the relevant particle dynamics have been for suspensions or dispersions of MNPs in aqueous media. The final section of this introduction focuses on the current status regarding our understanding for MNP exposure and toxicity to fish.

1.6.2.i Fish as targets for MNPs

Fish are fundamentally important as sentinels for the health of the aquatic environment, and are an environmentally realistic target species for exposure to MNPs. Potential routes of uptake for MNPs in fish include absorption via the gill epithelia, via the gut epithelia as a result of dietary exposure and/or drinking or via the skin (Figure 10). The studies that have been conducted to date have mostly been concerned with recognising MNP types that cause overt toxic effects, identification of target organs and to a lesser extent, biodistribution of nanoparticles in the bodies of exposed fish. Although these are key field of study there is very little research currently into early life stage sensitivity, characteristics of exposure media or, and essentially nothing developing rapid, high throughput screening systems to assess various aspects of MNP toxicity. A complexity of studying MNPs in the aquatic environment is the diversity of their interactions, which are dependent on the physicochemical properties of the water and the multiple potential routes of entry into exposed organisms (Figure 10). Exposure experiments, and their interpretation, for aquatic organisms are arguably only comparable in complexity to the equivalent exposure studies on soil organisms.
1.6.2.ii Carbon based nanomaterials

One of the first in vivo ecotoxicological based exposure studies of fish to MNPs was an exposure of fullerenes to juvenile largemouth bass (*Micropterus salmoides* Oberdorster, 2004). The study found that at both 0.5 and 1 mg/l there was increased lipid peroxidation in the brain. Despite subsequent studies proving that the dispersant, tetrahydrofuran, was toxic in its own right (Henry et al., 2007), this stimulated the scientific community to broaden their hazard assessment of MNPs in the aquatic environment. Fullerene studies have been undertaken on a number of fish species including fathead minnow (*Pimephales promelas*) and Japanese medaka (*Oryzias latipes*, Oberdorster et al., 2006) where a 42% down regulation of the peroxisomal lipid transporter protein, PMP70 was shown but traditional biomarkers for lipophilic xenobiotic exposure were unchanged. Molecular markers also showed oxidative stress production in goldfish (*Carassius auratus*) to fullerenes (Zhu et al., 2008b). Further acute studies to fullerenes have measured responses including; an increase in malformations, pericardial edema, mortality, a delayed embryonic development, reduced hatching success in and a light sensitive toxicological response in dechorinated zebrafish embryos (Zhu et al., 2007; Usenko et al., 2008). A recent critical review of the evidence of fullerenes induced toxicity indicated that
aqueous nano C<sub>60</sub> have minimal potential to produce ROS and that oxidative stress in fish is not induced by any environmentally relevant exposure (Henry <i>et al.</i>, 2011), however of current interest is their transport, and bioavailability modification of co-contaminants in aquatic environments (e.g. Park <i>et al.</i>, 2011)

A number of studies have assessed the effects of carbon nanotubes in fish models. Zebrafish embryos treated with SWCNTs and DWCNTs caused hatching delay at concentrations over 120 mg/l and 240 mg/l respectively however 99% of embryos hatched by 72 hpf and development was not affected (Cheng <i>et al.</i>, 2007). Transport across the chorion membrane is via pores which are nano-scaled, and may offer a protective barrier against penetration by the micro-scaled MNP aggregates. Embryos treated with MWCNTs exhibited dose dependent increases in mortality and decreased hatching as well apoptosis (Asharani <i>et al.</i>, 2008a). Here concentrations of 60 mg/l and above showed deformation of the notochord and increased mucus production in the intrachorion region. A further study by Cheng <i>et al.</i> (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. Sublethal effects included a build up of white blood cells in the trunk region of embryos in the early stages of the exposure as well as lysosome-like vesicles in the blastoderm cells. A life cycle study was undertaken on these embryos and although the injected larvae developed normally and displayed no decrease in egg production, the survival of the second generation larvae was 50% lower by than that of control fish at 14 days post hatch.

Studies in other fish models include a 10 day semi-static exposure of juvenile rainbow trout to SWCNTs, a dose-dependent (0.1-0.5 mg/l) rise in ventilation rate was observed with a visible accumulation of aggregates associated with gill mucus; 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 glutathione (GSH) were noted in the gills and livers, suggesting evidence of lipid peroxidation and oxidative stress in these tissues (Smith <i>et al.</i>, 2007). A similar study with rainbow trout and SWCNTs however only showed a transient increase in TBARS (Fraser <i>et al.</i>, 2011). In vitro studies also show agreement on a molecular level with increases in inflammatory gene expression for exposure of CNTs to a rainbow trout macrophage culture (Klaper <i>et al.</i>, 2010)
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. However, critically, despite the background levels in the environment being relatively low, these effects are seen at concentrations far beyond the predicted exposure concentrations modelled for the environment (Gottschalk et al., 2009).

1.6.2.iii Titanium-dioxide nanoparticles

Due to its prominent usage and ease to work with, TiO$_2$ MNPs have received some of the most extensive research. The crystalline structure of TiO$_2$ MNPs varies between two main forms of rutile and anatase, and its composition is a key factor in toxicity (Jiang 08). Exposures of zebrafish embryos to TiO$_2$ MNPs show relatively low toxicity when compared to other MNPs such as Ag. For example, 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 (Griffitt et al., 2008) and a further study by the same research group found exposure of adult zebrafish to 1000 μg/l of the same TiO$_2$ nanoparticles caused no change in molecular or histological parameters in zebrafish gills (Griffitt et al., 2009). Similar studies found TiO$_2$ nanoparticles showed no toxicity to zebrafish embryos or larvae along with no size related differences in effects between nanoscale and bulk material (Zhu et al., 2008a). In fathead minnows the LC$_{50}$ for TiO$_2$ MNPs also displays low toxicity with records for over 500 mg/l (Hall et al., 2009). However some toxicological results have been reported, with innate immune system responses and decreased neutrophil function in fathead minnows and respiratory distress and indications of oxidative stress, although no mortalities in juvenile common carp (Jovanović et al., 2011 ). Another in vivo study, a 14 day semi-static exposure of rainbow trout to TiO$_2$ MNPs, 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 in the food had no impact on growth and although an increase in TiO$_2$ content in the liver and spleen was observed in the early stages of the exposure but there were no biological ramifications recorded (Handy et al., 2008a). Another 8 week diet based study again by the same group showed an altered partitioning, for the same species, with Ti accumulated in the gill, gut, liver, spleen, and brain. In this instance however disturbances to Cu & Zn levels and a 50 % inhibition of Na+/K+-ATPase activity was seen in the
brain (Ramsden et al., 2009). Other dietary exposure studies, however, did not find any significant distribution of TiO$_2$ in the analysed tissues (Johnston et al., 2010).

With exposure conditions often a confounding factor when attempting to assess toxicity and biodistribution of a specific particle, experiments that remove environmental variation and nullify external changes in particle characteristics that might modify uptake, are useful. These direct exposure methods include injection studies, which have also been carried out for TiO$_2$ MNPs into rainbow trout, where localisation and biodistribution results indicated partitioning to tissue vesicles surrounding the kidney tubules, although kidney function was not compromised and there was no evidence of lipid peroxidation in blood, liver or kidney tissue (Scown et al., 2009). Injection approaches are also a common delivery vector for zebrafish embryos, and a gene microarray analysis on TiO$_2$ MNP microinjected zebrafish resulted in significant effects for genes involving in circadian rhythm, kinase activity, vesicular transport and immune response, (Jovanovic et al., 2011). To gain a more natural exposure method and analyse food chain bioaccumulation, Zhu et al (2010) exposed *Daphnia magna* to TiO$_2$ MNPs resulting in concentrations of up to 62 mg/g dry weight Ti, and then fed the contaminated daphnia to zebrafish but showed no biomagnification after 14 days.

Toxicity of TiO$_2$ MNPs can be exacerbated by UV radiation (Nakagawa et al., 1997) and the impact of illumination on TiO$_2$ toxicity to fish follows these findings. Xiong et al (2011) found that despite aggregation of particles to similar sizes of the bulk material, upon illumination for 96 hours, toxicity to zebrafish was significantly increased, with the authors suggesting a free radical based mechanism, these results are also mirrored *in vitro* using a rainbow trout gonadal cell line (RTG- cells), which showed no adverse effects to cells at concentrations of up to 50 mg/l TiO$_2$, however, this altered significantly under UVA radiation where increased levels of DNA strand breaks were reported (Vevers and Jha, 2008).

Two further studies, have also demonstrated an additional toxicity factor associate with TiO$_2$ MNPs in the form of enhanced accumulation of heavy metals, arsenic and cadmium in this instance, in the viscera and gills of carp when exposed in the presence of TiO$_2$ MNPs (Sun et al., 2007; Zhang et al., 2007).

1.6.2.iv Silver nanoparticles

Silver is one of the most widely utilised MNPs in current production and as such has generated one of the largest data bases on its likely eco-nanotoxicity. The predominant *in vivo* exposures
of Ag MNPs to fish have been in zebrafish models although there is a great deal of variation within the literature with regards effects due to differences in particle size, coatings, exposure media and delivery methods. Zebrafish embryos exposed to 5-20 nm Ag particles capped with starch or bovine serum albumin (BSA) to aid dispersion exhibited a dose-dependent increase in deformities, mortality and hatching delay. Distribution of Ag 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 mg/l 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 Ag MNPs as well as a decreased expression of a notochord development gene. These effects however were attributed to Ag ions present in the media (Yeo and Kang, 2008). This emphasises the importance of being able to trace the particle kinetics within an exposure system, which is exactly what Lee and collegues (2007) achieved as they traced Ag nanoparticles of 5-46 nm transported in and out of chorion pore channels by Brownian diffusion. The particles were observed inside exposed embryos at all developmental stages and dose-dependent toxicity characterized by developmental deformities and mortality was observed (Lee et al., 2007).

Differentiating between ionic and particle based toxicity, or a combination of both, is one of the challenges facing nanotoxicologists. This is especially relevant for silver where Ag ions are known to be extremely toxic to aquatic organisms (Hogstrand and Wood, 1998). A study investigating the effects of Ag nanoparticles in zebrafish fry found that although 26 nm silver particles exhibited toxicity, silver ions were over 300 times more toxic 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 with replicates exposed to the equivalent concentration of Ag ions calculated to dissociate from the particles. The results showed whole body burdens of Ag were significantly higher in nanoparticle exposed fish than in controls or fish exposed to dissolved Ag and analysis of global gene expression in the gills found differences in response between nanoparticle exposed fish and fish exposed to soluble Ag ions. This suggests that the biological effects of exposure to Ag MNPS do not appear to be driven solely by the release of silver ions (Griffitt et al., 2009). Another ion vs particle study in medaka for Ag MNPs gave similar LC50 values for Ag MNPs and AgNO3, however gene expression indicated different modes of toxicity (Chae et al., 2009). The differences in Ag particulate and ionic toxicity can also be seen in fathead minnows studies. The 96 h LC50 values for AgNO3 have been recorded at 6.7 μg/l (Holcombe et al., 1983) with good agreement in Karen et al (1999) whilst 96 h LC50 values for Ag MNPs exposed to fathead minnow embryos were 9.4 and 10.6 mg/l for unsonicated MNPs.
and 1.25 and 1.36 mg/l for sonicated 35 and ≤ 100 nm Ag MNPs respectively (Laban et al., 2010). This study also described the AgNO₃ toxicity to be 3 times higher than Ag MNP toxicity although these results are likely to be life stage sensitive. A size related toxicity has also been observed for Ag MNPs when zebrafish embryos were exposed to 5.9, 15.3, 51.2 and 108.9 nm Ag MNPs at 250 μM which resulted in a 80, 64, 36 and 3 % mortality respectively after 24 hours (Bar-Ilan et al., 2009). Also, Scown et al (2010b), showed low uptake and potential oxidative metabolism in the gills for the smallest sized Ag particles exposed to rainbow trout.

Different fish species may show differences in their vulnerability and sensitivity to MNPs. Their vulnerability relates to their ecological niche and sensitivity relates to the innate differences in their responses and the effects induced. The variation in ecological niche determines their exposure, with species like carp which associate with the benthos likely to encounter sedimented MNPs as opposed to trout. Early life stages may too be more vulnerable than others. This is particularly important when considering a novel pollutant and especially in the case of Ag where there is strong evidence of metal toxicity, as assessment needs to encompass all of the potential pathways and high risk organisms. Earlier studies on metal toxicity utilising AgNO₃ may be difficult to compare to Ag MNP concentrations despite the previously observed conformity in some of their responses. Hogstrand and Wood (1998) observed the effects of AgNO₃ in freshwater fish and seawater fish and discovered much higher 96 h LC₅₀ results for seawater fish than in freshwater. Earlier than this Davies et al., (1978) recorded mean 96 h LC₅₀ values in rainbow trout of 6.5 μg/l and 13 μg/l in soft and hard water with similar values generated by Hogstrand et al., (1996). For MNPs Shahbazzadeh et al (2009) reported a slightly lower 96 h LC₅₀ concentration of 2.3 μg/l for Ag MNPs to rainbow trout. The data for zebrafish is not so clear however with a 96 h LC₅₀ for AgNO₃ of 15 μg/l reported by Dolzelova (2008) and a 25 μg/l and 84 μg/l 48 h LC₅₀ for Ag ions and Ag MNPs respectively (Bilberg et al., 2011) compared to a 24 h LC₅₀ of 250 mg/l for Ag MNPs by Choi et al (2010). Despite the different time scales this is still a significant difference. Further differences in same species response are seen for Japanese medaka with 48 h LC₅₀ values of 1 mg/l Ag MNPs (25 nm) reported by Wu et al (2010) compared to 96 h LC₅₀ values of 34.6 μg/l Ag MNPs (49.6 nm) reported by Chae et al (2009), again the difference in time scales should not have this significant an effect.

The metal silver, in summary, is known to be very toxic to organisms, hence its large scale use as an antimicrobial agent. However, with regards to fish, Ag MNP’s reported effects are extremely varied, ranging from low levels of toxicity, to uptake and changes in molecular markers of toxicity at relatively low levels after only a short exposure period. The rationale for
this variation is the diversity of Ag particles under scrutiny, with different dissolution rates stemming from sizes and capping agent or the presence or absence of light.

1.6.2.v Other metal and metal oxide toxicities

Other studies on the less commercially relevant metal and metal oxide particles have been fewer in frequency. Many of the assessments for MNP toxicity to fish for a variety of particle types have employed the zebrafish model. These include uptake but no effects of Au MNPs into zebrafish embryos (Bar-Ilan et al., 2009; Asharani et al., 2011), no effects for Al MNPs to embryos after 48 hours (Griffitt et al., 2008), no acute toxicity for nickel particles exposed to embryos from 24 – 120 hpf at concentrations up to 1g/l (Ispas et al., 2009), low dose dependent toxicity of ZnO MNPs (Zhu et al., 2009) and 3 studies by Griffitt et al. (2009;, 2008;, 2007) on Cu. Cu MNPs were found to have a 48 hour LC\textsubscript{50} value of 1.5 mg /l (Griffitt et al., 2007), and despite up to 60% of the particles flocculating out of suspension the dissolution was not sufficient to cause the observe mortality. In this study histological examination revealed the gills to be a primary target organ with visible damage and a dose-dependent decrease in Na+ K+ -ATPase activity. Furthermore exposure to comparable concentrations of ions and particles, Cu MNPs produced greater hypertrophy of epithelial cells along with difference gene expression patterns compared to soluble Cu (Griffitt et al., 2007). A second study resulted in 48 h LC\textsubscript{50} values of 0.94 mg/l in adult fish and 0.71 mg/l in juveniles (Griffitt et al., 2008). The latter finding showed greater MNP toxicity to the larvae than soluble Cu which had a 48 h LC\textsubscript{50} value of 1.78 mg/l. In 2009 (Griffitt et al., 2009), similar levels of uptake were shown for ionic and particulate Cu and comparing gene expression profiles to those of an Ag exposure resulted in distinct mechanisms for the two nanoparticle types.

Other models have been utilised to assess fish exposures to MNPs but in some examples, such as CeO\textsubscript{2}, an environmentally relevant MNP, there are extremely few studies. A body of literature describes or investigates bioavailability and/or toxicity of CeO\textsubscript{2} MNPs to aquatic organisms (e.g. Cohen et al., 2007; Gaiser et al., 2009; Lu et al., 2010; Gaiser et al., 2011; Garcia et al., 2011), however specific fish studies are limited to two trout hepatocyte studies (Gaiser et al., 2009; Scown et al., 2010a). Trout hepatocytes were also utilised in another MNP toxicity assessment where Au MNPs caused a threefold elevation of ROS levels, but no cytotoxicity at low mg/l concentrations (Farkas et al., 2010). Au is another widely used, yet less studied particle, however in this instance, Au is known to be one of the least reactive elements. This is evidenced by a mesocosm study with Au-nanorods (65 nm length, 15 nm diameter) to sheepshead minnows (Cyprinodon variegates) where no acute toxicity was
observed nor accumulation of Au for any banchial uptake pathways and only limited uptake for oral routes (Ferry et al., 2009). A further MNP, zero-valent iron, is directly applied to the environment in large quantities and yet its toxicity to fish is relatively unstudied, which is surprising considering dietary Fe has previously been seen to cause lipid peroxidation in the liver and heart of fish (Baker et al., 1997). The little research into Fe MNP effects have shown various effects from acute lethally to sublethal toxic effects including histological, oxidative stress responses (Li et al., 2009; Chen et al., 2011).

Upon the commencement of this PhD, the field of nanotoxicology was still in its infancy and despite the recent surge in interest and research surrounding this field there is still a lack of standardized testing. This makes deriving confident results from the literature particularly difficult. There are a vast array of nanomaterials available with many nanomaterial types manufactured to different specifications and post production functionalisation allowing further diversification. In combination with varying exposure parameters, to draw conclusion on biological effects should be done with the utmost caution.

1.7 Aims and objectives of this PhD

The gap between what we know about new nanotechnology products and their environmental impacts continues to get wider. Thus, the development of a knowledge base for potential effects that MNPs may have on aquatic organisms is urgently needed in order to appropriately assess the associated risks. The main aim of these studies carried out in this PhD thesis was to begin to understand some of the key principles that influence the bioavailability of MNPs to fish. These include the behaviour of MNPs in the aquatic environment and potential routes of exposure, as well as developing and assessing screening techniques for MNPs. The particles studied are those that have particular ecological relevance due to their commercial use within industry and in consumer products and therefore their potential to pollute the environment. At the start of this PhD very little data were available on the fate and behaviour of MNPs, especially with regard to environmentally relevant conditions. Furthermore MNP levels in the environment are still largely unknown and have only been assessed on a broad scale with models. Within this PhD an aim has been to keep exposure MNP levels and concentrations within a range that can be considered environmentally relevant. To address the main aims outlined above, a series of experiments were conducted to assess the bioavailability of a suite of metal and metal oxide MNPs using fish as both a vertebrate model and a sentinel for the aquatic environmental.
The key objectives/hypotheses and the work undertaken for the respective chapters were as follows:

Chapter 2.

**Hypothesis I** – Fish hepatocytes are not suited for screening MNP uptake and toxicity

**Objective I** – Assessment of fish hepatocyte cultures to be developed as a high throughput screening system for MNP uptake and particle features conferring toxicity.

With a view to developing a high throughput screening assay suitable for assessing the uptake and toxicity of a wide variety of MNP types, primary trout hepatocytes cultures were established and exposed to a variety of commercially available nanoparticles and their bulk equivalents. Responses were analysed using a suite of well known toxicity assays: lactate dehydrogenase (LDH) release, measurement of thiobarbituric acid reactive substances (TBARS) and glutathione-S-transferase (GST). MNP uptake was assessed using coherent anti-Stokes Raman spectroscopy (CARS) imaging.

Study species: Rainbow trout.

Chapter 3.

**Hypothesis II** – Coherent anti-Stokes Raman spectroscopy (CARS) is not suitable as a method to trace uptake of MNPs into exposed fish cells, embryos and tissues

**Objective II**. To investigate the use of coherent anti-Stokes Raman spectroscopy (CARS) as a method to trace uptake of MNPs into a wide range of fish cells, embryos and tissues from experimental exposures to MNPs.

CARS was developed and adapted to assess the utility of the technique for tracing uptake and distribution of MNPs in a series of cells and tissues (principally fish). These findings, together were used as a major component of a review article critically assessing the use of this technique in nanotoxicology.

Study species: Rainbow trout (hepatocytes and gill tissue), zebrafish, *Daphnia magna*, *Corophium volutator*. 
Chapter 4.

Hypothesis III  MNP bioavailability to fish is not affected by NOM in the water column

Objective III. The main objectives here were to investigate the influence of NOM on MNP bioavailability in environmentally relevant conditions, and identify the main target tissues in exposed fish.

Common carp were exposed to two concentrations of cerium dioxide nanoparticles and bulk particles under conditions of high, low and no fulvic acid, a highly characterised component of NOM. Uptake and particle distribution within the organism were analysed using inductively coupled plasma mass spectrometry (ICP-MS) to measure cerium content in the potential target tissues of gill, kidney and brain. Water samples were characterised using dynamic light scattering (DLS), zeta potential and transmission electron microscopy (TEM) to inform on particle characteristics and behaviour dynamics within the water column that may have effected bioavailability in the presence of NOM.

Study species: Common carp.

Chapter 5.

Hypothesis IV. There is not a size related maternal transfer of silver into the developing larvae in a live bearing fish.

Objective IV. To investigate the potential for maternal transfer of MNPs into unborn offspring in an ovoviviparous fish species

The final study examined the maternal transfer potential of Ag nanoparticles into the developing offspring via dietary exposure of adult guppies (Poecilia reticulata). Citrate coated 7nm and bulk particles were incorporated into feed and Ag content of guppy larvae and the maternal sires were measured using ICP-MS. Survival and birth weight of larvae and toxicity indices to adult fish were also assessed.

Study species: Guppy
CHAPTER 2

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 throughput 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-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;.
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 characterizing 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 nanoscale. A number of studies have turned to electron microscopy to determine nanoparticles 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 nonlinear 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 stainfree 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 nanoparticles 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. Perfusion 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$_2$O) and then 100 mL perfusion solution (Hank’s solution containing 358 mg L$^{-1}$ CaCl$_2$ x 2H$_2$O 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$_2$O). 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 10 μg 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 10⁶ 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 10⁷ 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 TiO₂, ZnO, CeO₂ 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 hepatocytes lysed with Triton® X-100.

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 0.625 μM 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 H₂O₂ 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. 100 μL of supernatant and standards 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 hour and then cooled to room temperature under a stream of flowing tap water. 600 L 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 532 nm 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 nanoparticles 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\(_2\)O\(_2\) 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, \(\varepsilon_{340} = 9600 \text{ L mol}^{-1} \text{ cm}^{-1}\)).

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 centered 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 a 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
TEM images of particles (a selection of which are shown in Fig. 1), suggest that none of the ENP types are present as uniformly distributed particles, and there is extensive polydispersity of and aggregation of particles into larger aggregates in the nanoparticulate forms even as a dry powder. We, therefore, expect the ENPs to behave as particles much larger than the suggested manufacturer’s size, which had limited accuracy. 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. TiO2 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. 2.). 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. 3.). Cells treated with 500 μg mL$^{-1}$ H$_2$O$_2$ 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 fourfold 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 μg mL$^{-1}$ (ANOVA with Tukey’s post-hoc test; df=13,59; F=5.276; p<0.001) (Fig. 4.). Treatment of cells with 500 μg mL$^{-1}$ H$_2$O$_2$, 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 5. 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 (Fig. 5.) were taken at 924 nm, whereas images C and D were taken at 919 nm. These differences between the two wavelengths are critical to the identification of the ENPs dosed to the hepatocyte cells. At 924 nm C-H bonds are specifically excited and thus C-H rich structures such as lipids within the cell fluoresce (Fig 5. A, B). At 919 nm lipids are negatively contrasted and appear as dark areas within the cell (Fig. 5 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 (Fig. 6.). Here we show signals from CARS images derived from primary hepatocytes dosed with CeO$_2$, 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 (TiO$_2$ in this instance) within the hepatocyte cells (Fig. 7.), 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], TiO₂
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 5 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 pointspread-function of the imaging system exceeding the size of an individual nanoparticle [38]. This is best illustrated in Figure 6C 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 nanoparticles 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 sulphydryl 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 nanoparticles 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\(^{-1}\) \(\text{H}_2\text{O}_2\), 10 ng mL\(^{-1}\) and 500 ng mL\(^{-1}\) \(\text{AgNO}_3\). 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.

Funding

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Acknowledgements

We would like to thank Chris Pook for help with the GST assay, Anke Lange and Lisa Bickley for help with the hepatocyte isolations. All investigations were performed in accordance with the Animals (Scientific Procedures) Act, 1986 (UK).
Table 1. Size and charge characterization data for nano-sized and bulk particles suspended in HPLC-grad water and M199 cell culture medium.

Figure 1. TEM images of the of nano- and bulk particles used for this study 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.

Figure 2. LDH release from primary trout hepatocytes into the culture medium after 24h treatment with nanoparticles. Results are given as the mean and each carried out in triplicate. Data are means ± S.E.

Figure 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 (H$_2$O$_2$), Ag 10 nm, TiO$_2$ bulk and CeO$_2$ bulk (ANOVA, Tukey’s p=0.006).

Figure 4. 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. * - Significantly higher than control (ANOVA, Tukey’s p<0.001).

Figure 5. Hepatocytes imaged with MPM/CARS at different wavelengths. The epi-signal (ECARS) allows detection of small cellular features. Forward detection (F-CARS) is required for detail of larger cellular structures, such as the nucleus and cell membrane. All the frames in Figure 4 are the same image, but visualised differently. (A) E-CARS at 924 nm. (B) F-CARS at 924 nm. (C) E-CARS at 919 nm. (D) F-CARS at 919 nm.

Figure 6. 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 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 7. A 2-dimensional representation of a 3-dimensional z-stack showing a cluster of hepatocytes dosed with TiO$_2$ ENPs at 1000 ng L$^{-1}$. Frames are representative images of a 360º rotation, illustrating the source of the ENP signal as within the cell membrane in all three dimensions.
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Figure 1.
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Table 1.
Chapter 3

Tracing Engineered Nanoparticles in Biological Tissues using Coherent Anti-strokes Raman Scattering – A Critical Review
Tracing Engineered Nanoparticles in Biological Tissues using Coherent Anti-stokes Raman Scattering – A Critical Review

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Abstract

Manufactured nanoparticles (MNPs) are used in an extremely diverse range of products and increasingly entering into the environment driving a need to better understand their potential health effects in both humans and wildlife. Imaging methods applied for locating nanoparticles in exposed organisms enable more targeted health effects analyses. Current imaging methods used include electron microscopy, dark field single optical microscopy, and confocal microscopy, but each of these techniques is limited because they require either extensive processing of the material, staining, use of high intensity illumination that can lead to photo damage and/or have limited tissue penetration. Coherent anti-Stokes Raman scattering (CARS) microscopy is a label-free imaging technique that is capable of real-time, non-invasive imaging of living cells and organisms based on molecular vibrational spectroscopy and has recently been applied effectively for determining localisation and fate of nanoparticles in a wide variety of biological matrices, from individual cells to whole organisms. In this review we give an overview of the CARS process and describe the contrast mechanisms involved with imaging various nanomaterials. We then present a series of illustrative examples to demonstrate the application of CARS microscopy and critically discuss the advantages and limitations for its use in nanotoxicology. CARS was shown to provide excellent contrast for a variety of MNPs and demonstrated effective localisation of a wide range of MNPs into cells, tissues and even whole organisms. Advantages of this technique include imaging with near-infrared excitation wavelengths which minimises photo-damage and allows live sample imaging. Additionally the label-free nature of the technique requires no modification of MNPs that could alter the transport kinetics and cellular uptake of particles.
Introduction

The potential health effects associated with exposure to nanomaterials (NMs) is now of major international concern and with a huge expansion in nanomaterial applications (global sales were predicted to top € 450 billion in 2010; Grieger et al., 2010), precautionary approaches are being called for until the same desirable properties these materials offer to medicine and industrial applications are better defined in terms of their associated potential risks (Oberdorster, 2010). As a consequence, there has been a surge in funded research programmes internationally to investigate the potential hazards associated with exposure to NMs. Although exposure studies have been conducted in many organisms and model systems, most have been acute in nature and at exposure regimes that far exceed levels in present environments. Findings from these studies have been variable and often lack in any consensus for any one NM. For some NMs, however, there does appear to be causes for concern (e.g. Schrand et al., 2010) whereas for others, the predominant data would suggest there is no concern e.g. titanium dioxide (TiO₂, reviewed in Menard et al., 2011). It should be recognised, however, that there are extremely few studies that have considered chronic health effects and relatively few NMs have been thoroughly tested. It is also the case that many of the reported eco(toxicology) studies have provided limited information on the associated NM characteristics in the exposure system, and in turn it has been difficult to establish with any precision the nature of the exposure material, making comparisons across studies difficult. Amongst the plethora of emerging exposure data, even less attention has been direct towards establishing the target sites for NM in the exposed animals, and this is fundamental to advancing our understanding on what the (likely) associated health outcome(s) might be. An exception to this is for airborne exposures to selected NMs (e.g. carbon nanotubes) which were some of the earliest particles studied (Tantra and Cumpson, 2007) and have been shown to contribute to pulmonary diseases, inflammation, fibrosis, and biochemical/toxicological changes in the lungs inflammatory responses by novel mechanisms (reviewed in Lam et al., 2006).

Techniques applied for the quantification of NM uptake into biological systems have include inductively coupled plasma mass spectroscopy (ICP-MS) on tissues extracts after NM exposure (Ferry et al., 2009; Scown et al., 2009) and the use of radioactive labelling of particles to determine their presence and transport (Lu et al., 2010; Sumner et al., 2010). Although techniques such as these enable accurate quantification of NM distribution, the particles have been localised only to the tissue or organ. Direct imaging techniques offer powerful methods for visualising NMs within tissue, at cellular or even sub-cellular levels. There are few well-
defined techniques, however, for accurately imaging or characterising nanoparticles, especially within a biological matrix. Electron microscopy (EM) has sufficient resolution to determine nanoparticle localisation within a tissue (Soto et al., 2005; Cheng et al., 2007; Mouchet et al., 2008), and high resolution transmission electron microscopy (TEM) has capacity for structure identification of individual nanoparticles (Petri-Fink et al., 2008). Furthermore, EM techniques can be combined with energy-dispersive X-ray spectroscopy (EDX) analysis to identify the nature of any metal constituents of the NMs. However, samples for both TEM and Scanning Electron Microscopy (SEM) require preparation methods and imaging conditions which can create artefacts within the sample, such as NM aggregation, that do not have an exposure related aetiology. These techniques also do not allow for live imaging. Furthermore, TEM can generally only be used for metallic particles that are electron dense as non metal coatings or shells lacks adequate contrast (Fan et al., 2007). An exception to this is energy filtered transmission electron microscopy with electron energy loss spectrum imaging that has been used successfully to image single-walled carbon nanotubes (SWCNTs) in cells (Porter et al., 2007a; Porter et al., 2007b).

Dark-field single optical microscopy has been applied successfully to track uptake of silver NMs in real time into zebrafish embryos (Lee et al., 2007) and into nematodes (Roh et al., 2009) and this technique has nanometer resolution. However, the need for high intensity illumination can lead to photodamage, and the sample has to be extremely pure as contaminants can cause major interference. Furthermore, this technique has minimal tissue penetrance.

Confocal microscopy, a linear optical technique in which single photon interactions with a sample depend on incident light intensity, has been applied successfully to visualize the biodistribution multi-walled carbon nanotubes (MWCNTs) into zebrafish (Danio rerio) embryos (Cheng et al., 2009) and polystyrene nanoparticles into hepatocytes (Johnston et al., 2010c). The disadvantage of standard confocal microscopy, however, is the requirement to stain the sample to provide contrast or, fluorescently label the nanoparticles (as was done in the studies of Cheng et al., 2009 and Johnston et al., 2010c), which may change the nature of the particle (including their toxicity). Confocal techniques also lack spatial resolution (approximately 0.5 μm) and although they have sufficient depth penetration for studies on cultured cells, they are limited in depth penetration for most applications to whole tissues and in vivo imaging.

Some of the most recent techniques for visualising nanoparticles utilise the specific intrinsic properties of the nanoparticles themselves to assist visualisation. Examples of these properties include the plasma oscillations resonating within the electromagnetic spectrum for gold
nanoparticles (Murphy et al., 2008), and low activation energy from the valence to the conductance band and subsequent high quantum yield for quantum dots (Gonda et al., 2010; Jia et al., 2010). Of course, these methods are limited to specific types of nanoparticles with conductive properties.

A recent paper reviewing imaging techniques for studies on uptake and fate of engineered nanoparticles listed the following key criteria for imaging tools (Tantra and Knight, 2011): high selectivity (or contrast) for nanoparticles, high sensitivity for nanomaterials, the ability to image whole and sectioned cells, an ability to visualise cell structure, label free detection, to be non-invasive, yield complimentary chemical information, high accuracy, reproducibility and high throughput. In theory CARS offers all of these advantages. In this review we first briefly provide technical details to describe how CARS works, as this is essential in informing on the strengths and limitations of the method. We then explore CARS imaging of NM in a variety of biological tissues, spanning isolated cells to intact live organisms and discuss critically the applications, strengths and limitations of this technique applied to the field of nanotoxicology.

**CARS Microscopy Background**

Microscopy techniques based on vibrational spectroscopy offer intrinsic chemical selectivity, as different molecules have specific vibrational frequencies. Infrared microscopy has seen widespread development for biological applications (Rodriguez et al., 2006), but is limited by poor sensitivity due to water absorption and low spatial resolution associated with the long infrared wavelengths. Raman microspectroscopy has been extensively explored to map the chemical composition of biological samples and has found a broad range of biomedical applications (Evans and Xie, 2008) including, tumour diagnostics, DNA detection, bone mineral density and microendoscopy. Despite the wealth of chemical information that can be obtained from Raman scattered light, the technique suffers from a major limitation and this is that Raman scattering is extremely weak (typical photon conversions for Raman scattering are lower than 1 in $10^{18}$). Therefore, image acquisition times are long and often high laser power are required. These factors have severely limited the application of Raman microscopy to the study of living systems due to the combined factors causing photo-damage. Far stronger molecular vibrational signals can be obtained using coherent anti-Stokes Raman scattering (CARS), which was first demonstrated in 1965 by Maker and Terhune (1965) for analysing the molecular vibrational spectra of combustion gases. In 1999 developments in ultrafast infrared lasers revived CARS microscopy as a tool for biological imaging (Zumbusch et al., 1999).
CARS Theory

CARS microscopy derives its contrast from intrinsic molecular vibrations in a sample. A pump beam, of frequency \( \omega_p \) and a Stokes beam, \( \omega_s \), interact with the sample via a four-wave mixing process (Figure 1a). When the beat frequency \( \omega_p - \omega_s \) is tuned to match a Raman active vibrational mode, molecules are coherently driven with the excitation fields resulting in the generation of a strong anti-Stokes signal. CARS is generated via a four-wave mixing processes in which the signal intensity scales with the squared modulus of the third-order susceptibility at the anti-Stokes frequency. Third order nonlinear susceptibility, \( \chi^{(3)} \), can be expressed in the following general form (Cheng and Xie, 2004):

\[
\chi^{(3)} = \frac{A_R}{\Omega - (\omega_p - \omega_s) - i\Gamma_R} + \chi^{(3)}_{NR} + \frac{A_T}{\omega_T - 2\omega_p - 2\omega_s - i\Gamma_T}
\]

Where \( \Omega \) is the vibrational frequency of the Raman active vibrational mode; \( A_R \) and \( A_T \) are constants representing the Raman scattering and two-photon absorption cross-sections; \( \omega_T \) is the frequency of the electronic transition; and \( \Gamma_R \) and \( \Gamma_T \) are the widths of the Raman line and the two-photon electronic transition respectively.

Only the first term in equation 1 corresponds to the contribution to the FWM signal via the CARS process. The second and third terms correspond to non-resonant FWM related to the electronic structure of the sample and are spectrally indistinguishable from the CARS signal. These non-resonant components are independent of the frequency difference \( \omega_p - \omega_s \) and therefore do not provide any vibrational contrast and thus no chemical specificity. For most biological imaging applications investigators strive to minimize the non-resonant contributions, however, these second terms can be exploited to derive image contrast from sub-wavelength nanomaterials.

Term two corresponds to the non-resonant electronic contribution to the FWM signal derived the third-order electronic susceptibility of the sample. Local variations in this term leads to a non-chemically specific FWM image that is essentially a map of the electronic polarisability and density. This in its self offers a useful structural contrast. An effect of nanoscale materials is an enhanced nonlinear electronic susceptibility, therefore this term can be particularly useful for the location of nanomaterials.

Term three corresponds to two-photon enhanced electronic contribution to the FWM signal. Large non-linear susceptibilities result when the energy of the pump photons are matched to
resonantly excite electrons into an excited state via two-photon absorption. This term can often be ignored for biological samples but for certain materials provides a useful contrast mechanism. For example, many metal oxides are defined as wide bandgap semiconductors, with bandgaps in the optical region. This allows selective enhancement of (3) $\chi$ by tuning $\omega_p$ on or near the two-photon electronic resonance of the band-gap transition, and has been used to derive vast FWM signals from metal oxide nanoparticles.

Terms 2 and 3 can be used to generate strong FWM signals from particles that are over an order of magnitude smaller in size than the excitation wavelength. Moreover, the combination for FWM and CARS contrast offer an excellent solution for subcellular location of nanoparticles within biological structures deep within highly scattering tissues (see later).

Intrinsic Properties of Nanomaterials that facilitate CARS Imaging

The problems associated with non-resonant contribution from solvents in a sample, namely the generation of a background to the image, has instigated a number of methods to reduce this non-zero background signal. One of the most effective involves detecting the CARS signal in the backwards direction, or epi-detected CARS (E-CARS, Volkmer et al., 2001). This means that a small scatterer or imaging target (with an axial length less than the anti-Stokes wavelength, approximately 300nm) has a backwards signal that is comparable to the forwards signal (F-CARS) due to a lack of constructive and destructive interference (Figure 1b, Cheng et al., 2001). This means that small scatterers have a significantly higher contrast in the epi-direction than in the forwards direction. Conversely, only the larger cellular structures appear in the F-CARS images. When comparing the (identical) frames, the smaller scatterers present in the epi-generated image relate to black-holes or are not present at corresponding locations in the forwards-images, highlighting their complementary properties. An additional advantage of detecting two signals (F and E CARS) is the elimination of background noise present in the forward image that arises from the non-resonant electronic contribution to the CARS signal discussed earlier (see Scown et al, 2010 for illustrative example).

Various aspects and elements of the CARS system can be exploited to enhance different physical properties of NMs. Features of the CARS process can amplify various components of NMs that facilitated specific imaging of the material in question. Although some non-resonant contributions reduce image contrast some, however, are useful. Materials with naturally large non-resonant non-linear susceptibilities provide brilliant contrast using CARS spectroscopy due to their strong electronic resonances and generate both extremely strong F- and E-CARS
signals. This occurs for metal oxide NMs as demonstrated by Moger et al (2008). In addition to exhibiting markedly different chemical properties from their bulk materials, NMs can also exhibit enhanced optical properties that are often accompanied by an enhancement of the nonlinear optical properties (Wang et al., 2011). Moger et al (2008) showed that this enhancement can be exploited in metal oxide NMs to produce large FWM signals. Due to metal oxides being wide band-gap semiconductors, the combination of innate large two photon resonance and the ability to enhance their third order susceptibilities allowed the authors excellent visualisation of metal oxide NMs. Thus the heightened nonlinear response of some NMs allows the location of particles far smaller than the linear scaling of the signal from bulk material would suggest which aids their visualisation without any need for modification to the sample. Using zinc oxide as an example for a typical metal oxide response, here the F-CARS (Figure 2a) and E-CARS (Figure 2b) images show that the non-resonant susceptibilities of the metal oxide NMs are enhanced in the epi-direction, providing a stronger signal without the background interference present in the forwards image.

Quantum size effects of NMs are also suggested to contribute to an enhanced third-order response as well. Silicon, for example, has third order nonlinear susceptibilities that are among the highest known for solid state materials (Wynne, 1969) and the properties of nano silicon have been highlighted for their potential in imaging applications (Park et al., 2009). Further strong third-order responses have also been established for quantum dots (QDs, Jain and Lind, 1983). Some materials, such as gold, do not have any Raman active bands and the FWM signal cannot be enhanced by vibrational resonance. However, the optical properties of several metals, including gold, are dictated by the presence of surface plasmons. Therefore, as exemplified by gold nanorods, FWM occurs strongly when the pump laser wavelength is tuned to be resonant with the longitudinal plasmon resonance wavelength (Jung et al., 2009, Figure 3a), here the coherent FWM signal arises from a plasmon-induced enhancement of gold’s third order nonlinear susceptibility. This leads to an intense signal that can be readily isolated from any resonant CARS signals in the surrounding medium by tuning the pump and Stokes away from the resonant wavelengths. This is a property which makes gold very attractive as contrast agents in live biological samples (Garrett et al., 2011).

Polymer NMs have had wide research effort into their biomedical applications. Unlike other optically unique NMs such as gold, polymer NMs are often fabricated for drug delivery purposes as opposed to contrast or targeting agents. Precise imaging of the NM location is highly critical for assessment of drug delivery success. Some polymeric NMs, depending on their construction, are typically difficult to separate from natural lipid sources within a
biological matrix, however, other polymers may have properties that CARS can tune in to. Poly(lactic-co-glycolic acid) (PLGA) particles, for example, can be chemically distinguished using CARS by tuning into CH$_3$ bonds at 2940 cm$^{-1}$ away from the CH$_2$ vibrational mode of 2840 cm$^{-1}$ (Xu et al., 2008, Figure 3b). Polystyrene nanoparticles can also be distinguished at 3045 cm$^{-1}$, which corresponds to their aromatic C-H stretching vibration (Tong et al., 2007). Additionally at the nano scale, biological molecules such as DNA have also been visualised with CARS, the resonant frequency of 1337 cm$^{-1}$ can be assigned to the ring-breathing mode of a diazole adenine molecule in the DNA (Ichimura et al., 2004).

Other particles that give a strong FWM include carbon nanotubes, which have been imaged using CARS (Lefrant et al., 2008). FWM is enhanced by one-photon resonance and is thus a sensitive probe for the nonlinear electronic response of nanoscale objects. Strong signals are expected from SWNTs which will generate FWM when the excitation beams are resonant with electronic transitions of the nanotube (Kim et al., 2009). In contrast to SWNTs, the optical nonlinearity of graphene does not show resonant behaviour as a function of excitation wavelength, but exhibits a very strong optical nonlinearity from the interband electron transitions in the near-infrared spectral region (Hendry et al., 2010).

One of the restrictions on CARS is that its quantitative capabilities are limited with less abundant scatterers. Therefore visualising small targets such as NMs with CARS provides a great challenge to its applicability. Despite the fact that most of the NMs imaged from environmental samples are aggregates, it is still 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 (Rodriguez et al., 2006). This leads to variation in intensity of the signal from particles appearing to be of similar size. This complication could be alleviated if it were possible to image a single nanoparticle with CARS and subsequently record its brightness; however, this is obviously not without difficulties.

In summary of this section, the various technical parameters that are considered when imaging with CARS, can be used to exploit different susceptibilities in a broad range of particle types, including, polymer, carbon and metallic NMs. These unique imaging properties of NMs that can be enhanced by the CARS process consequently provide excellent contrast and visualisation targets for localisation within a biological matrix.
CARS Microscopy of Living Cells

There are a wide range of techniques and methods available for preparing cells for visualising cellular and sub-cellular features that range from staining to isotopic labelling. However, these methods have several drawbacks; from being relatively unstable to being cytotoxic to some degree. CARS utilises the intrinsic properties of a sample to derive contrast and generate a signal, which provides more information than the transmitted light image would, due to the wavelength determined contrast of the bonds present. This chemical selectivity has been demonstrated with the excitation wavelength tuned to phosphate groups, C-O bonds of DNA, C=C and C-H bonds, and amide bonds (Hashimoto et al., 2000; Volkmer et al., 2001; Cheng et al., 2002a; Cheng et al., 2002b). This enables the imaging of different structures within a cell or cellular component such as the cytoplasm or nucleus removing the need for any non-native contrast agent or pre-processing of the sample which could interfere with any of the endogenous biological processes of interest. This advantage of CARS is epitomised in Figure 4a where the predominant cellular features of an embryonic fibroblast cell are immediately discernable when the system is tuned to excite C-H bonds. An important point to note when imaging particular molecular species is that similar functional groups appear in different biomolecules, so despite the chemical selectivity of CARS care must still be taken when interpreting the image.

The cellular resolution capabilities that CARS has demonstrated enables the isolation and imaging of single cells from a culture and discern some of the sub-cellular features. This also facilitates examination for cell uniformity (within a culture) and morphological aberrations. Cellular scale imaging with CARS, however, is not limited to cell cultures. Figure 4b displays a close up image of the gill arch from a rainbow trout (Oncorhynchus mykiss) and reveals the resolving ability of the CARS technique to characterise structural and cellular differences within a tissue sample; in this instance the blood cells circulating the tissue are visualised in a false blue colour. However, whilst the CARS technique has demonstrated its capabilities in imaging single cells, the spatial resolution within a relatively thick sample means organelles smaller than the diffraction limit of the system cannot be resolved. The spatial resolution in the EX plane is 200 nm which prevents imaging of the majority of organelles within a cell, reducing the detail of the image.

A significant limitation of CARS is the generation of a non-resonant background signal (e.g. Figure 5). Arising from electronic contributions to the third order susceptibility from any surrounding solvent or objects. These non-resonant contributions to the signal are not due to
stimulated resonance of a specific chemical bond. This background can reduce contrast from an image and can complicate spectral assignment. In some cases this limits detection sensitivity, especially for the less abundant molecules targeted, due to the quadratic dependence on their concentration (equation 1).

Despite the limitations of resolution and non-resonant contributions, further advantages of CARS provides strong motivation for non-invasive and chemically selective microscopic techniques. These include the imaging of live or delicate structures (Figure 4) where ongoing biological processes and structural integrity are preserved. The predominant feature of CARS that enables this is the use of infrared excitation and the fact that the CARS radiations produce large and directional signals which reduces the average excitation power needed and consequently causes less photodamage to cells. The wavelengths utilised by CARS are also less phototoxic owing to the lack of significant endogenous absorbers in most tissues (Svoboda and Block, 1994). This minimises the risk of photobleaching, common in fluorescence based microscopy, which could affect the viability of live cells or irreversibly damage fresh tissue samples. The near infrared wavelengths also give CARS an increased depth penetration over conventional optical microscopy (Helmchen and Denk, 2005). These factors contribute to a significant advantage of CARS when imaging, especially for live or delicate structures where the non-destructive technique, and lack of necessity to section a sample, allow highly focused and detailed yet sympathetic visualisation of the relevant cells or tissue.

**CARS imaging of Nanomaterials within Live *in vitro* Systems**

The kinetics of NMs, due to their varied properties, differ not only from their bulk counterparts but also within their various functionalisations, elemental constitutions and capping agents, vastly increasing the multiplicity of their interactions within the environment. As such, to visually ascertain the localisation, mode of uptake and fate of NMs within a sample potentially provides a great detail of information that is currently lacking or difficult to attain from the current literature (Tantra and Knight, 2011).

Precise localisation of NMs within a biological matrix places numerous demands on the imaging technique. This includes having sufficient spatial resolution to locate particles within a cell, combined with a 3-dimensional sectioning capability (which CARS microscopy possesses, see later section) and finally an imaging modality that can detect and derive sufficient contrast from both the biological matrix and the NMs, which are highly varied targets for imaging. Important in this respect is the accuracy of CARS imaging compared to tracing NM localisation
using fluorescent tags or markers. This is because CARS directly images the particle itself and not just the visual label, therefore precise localisation of a NM can be established. This is emphasised by the false positive results that can occur when using dye markers with particles. In a study by Xu et al (2008) a lipophilic dye was shown to be released from nanoparticles into the surrounding extracellular medium before being taken up by cells or being transferred onto the membrane after direct contact, in this instance confocal microscopy and data from a fluorescence activated cell sorter (FACS) produced data that suggested nanoparticle uptake, yet CARS assessment showed the absence of particles in the cells and therefore the fluorescence observation was not indicative of nanoparticle uptake.

The FWM process of CARS is particularly pertinent when imaging within a biological matrix, where the non-resonant susceptibilities of NMs can be used to avoid confusion with stimulated vibrational contrast from targeted molecules such as lipids. This is because the C-H bonds will not produce non-resonant contributions towards the image (Akimov et al., 2009). In combination with the optical properties of NMs, the ability to tune the excitation wavelength away from the natural vibrational resonance of a sample, for example by presenting the fluorescing C-H bonds as negatively contrasted, allows affirmation of the NMs which produce a strong signal across a broad wavelength spectrum. Using CARS we have already shown the uptake of various NMs into primary cultured trout hepatocytes (Scown et al., 2010). NM identification was established by tuning the excitation wavelength off resonance for C-H bonds which confirmed the remaining signals as NMs. The CARS NM signals in the images were determined on the same visual plane as the cell nucleus giving clear indication of their subcellular location. Figure 8 highlights the utility of imaging at two different Raman vibrational frequencies to determine nanosized cerium dioxide (CeO₂) particles presence in primary isolated rainbow trout hepatocytes. Imaging at the C-H exciting wavelength of 2840 cm⁻¹ (Figure 6a, b) lipid dense structures are present in both forwards and backwards directions as white spots. When imaging at a wavelength of 2955 cm⁻¹ (Figure 6c, d) this negatively contrasts C-H bonds which appear black spots in the forwards direction or not at all in the backwards direction. This facilitates the ability to distinguish between endogenous scatterers such as lipids and NMs where the strong excitation signal detected in the system is generated across both wavelengths. These images can be merged in false colour to highlight the different signals and crucially identify NM presence (Figure 6e); the yellow colouration represents the signal obtained from excited C-H bonds in lipid dense structures, the green depicts the cellular structure and the blue is generated from strong E-CARS signal of the broad spectrum NMs. This technique facilitates accurate interpretation of the CARS image and removes the possibility of confusion between native and non-native scatterers within the
sample. This technique can be applied for the identification of any NM within the system, although, particles that have specific properties which are exploited for imaging, such as Au’s plasmon resonance, will only be visible at their particular stimulated wavelength and as such possessing a distinctive signal which enables their identification.

**CARS Microscopy of Nanomaterials in Tissues and Live Organisms**

CARS is able to delineate cellular structure within a fragile sample as demonstrated by Moger and colleagues (2008) and Johnston *et al.* (2010a) to relate the position of TiO$_2$ NMs on the gills of exposed rainbow trout (Figure 7a). Figures 7b and c further exemplify the *ex-vivo* imaging capabilities of CARS for fresh tissue samples, subsequent to a nanoparticle challenge. Rainbow trout kidney tissue (Figure 7b), dissected after an injection trial with TiO$_2$ NM, provided a clear demonstration of particle presence in the cellular environment, which then allowed targeted transmission electron microscopy (TEM) of the tissue with the knowledge that TiO$_2$ was present. Particles were subsequently shown to accumulate in the kidney by TEM (Scown *et al*., 2009). Galloway *et al* (2010) confirmed TiO$_2$ nanoparticle uptake into *Arenicola marina* following observation of CARS images (Figure 7c). CARS images of the target tissues showed particle aggregates in the gut lumen and allowed subsequent biological measurements to be interpreted with the knowledge and confirmation of NM presence. Figure 7d shows polymer drug delivery particles in a mouse brain slice after intravenous injection (Garret *et al*., in press). Visualising the distribution of the particle within surrounding cellular structures allows for a greater understanding of nano-drug delivery potential, with great potential benefits for measuring the distribution distance achieved of drug delivery systems from a blood vessels using CARS. The critical aspect of the CARS system that allows imaging deep into scattering tissue are the excitation wavelengths being near infra-red, this allows low scattering and thus increased penetration deep, up to several hundred microns, into the sample. Its increased depth penetration compared to standard fluorescence and confocal microscopy techniques, allows localisation and fate data to be derived from intact tissues not normally possible, highlighting the utility of the CARS method. Importantly, from these examples, further and more directed studies can be made.

*In vivo* systems would normally be extremely difficult to image live and determining uptake or biodistribution after an exposure to NMs a very technically challenging process. The non-invasive CARS system allows imaging of live samples, not just at a cellular levels as depicted by previous figures, but also at the organism level (Figure 8). The fact that the sample is still live means the biological processes that interact with or are stimulated by the presence of NMs are
still functioning. This makes the real-time image a perfect snap-shot of the true functionality and behaviour of the NMs in situ. Figure 8a illustrates this well, with an image of a zebrafish larvae. Widely used as a model species in toxicology and developmental research, the imaging of a live zebrafish embryo or early stage larvae with all the advantages that CARS provides, presents great opportunity for nanotoxicology studies. In this instance the distribution of microinjected silver nanoparticles into the 1 cell stage are ascertained 24 hours post fertilisation (hpf). Live sample visualisation offers many benefits when assessing localisation of NMs after exposure, including cellular partitioning and excretion routes. Figure 8b portrays zinc oxide nanoparticles uptaken and retained in the gut of a daphnid. This image permitted viable assessment of further propagation of these NMs from the gut into other tissues (Fabrega et al., unpublished data). Thus, CARS presents superior imaging capabilities where fluorescence-based imaging techniques are limited or not applicable due to their scattering, e.g. on very superficial regions or a lack of depth penetration for in vivo systems.

The envisaged incident of NMs being bioavailable to many organisms presents many potential pathways of uptake after exposure due to their diverse nature. Critical to this is the understanding and tracing of NM uptake within different systems. As one recent review on NM hazard assessment concluded, the fate and behaviour of NMs that modulate their environmental impact is the “one black box (that) remains to be opened and understood” (Kahru and Dubourguier, 2010). The versatility of CARS allows this to be assessed both in vitro and in vivo with precision.

Precise localisation of NMs using CARS allows a more detailed insight into particle dynamics within a system and thus allows for extrapolation regarding toxicokinetics and subsequent target cellular structures or tissues. This may vary between NM types depending particle characteristics, exposure route or specific functionalisations. Direct imaging is an excellent method to distinguish individual NM type effects on partitioning and cellular/tissue targets. Furthermore, to achieve this in situ facilitates even greater understanding of real-time NM kinetics. The ability of the CARS technique to achieve this is highlighted in Figure 9 where microinjected 5nm CeO$_2$ NMs into the 1 cell stage of zebrafish embryos associate with the chorion (Figure 9a) and are not seen to be retained in the embryo at 3.5 hpf. This is not the case however for 7nm silver particles (Figure 9b), which are seen to remain internalised in the embryo structure and integrated throughout dividing cells at 6 hpf.

Further accurate particle localisation within a live sample is achievable with a different CARS technique. For the previously mentioned hepatocyte exposures, the ultimate clarification of
internalisation of the NMs, copper in this instance, came in taking a series of 2-dimensional images through the x–y plane, each separated by 0.25 μm in the z-direction. By creating this ‘z-stack’ the origin of the NM signal was found to be centred within the boundaries of the cell. Subsequently combining the slices, it was possible to produce a 3-dimensional interpretation of the image to illustrate this (Figure 10a). This method to ascertain internalisation is not restricted to single cells. Moger and colleagues (2008) used this technique with gill tissue and Fabrega-Climent and colleagues (unpublished data) showed the retention of zinc oxide bulk sized particles within the gut of a corophium determined by a 3-D reconstruction of the gut and hepatopancreas (Figure 10b). As with the earlier presented images of NM uptake into Daphnia (Figure 8b), localisation provides excellent knowledge of fate and behaviour within the organism itself, with the addition of a further dimension to create a volumetric attestation of NM distribution and so eliminate any uncertainty of uptake and inform on further biodistribution of particles.

Future applications

Future development of the CARS systems is constantly on-going. Since the re-emergence of CARS over 10 years ago non-linear label-free imaging based upon Raman scattering has since been improved upon, with the development of stimulated Raman scattering (SRS). This technique eliminates non-resonant background, Raman shift does not match any vibrational resonance, there is no generated signal (Freudiger et al., 2008). This is in contrast to CARS, which exhibits a strong spectral background independent of the Raman shift. However, as previously discussed, it is precisely this non-resonant spectral background of CARS that provides the contrast for imaging metal NMs. An ideal scenario would be forwards detection of SRS with backwards detection of E-CARS. Further applications of CARS beyond NM uptake assessment are especially exciting in the area of nanotherapy and drug delivery techniques that utilise the nano-sized properties of the NMs to improve efficacy, safety and targeting (reviewed in Caldorera-Moore et al., 2010). CARS provides a system that would also be able to effectively assess the performance of drug delivery particles in terms of uptake and distribution throughout a tissue. CARS also has the potential to assess markers of toxicity, with the ability to detect chemical changes as a result of NM exposure. These might include some common toxicological endpoints measured subsequent to a NM challenge, such as changes in protein concentrations in oxidative stress reactions or as by-product of free radical generation.

Conclusions
In summary we have shown the CARS technique to be advantageous over other conventional microscopies and detection methods for the following reasons:

- CARS provides contrast based on the intrinsic molecular vibrations of a specimen, circumventing the need for chemical perturbation by exogenous labels.
- CARS uses near infra-red excitation wavelengths which allow microscopy at depths of several hundred microns into intact tissues.
- The near infra-red excitation beams minimise photo-damage to live and delicate samples.
- The label-free nature of the technique eliminates the potential perturbation seen when using fluorescent labelling of NMs, which can modify their transport kinetics and bioavailability.

Every imaging technique has its strengths and limitations, and although the above examples highlight the uses of the CARS setup, there are a number of limiting factors in its usage which the authors have tried to outline throughout the review to the best of their ability. These include: limited spatial resolution, a lack of quantitative capabilities and a non-resonant background to the images and these shortfalls may restrict the application of CARS in a number of circumstances.

In this review we have presented localisation data for a range of NM types for a variety of biologically relevant samples widely used in nanotoxicology. We have demonstrated the advantages of CARS as well as the gap it can fill in the current deficit of suitable imaging systems. These advantages can be adopted for a range of research questions from the cellular to organism level and for samples of high uptake and toxicological relevance. CARS, therefore, can potentially be an extremely useful tool in providing desperately needed information on the fate and behaviour or NMs both in the environment and within susceptible organisms and their tissues.
Figure legends.

**Figure 1.** Schematic depicting the four wave mixing process of CARS (a). Utilising a pump beam, of frequency $\omega_p$ and a Stokes beam, $\omega_s$, when the beat frequency ($\omega_p - \omega_s$) is tuned to match that of a Raman active vibrational mode, $\Omega$, this generates a strong anti-Stokes signal at $\omega_{AS} = 2\omega_p - \omega_s$. (b) Diagrammatic representation for generated signal size and direction from CARS signals of different sized particles.

**Figure 2.** The F-CARS (a) and E-CARS (b) images of zinc oxide nanoparticles, here the non-resonant susceptibilities of the nanomaterials are enhanced in the epi direction, providing a stronger signal without the presence of background interference. Reproduced from Moger et al (2008). Scale bars represent 50 µm.

**Figure 3.** Energy schematic of FWM from gold nanoparticles (a) when the plasmon resonance wavelength matches the pump ($\omega_p$), Stokes ($\omega_s$), or anti-Stokes wavelength ($\omega_{AS}$), FWM is expected to be enhanced, reproduced from Jung et al (2009). (b) CARS spectra of PLGA and lipid bodies where the peaks at 2940 cm$^{-1}$ and 2840 cm$^{-1}$ arise from CH$_3$ stretching in PLGA and CH$_2$ stretching in lipid bodies, respectively. Reproduced from Xu et al (2008).

**Figure 4.** (a) F-CARS image taken at 2,845 cm$^{-1}$ of 3T3-L1 (mouse embryonic fibroblast) cells undergoing differentiation. Here the image is taken in the z-plane that runs through the centre of the cell as the nucleus is in focus (arrowed), reproduced from Nan et al (2003); scale bar represents 15 µm. (b) A close up off a gill arch excised from a rainbow trout reveals the resolving ability of the CARS technique to deliniate structural differences within a tissue sample, here the blood cells (indicated by arrows) circulating the tissue are visualised in a blue false colour to differentiate from the surrounding tissue. Reproduced from Moger et al (2009), scale bar represents 50 µm.

**Figure 5.** A431 carcinoma cells. (a) showing positive resonant contrast and (b) without resonance. Only lipid droplets show up as resonant contrast and thus a stronger signal in plate (a), however, the additional non-resonant background present, significantly reduces the contrast available for detailed imaging. Scale bar represents 50 µm.

**Figure 6.** Rainbow trout hepatocytes after exposure to CeO$_2$ NMs. Imaging at the C-H exciting wavelength of 2840 cm$^{-1}$ (a, b) lipid dense structures are present in both forwards (a) and backwards (b) directions as bright white spots (i). The same images at a wavelength of 2955
cm$^{-1}$ (c, d) negatively contrasts C-H bonds which appear black spots in the forwards direction (c, ii) or not at all in the backwards direction (d). Thus the bright spots that are still generating a strong signal at both wavelengths are NM (iii). These data can be combined to present an image where the lipid fluorescent and the signal from the NMs are distinguished (e), here lipids are presented in yellow and CeO$_2$ NM as blue. Additionally uptake of NMs is ascertained by the CARS signal being present in the same imaging plane as cellular structures such as the nucleus. Scale bar represents 20 µm.

**Figure 7.** CARS images of (a) the gill arch of a rainbow trout, (c) rainbow trout kidney tissue and (c) gut of *Arenicola marina* each showing the presence of titanium dioxide nanoparticles (indicated by arrows), contrasted white in (a) and light blue in (b, c), associated with the tissue. Scale bars represent 100 µm. (d) A composite of epi-CARS images of mouse brain cortex after intravenous dosing with a drug delivery polymer nanoparticle, imaged at different vibrational bands to gain contrast from the particles (yellow, indicated by arrows) and then the surrounding tissue to illustrate the polymer particles against the brain structures, such as the capillaries. Scale bar represents 10 µm.

**Figure 8.** Demonstration of CARS imaging capacity for whole organisms, depicted here by a zebrafish embryo 24 hpf (a) injected with Ag nanoparticles at the 1 cell stage. Here the green components of the image are the Ag NMs dispersed throughout the organism; with the eye (i), ear (ii) and yolk sac (iii) apparent. Live sample visualisation offers many benefits to the user when assessing localisation and biodistribution of NMs post exposure, (b) zinc oxide NMs, here depicted in red (arrowed), are shown to be retained in the gut of a daphnia. Scale bars represent 100 µm.

**Figure 9.** Zebrafish embryos microinjected at the 1 cell stage with (a) CeO$_2$ nanoparticles (i, contrasted blue), imaged 3.5 hpf and (b) silver nanoparticles (i, red) imaged 6 hpf. Both images are visualised at 2840 cm$^{-1}$ the C-H excitation frequency. (ii) Indicates the mass of dividing cells and (iii) the yolk sac. Scale bars represent 100 µm.

**Figure 10.** Z-stacks of both cells and whole organisms can be reconstructed to provide 3 dimensional analysis of a sample. An extremely useful method of establishing nanoparticle localisation and biodistribution within a complex sample, here (a), the boundaries of hepatocyte cells contrasted in green (i) with Cu NMs imaged in orange (ii). Zinc oxide bulk particles provide the strong contrast in white and are shown not partition into the hepatopancreas (i) surround the gut (ii) in a *corophium* (b). Scale bar represents 100 µm.
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medium does not prevent uptake and toxicity toward a rainbow trout gill cell line. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology* **153A**, S87-S88.


Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 8.
Figure 9.
Figure 10.
Chapter 4

Natural organic matter affects bioavailability of cerium oxide nanomaterials to fish

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Natural organic matter affects bioavailability of cerium oxide nanomaterials to fish

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ABSTRACT.

In the aquatic environment natural organic colloids affect the fate and behaviour of nanoparticles. Nothing is known about how these interactions affect the bioavailability of nanoparticles to exposed organisms, which is a fundamental knowledge gap in risk assessment analysis. In this study we investigated interactions of cerium dioxide (CeO$_2$) nanoparticles with fulvic acids, representing natural organic matter (NOM), and their bioavailability to fish for exposure via the water over 28 days. Our main and novel finding was an enhanced uptake of cerium into body tissues (gill, kidney, brain) of fish in the presence of fulvic acids. For exposure to 50 μg CeO$_2$ /l there was a 2.5-fold and 6-fold higher Ce content in the gills with the addition of 50 μg and 250 μg fulvic acid /l, respectively, and in the brain a 5.5-fold higher Ce content with the addition of 250 μg fulvic acid /l. There was a trend for a reduced CeO$_2$ aggregate size and a more stable suspension with the citrate coating and the highest fulvic acid concentration as determined via dynamic light scattering (DLS) and zeta potential particle characterisations. Transmission electron microscopy (TEM) analyses however, did not identify differences in size measurements of the CeO$_2$ particles in the exposure water between the treatments. These findings indicate that the presence of NOM effects the fate, behavior of CeO$_2$ particles in water to fish and highlights the importance of considering the conditions in natural waters for understanding bioavailability, and thus potential biological effects, of nanomaterials to aquatic organisms.

KEYWORDS: cerium oxide, nanoparticles, fulvic acid, uptake, nanotoxicology
The properties of nano form materials that are manipulated and exploited widely for commercial use, can also potentially give rise to adverse health effects and there is now concern for exposures in both humans and wildlife. Most nano materials, as with almost all other contaminants discharged as a consequence of anthropogenic activities, will find their way into water courses and the exposure of aquatic organisms must be considered in order to evaluate potential environmental impacts. Metal and metal oxide manufactured nanoparticles (MNPs) are now some of the most widespread nanomaterials in use, with applications spanning antimicrobials (silver (Ag), reviewed in Ref.) to fuel additives (cerium dioxide (CeO₂), reviewed in Ref.). Modelling studies have predicted environmental concentrations for some metal and metal oxide MNPs, notably titanium dioxide (TiO₂) and Ag close to, or above, that of the proposed no effect concentrations. Empirical data on environmental concentrations of MNPs are lacking, but TiO₂ particles in run-off derived from a newly painted (experimental) façades have been measured at concentrations up to 600 μg/l. Wastewater treatment works (WWTWs) are anticipated to receive significant amounts of MNPs and although much of this is expected to precipitate into sludge, inevitably some will enter freshwaters through WWTW effluent discharges, as has been reported for C₆₀ and C₇₀ fullerenes measured in the parts per billion (ppb) range. Further to this, a Ag spiked system demonstrated that about 5% of the Ag-NP passed through the treatment works and into the effluent discharge. An additional, and in some case very significant, source of MNPs to the aquatic environment is through their use for remediation purposes. For example, the US Environmental protection agency (EPA) funds programmes applying MNPs to combat problems associated with organic pollutants that is worth almost $1 billion (reviewed in Refs.).

CeO₂ has been prioritised for study by the Organisation for Economic Co-operation and Development (OECD) due to its extensive commercial use in applications
including computer chips, solar cells, polishing agents, precision optics, gas sensors, UV blocking agent for cosmetics and as an additive in cigarettes, capitalizing on its high oxygen storage capacity, low redox and UV absorbing potential \(^5, 16\). However, a more immediate environmental concern comes from its use as a catalyst in the automotive industry and as a fuel additive. The environmental toxicity and human health effects of CeO\(_2\) MNPs, however, are still uncertain \(^5, 17-19\).

Particle size has been reported to affect toxicity \(^20-23\) but there is a lack of consistency in this regard in the literature for CeO\(_2\) and indeed for metal oxide MNPs more generally. For CeO\(_2\) MNPs, reports on their toxicity span those which indicate they can be far more toxic than other metal or metal oxide particles \(^24\), to those that show little if no toxicity at all \(^25\). Some studies have indicated that CeO\(_2\) nanoparticles can actually provide cellular protection against free radical species, mitigating oxidative stress challenges \(^26\).

Some of the resulting uncertainty for CeO\(_2\) MNP toxicity relates to the lack of knowledge on their bioavailability and the form of the material available to the exposed organism (or cell system). The variable dynamics in the fate and behaviour of MNPs in natural systems that affect their form, function and bioavailability, creates an urgent need for more \textit{in vivo} studies to better identify target tissues and direct more intelligent approaches in toxicity testing \(^1\). This includes more environmentally realistic exposure scenarios than, for the most part, those reported in the literature. A recent study attempting to mimic a real world scenario for exposure to CeO\(_2\) \(^27\) (using radioactive CeO\(_3\)) in a simulated ecosystem, hypothesized that the predominant location for the ceria particles was at the water-sediment interface and demonstrated uptake into a number of aquatic species.

There has been a great deal of attention given recently to the role natural organic matter (NOM) in the aggregation and stability of MNP suspensions. NOM is a
ubiquitous substance in the environment predominantly made up from a composition of humic acids and fulvic acids with a hydrophilic fraction. With aggregation and sedimentation known to have a large influence on the fate and transport of particulate matter, including MNPs \(^{28}\), it is hypothesized that NOM and its ability to chelate metals will affect the colloidal stability and subsequent bioavailability of MNPs to aquatic organisms. This aggregation state is also an important consideration for the impact on MNP reactivity \(^{29}\) and potential toxicity \(^{30-32}\). Although MNP suspension stability relies on a multitude of factors such as pH, ionic strength and composition, various studies give evidence of increased stability \(^{33-35}\) and decreasing aggregation \(^{36, 37}\) of MNP suspensions upon addition of NOM. Von der Kammer and colleagues \(^{33}\) suggested the availability of organic molecules adhering to the metal oxide particles can provide a barrier to aggregation, and they displayed a very low sedimentation rate for TiO\(_2\), ZnO and CeO\(_2\) nanoparticles at high (mg/l) concentrations. Other studies suggest NOM adsorption can also decrease the zeta potential of nano-oxide suspensions and increase the electrostatic repulsion between particles with its adsorption to the surface area indicating that NOM-coated nano-oxides could be more easily dispersed and suspended \(^{34, 38}\). Quik and colleagues \(^{39}\) found that increasing the concentration of NOM increased the amount of CeO\(_2\) particles remaining in suspension and the addition of NOM reduced the zeta potential sufficiently to reduce the occurrence of aggregation. Most laboratory experiments assessing the toxicity of MNPs in aquatic environments have not considered the presence of NOM in their testing regimes, which could have fundamental implications for effects assessments.

In this study we examined the behaviour and subsequent uptake potential and tissue partitioning of citrate coated CeO\(_2\) MNPs (nominally, 20-30nm in size) in common carp (\textit{Cyprinus carpio}) for exposures via the water in the presence of fulvic acid over a period of 28 days. Citrate has been widely used as a dispersant for MNP preparations \(^{40}\),
including for highly characterised particles used in toxicity testing and was applied to the particles to aid their dispersion. Fulvic acid was used as a standardised NOM to produce exposure conditions for the fish more relevant to natural waters. We show for the first time that the addition of natural organic matter by the way of fulvic acids can increase the bioavailability of MNPs to an exposed aquatic organism.

RESULTS AND DISCUSSION

It is widely accepted that some MNPs have the potential to induce biological effects and robust testing of MNPs is vital to ensure the sustainable development of this very large and expanding industry. Knowledge on the toxicity of MNPs, however, is still very limited, especially for environmentally realistic exposure scenarios. This is due, in part, to lack of data accurately establishing the behaviour and fate of particles and a paucity of information on biological effects of chronic low levels exposures with environmental realism. Here we investigated the fate and behavior of nano and bulk CeO$_2$ particles in simulated natural water and show enhanced bioavailability to fish of CeO$_2$ particles in the presence of fulvic acids, with fundamental implications for the risk assessment process for MNPs in the aquatic environment.

Ce levels in exposure medium

Measured water concentrations, determined by inductively coupled plasma mass spectrometry (ICP-MS), for the CeO$_2$ particles over the duration of the experiment are shown in Figure 1. Measured levels of Ce in the different treatments were higher than the nominals. In the 5 $\mu$g/l treatment the mean concentration was $20.45 \pm 0.2$ $\mu$g/l and in the 50 $\mu$g CeO$_2$ /l treatment, although close to nominal for the first 72 h, they were subsequently also 3 to 4 fold higher than nominals (mean measured, $173.48 \pm 9.4$ $\mu$g/l). All water samples for ICP-MS analysis were collected from the middle region of the
tank and it is possible that an uneven distribution of particles accounted for the differences in the measured vs nominal concentrations for the nanomaterial. In our studies (in bare tanks) we found no major sedimentation of Ce in the nanoparticle exposures, as has been reported for a more complex exposure system that simulated an ecosystem with aquatic plants, water snails, fish and sediment \(^{27}\), which would indicate that the citrate coating (and/or presence of fulvics) provided some stabilization of the CeO\(_2\) nanoparticles to help maintain them in the aqueous phase. The activity and motion of the fish in the tanks may also have served to help maintain particulates in the aqueous phase in the exposure tanks. Quik and colleagues \(^{39}\) also described a decrease in sedimentation of CeO\(_2\) nanoparticles with increasing NOM in an algae growth medium.

Our study shows that despite variation in the measured versus nominal levels, the semi-static exposure dosing method provided an effective and relatively stable exposure system for delivery of citrate coated CeO\(_2\) nanoparticles in the aqueous phase to fish, over a period of 28 days (Figure 1).

In contrast with the nanomaterial treatments in the bulk ceria treatment there was a progressive decline in the exposure material in the water column. The measured concentration of Ce in the bulk treatment (nominal 5 \(\mu\)g/l) was in the region of 23.7 \(\mu\)g/l at its highest level in the initial phase of the study, dropping to only 0.22 \(\mu\)g/l at the end of the study. These data presented illustrate the importance of measuring dosing levels of exposed materials in the aqueous phase throughout exposures to enable relevant comparisons on effects analysis to be drawn between the materials that clearly have very different fate and behaviour dynamics.

**Bioavailability of CeO\(_2\)**

The uptake of ceria into fish tissues, determined via ICP-MS, showed that the CeO\(_2\) MNPs were bioavailable to exposed fish (Figure 2). Concentrations of Ce in the gill, brain and kidney tissues, demonstrated significantly enhanced uptake of Ce with the
addition of fulvic acids, compared to treatments without fulvic acid (gill tissue; $F_{2,3} = 23.706$, $p = 0.015$, kidney tissue; $F_{2,3} = 83.801$, $p = 0.002$, brain tissue; $F_{2,3} = 36.29$, $p = 0.008$, Figure 2a,b,c). Ce levels in the blood were below the threshold for accurate measurement (data not shown). In the kidney and brain tissue, Ce levels for bulk material exposures were lower compared with the corresponding concentrations of nano and fulvic acid (5 μg CeO$_2$ / l and 50 μg fulvics / l). For the gill tissue there were no apparent differences in Ce concentrations for nano compared with bulk material at the same concentrations. Enhanced uptake of Ce (at exposures of 50 CeO$_2$ μg/l) occurred for the higher concentration of fulvic acid. Uptake levels were highest in the brain (232 ppb for exposures to 50 μg CeO$_2$/l and 250 μg fulvic acid/l, Figure 2c), then kidney (92 ppb for exposures to 50 μg CeO$_2$/l and 50 μg fulvic acid/l, Figure 2b) and then gill (48 ppb for exposures to 50 μg CeO$_2$/l and 250 μg fulvic acid/l, Figure 2a). Overall, Ce concentrations for the nanoparticle exposures were significantly higher in the brain, kidney and gills for exposure conditions containing fulvic acid (Figure 2). These data suggest the presence of fulvic acids had an enhancing effect on the bioavailability and uptake of Ce into the exposed fish.

The gills showed the clearest effect of fulvic acid on the uptake of Ce; in the 50 μg CeO$_2$ /l treatment there was a 2.5-fold higher content Ce in gill tissue with the addition of 50 μg fulvic acid /l and over a 6-fold increase with the addition of 250 μg fulvic acid /l, whilst there were no changes in Ce content in the gill of exposed fish to 5 μg CeO$_2$ /l with addition of fulvic acid (Figure 2a). Potential routes of uptake of Ce into this tissue include paracellular diffusion through the tight junctions between gill cells into the blood or transcellular uptake, although direct diffusion through damaged tissue caused by inflammation of the gill by the MNPs is also possible.

The finding that levels of Ce in the kidney and brain for selected exposures were higher than for those in the gills raises interesting questions relating to their route of
entry into these tissues. The most obvious route to these tissues, given we exposed via the water, is via the gills and the presence of Ce in the gills would support this as an entry route. The fact that Ce concentrations were higher in the brain and kidney may reflect a greater capacity for accumulation and slower clearance rates in these tissues compared with the gills, as previously seen for Ce in carp

However, other possible routes of entry in our exposure system include via the skin, gut and olfactory epithelium. The skin is an unlikely route of entry for MNPs, as the mucosal surface is sloughed away continuously and a previous study exposing fish to zinc oxide (ZnO) nanoparticles via the water found no significant quantities of Zn were taken up into the skin. Uptake via the gut may occur due to stress induced drinking and/or via active foraging; carp readily feed on organic particulates and thus may have ingested aggregates of the CeO₂ particles. If this were a major route of uptake, however, we might have expected to see evidence of uptake into body tissues for the bulk CeO₂ material also (especially for kidney tissue) and this was not the case. In other animals, the olfactory epithelium has been proven to be a route of uptake for particles. In rats, translocation of manganese oxide and iron oxide via the olfactory neuronal pathway to the olfactory bulb have been demonstrated in inhalation studies and TiO₂ instilled nasally to mice has been shown to enter the brain directly through the olfactory bulb.

Metals exposed via the water to fish have the potential to circumvent the blood-brain barrier and gain direct access to the central nervous system via the olfactory neurones, as demonstrated for manganese by Tjalve et al. Thus, uptake routes of MNPs into fish from exposure in aqueous may occur potentially via a number of routes (gills, gut and olfactory surfaces) and we are not able here to identify definitively which of these (possibly all) acted as uptake routes in our studies. Importantly, however, addition of fulvic acid may affect uptake into the organisms differently for the different exposure routes (and at the different exposure concentrations). For example, induced aggregation
of particles is likely to facilitate uptake via the gut as carp feed on particulate matter, whereas smaller (nano) sized particles will likely have an enhance uptake into the fish via the gill and olfactory surfaces (a detailed discussion of particle size, presence of NOM and experimental exposure dynamic in relation to uptake is given below). This serves to further indicate the complexities of some of the potential pathways and toxicokinetics for metal MNPs to aquatic organisms exposed via the water in an environmentally realistic scenario.

**Possible functional consequences of Ce uptake**

Given the apparent effects of NOM on uptake of Ce into the gill, this suggests a significant bearing on operatives in models used to predict toxicity effects in exposed fish such as the biotic ligand model (BLM) which places assumptions on the dominance of free ion activities in determining bioavailability as well as the supposition that aqueous phase chemistry is at equilibrium. Reports in the literature on how NOM may affect metal toxicity are fairly consistent, with the majority of studies reporting a protective effect of NOM. These include studies on nano-sized zero valent iron showing reduced toxicity to bacteria, a reduced toxicity and bioavailability of quantum dots to *Daphnia magna*, a reduced toxicity of silicon dioxide (SiO$_2$) to algae and a reduce copper oxide (CuO) toxicity to daphnids all in the presence of NOM. For CeO$_2$ MNPs Van Hoecke and colleagues described an increase in stability and decreased toxicity towards algae, hypothesizing a reduced bioavailability of CeO$_2$ particles due to NOM adsorption to the surface. In fish too, a number of studies indicate reduced toxicity of metal in the presence of NOM (e.g. for Ag, and nickel). Nevertheless there are a few studies indicating NOM can have enhancing toxicity effect for nanomaterials and other contaminants. Examples of this include a reduced EC$_{10}$ for both uncoated TiO$_2$ and coated ZnO nanoparticles in daphnia, when exposed in natural water and an increase in trichlorfon toxicity in the presence of NOM. A number of studies
indicate the decrease in toxicity as a consequence of binding of the ionic forms of the metal by NOM, thus restricting its uptake\textsuperscript{56, 57, 61}. These data would imply that the Ce detected in our studies is likely to have a nanoparticle aetiology as opposed to an uptake of dissolved ions. An \textit{in vitro} study exposing a gill cell line to Ag nanoparticles has shown that the particles themselves can penetrate through the cell epithelium\textsuperscript{62}.

The uptake and tissue distribution of Ce in our study for the different exposure regimes indicate the potential for biological effects in the exposed fish. An enhanced uptake of MNPs into the gill in the presence of NOM suggest that gill tissues may be more susceptible to any adverse effects of MNPs in natural waters compared with aqueous systems without NOM. Gills have been shown previously to be a target for a range of metal and metal oxide MNPs for uptake via the water, inducing a range of histopathological effects, oxidative damage, and induction of a suite of genes involved in cellular processes including apoptosis, cell differentiation and proliferation\textsuperscript{63-67}. Nothing is reported however for toxicity in this tissue relating to ceria.

The presence of Ce in the kidney (Figure 2b) is consistent with this being a target tissue for MNPs, as established in other studies. For example, Ag MNPs have been shown to accumulate in the kidney in rainbow trout (\textit{Oncorhynchus mykiss}) after intravenous injection with a subsequent slow rate of clearance\textsuperscript{68} and in the fathead minnow (\textit{Pimephales promelas}) where intraperitoneal injection of TiO\textsubscript{2} particles resulted in a reduction in neutrophil function and presence in the kidneys\textsuperscript{69}. Other studies on different MNPs have shown little uptake or accumulation in the kidney. Examples of this include for latex nanoparticles where there was minimal uptake into the kidneys of rats (0.083 % of total recovered particles) after aspiration\textsuperscript{70} and for organically modified silica particles where there was highly efficient clearance of particles from the kidney of mice after intravenous injection\textsuperscript{71}. Differences in findings between these are hard to align and may relate to difference in exposure methods, test
organisms (rodent versus fish models) and axiomatically, differences in uptake routes, as well as factors such as particle type, coating and dosage.

TiO$_2$, Ag and Cu MNPs have been shown to induce kidney damage in rodent models for exposures via injection or gavage $^{72-74}$, and nephrotoxicity has been shown in vitro in cultured kidney cells for MNPs including carbon nanotubes, ZnO, silica, TiO$_2$ and CdS $^{75-78}$, however, dosing levels from the above studies are often in the ppm range (an order of magnitude higher than those in this experiment (ppb)), with little environmental relevance.

Our findings for uptake of Ce into the brain (Figure 2c) may give some cause for concern, although uptake was observed only in the 50 μg CeO$_2$ /l with 250 μg fulvic acid /l treatment group. Furthermore, there was high variability in uptake into the brain between exposed individuals in this treatment group. One of the first in vivo studies involving exposure of fish to nanomaterials, reported effects in the brain (for fullerenes, $^{79}$), and there have been other studies that provide evidence for uptake of MNPs into, and effect on, the brain for in vivo exposures. Equally, however, there are studies that argue to the contrary. Findings include, oxidative stress in the hippocampus for rats exposed to CeO$_2$ MNPs introduced intravenously $^{80}$. Frederici et al. $^{63}$ recorded tissue depletion of Cu and Zn levels and a concentration dependent increase in a marker for oxidative stress in the brain of rainbow trout after water exposure to 21 nm TiO$_2$ MNPs. One study reported that dietary exposure of TiO$_2$ to rainbow trout resulted in uptake into the brain with subsequent low clearance of the metal $^{81}$. Further recent investigations have shown that TiO$_2$ nanoparticles injected into the abdominal cavity of rats caused brain injury $^{82}$, intraperitoneal injections of Ag NPs cause apoptosis and free radical induced neurotoxicity in the brain $^{83}$ and injection of AlO$_2$ nanoparticles affected the innate immune system of the rat brain $^{84}$. Other studies, however, have failed to provide any evidence for the ability of MNPs to cross the blood brain barrier. Examples
include a study in rats where CeO$_2$ nanoparticles, despite observations of biological effects in the brain \cite{80} and a follow up study with smaller sized particles of 5 nm, did not show that particles were able to penetrate but were rather seen to aggregate on the periphery of the brain \cite{20}. Shinohara et al. \cite{85}, exposing common carp \textit{in vivo} to a fullerene suspension, found that none of this material reached the brain tissues as a result of the exposure. There are very little data regarding CeO$_2$ nanoparticle interactions in the brain, and the available studies give conflicting results. The lack of consistency for evidence of MNP penetrating into the brain again highlights the variation that route of exposure and particle type can have on a multiplicity of biological interactions related to uptake. Nevertheless, our study would suggest that interactions of MNPs (here CeO$_2$) with natural colloids may facilitate uptake into the brain. Interestingly, a neuroprotective effect of CeO$_2$ has been shown in an \textit{in vitro} human Alzheimer disease model \cite{86} and in a mouse hippocampal brain model \cite{87}.

\textbf{Understanding the fate and behaviour of CeO$_2$ MNPs mediating their bioavailability}

Critical to an informed interpretation for any MNP biological effects study, and for comparison across studies, is the characterization of the particles employed, both in their raw state and in the exposure medium. Here this was done using a multifaceted approach and variety of techniques in an attempt to establish the size, shape and dispersion traits of the particle under scrutiny.

Dynamic light scattering (DLS) data obtained from experiment II (performed under identical conditions to the initial experiment and run to obtain detailed particle behaviour analysis, see methods section) found the smallest average sized CeO$_2$ particle aggregates occurred for the 50 $\mu$g CeO$_2$/l treatment in combination with the highest (250 $\mu$g/l) fulvic acid concentration on both sampling days (days 0 and 10 of the study (Figure 3d). The particle diameter for this treatment at day 10 was 3 fold lower than any
other treatment at the same time point. This reduction in size from the addition of citrate and fulvic acid, has been reported previously in the literature \(^ {34, 35}\) and would suggest that the NOM at the highest exposure level served to reduced particle aggregation. The high degree of variation in the DLS measurements within some treatments is likely due to the polydispersed nature of our exposure medium and this can result in a bias in DLS measurements towards larger particle sizes or aggregates (Ju-Nam et al, In Review). Indeed this bias reduces the utility of the method for accurate sizing of individual particles across of wide spectrum of sizes (Ju-Nam et al, In Review). A further drawback of the DLS technique is the insensitivity of the method for measuring the smaller particles and particles at low concentrations, in complex environmental media, as in this study, (see Domingos et al. \(^ {88}\)). Due to these limitations of the DLS method for our exposure samples we conducted some further analyses on the fate and behaviour of the CeO\(_2\) to establish the effect of citrate and citrate with fulvic acid on hydrodynamic diameter at higher exposure concentrations (1, 10 and 50 mg CeO\(_2\)/l see Figure S1). These analysis clearly demonstrated that addition of citrate and citrate with fulvic acid reduced the average particle diameter (13-fold and 11-fold reductions in average particle size respectively, when compared to the particles in aquarium water alone). These data, however, suggest that the main stabilising factor for the MNPs within our exposure treatments was related to the citrate coating rather than the addition of NOM. Zeta potential readings for particles in the media containing 1, 10 and 50 \(\mu\)g CeO\(_2\)/l were between 0 and -3.44 mV when in aquarium water, between -0.76 and -18.5 mV in aquarium water with citrate and between -0.69 and -12.1 mV in aquarium water with the addition of citrate and fulvic acids, indicating that none of the solutions were stable (charge readings were within the ± 30 mV domain, indicating an unstable solution). Nevertheless, the addition of the citrate/NOM showed a distinct tendency to produce a more stably dispersed suspension (S2).
Transmission electron microscopy (TEM) micrographs of the raw CeO$_2$ MNPs (S3) as a dry powder and when dispersed in aquarium water (Figure 4a) illustrate a significant aggregation of the MNPs in aqueous medium, with a reduction in the size of the aggregates formed when the suspension of CeO$_2$ MNPs was made up in the citrate solution (Figure 4b,c) compared to with the suspension dispersed in aquarium water alone (Figure 4a).

The TEM PSD data (direct measurements taken from TEM micrographs) provided strong evidence that the modal size of particles in the water column in all exposures were nano-sized (Figure 3a-c) with the most common size group 50-100 nm. TEM images derived from fulvic treatment groups suggested a higher frequency of smaller particles (Figure 4), however, quantitative analyses of average diameter measurements for particles, with and without the addition of fulvic acids, showed that they were not significantly different (Figure 3a-c), contrasting with the DLS data (Figure 3) from the same experiment. It was not possible to construct PSD for the other samples containing 5 µg CeO$_2$/l due to the limited number of particles observed in the TEM grids at the low concentration.

No PSD analysis was run on the bulk sized material, however micrographs of the bulk CeO$_2$ found particle sizes greater than 500 nm and with the aggregates being larger than those constructed from the nano material (Figure 4h). All measured particles were verified to be CeO$_2$ particles by X-ray diffraction (XRD) analysis (S4). We would emphasise that the ultra-centrifugal harvesting method used to eliminate artefacts of the drying process and facilitate capturing more material on the grid for TEM analysis (compared with the drop deposition method) involves actively forcing all particles in suspension onto the TEM grid and this can result in agglomerated particles, which in turn not necessarily give a wholly accurate representation of the particles in the exposure medium. Nevertheless, all sample treatments for these analyses were handled
in exactly the same way and despite these caveats TEM offers accurate measurement of primary particle sizes for comparing the state of agglomeration between the different treatment regimes.

Conclusion

In the final analysis we show that the semi static exposure system adopted provided a consistent delivery of CeO$_2$ particles via the aqueous phase. Detailed characterisation of the exposure particles illustrates the complexity and dynamic nature of the particles in the exposure medium and we highlight some of the challenges in comparing between treatment regimes for exposures considering more environmentally realistic scenarios (relatively low exposure levels and in combination with NOM). We emphasise too that studying the basic properties of MNPs, within an environmentally relevant scenario, is complicated by the dynamic nature of the particles themselves and their interactions with the exposure medium, and interpretations on measured particle sizes and characters may differ depending on the technique(s) employed, emphasising the need for employing multiple analytical approaches (discussed further in Ju-Nam et al. In Review).

Nevertheless, we provide evidence for uptake of CeO$_2$ from the aqueous environment into exposed fish and an enhanced uptake in the presence of NOM. Despite the limitations of the analytical methods applied to the interpretations on particle fate and behaviour in the exposure scenarios, collectively the particle size and stability measurements indicate that citrate and fulvic presence show a tendency to reduce the amount of aggregation within the system and provide a more stable system than for raw particles alone. We hypothesise that this decrease in aggregation, especially within the higher CeO$_2$ and fulvic concentration treatment groups, could lead to an increased amount of smaller particles being bioavailable to fish.
Our data emphasises the importance of considering conditions in natural water, rather necessarily than those used in standardized test conditions, to understand the bioavailability (and thus potential biological effects) of metal oxide MNPs, and possibly other MNPs, to aquatic organisms. To our knowledge this is the first study to show the increased bioavailability of MNPs to an aquatic organism as a consequence of the presence of NOM in the water.

METHODS

Materials

CeO\textsubscript{2} MNPs used in these studies were obtained from a commercial supplier (Alfa Aesar, www.alfa.com) with a nominal size of 20-30nm. ‘Bulk’ ceria (<5 μm) was obtained from Sigma Aldrich (www.sigmaaldrich.com). Standard Suwannee River fulvic acid 1S10F was acquired from the International Humic Substances Society (IHSS, St. Paul, MN).

Experimental set-up

The experimental set-up for the fish exposures is shown in the supplementary material (S5). Briefly, 60 litre tanks were half filled with mains tap water filtered by reverse osmosis (RO) and reconstituted with Analar grade mineral salts to standardized synthetic freshwater (Na\textsuperscript{+} = 8.27 mg/l, K\textsuperscript{+} 2.07 mg/l, Mg\textsuperscript{2+} = 4.38 mg/l, Ca\textsuperscript{2+} = 24.50 mg/l). There were 11 different exposure treatments; 4 control tanks containing; citrate, 50 μg fulvic acid /l, 250 μg fulvic acid /l and a RO water only and 7 further tanks containing CeO\textsubscript{2}, with and without fulvics; 5 μg nano CeO\textsubscript{2} /l, 5 μg nano CeO\textsubscript{2} /l + 50 μg fulvic acid /l, 5 μg nano CeO\textsubscript{2} /l + 250 μg fulvic acid /l, 50 μg nano CeO\textsubscript{2} /l, 50 μg nano CeO\textsubscript{2} /l + 50 μg fulvic acid /l, 50 μg nano CeO\textsubscript{2} /l + 250 μg fulvic acid /l, 5 μg bulk CeO\textsubscript{2} /l + 50 μg fulvic acid /l. The dosing regimes for nano CeO\textsubscript{2} were adopted to
represent predicted nano metal oxide values \textsuperscript{6} and the fulvic acid concentrations are below those occurring in many natural waters. The dosing regime followed a semi-static exposure scenario with 15 litre water changes every 48h. Ten juvenile common carp \textit{(Cyprinus carpio)} (length $8.4 \pm 0.12$ cm, weight $12.6 \pm 0.54$ g) were deployed into each tank and the exposure was run for 28 days. Tank water conditions monitored (daily) included temperature and pH (see Figure S6). Fish were not fed throughout the period of the exposure to reduce the level of tank contamination with faecal matter. The primary objectives of this experiment were to quantify the uptake and tissue distribution of Ce in the body tissues of the exposed fish, and to assess the stability of the dosing using the semi-static water column based delivery system.

A further experiment (experiment II) was subsequently run replicating the first, but over a shorter period of 10 days (exposure conditions were stabilised at this time - see Figure 3), to investigate further the fate and behaviour of the CeO$_2$ particles in the different exposure systems to help inform on the uptake dynamics in the exposed fish in the first exposure.

\textbf{Characterisation of CeO$_2$ nanoparticles and experimental exposure suspensions}

The CeO$_2$ particles were made up for both the experiments by suspending the received powders in Milli-Q water containing 5\% tri-sodium citrate in 1 litre bottles. These subsequently underwent sonication for 1 h before being added to the tanks with or without Suwanee river fulvic acid, depending on the treatment.

DLS and Zeta potential measurements were taken to obtain a spectra of size distributions at different concentrations of the raw particles in pure aquarium water, in the citrate solution and with the citrate and fulvic acid. This was undertaken to assess the effect of varying CeO$_2$ concentrations, on particle diameter and surface particle charge respectively, in the different exposure treatments. Further DLS measurements were taken at the beginning and end of the second experiment (II), to establish particle
behaviour and fate/size dynamics under the exposure conditions. Samples taken for the measurement of particle size distribution were undertaken in accordance with ISO 13321 (1996), using a Malvern nanoseries Zetasizer (UK). This instrument was also used to determine zeta potential. Calibration standards were implemented using Malvern -68 mV ±6.8 mV solutions. Measured electrophoretic mobility was converted to zeta potential using the Smoluchowski approximation.

TEM analysis was run on the raw particles both as dry powder and in a water suspension and on the water samples collected from each experimental treatment group from experiment II. To collect the material for TEM analysis, the ‘ultra-centrifugal harvesting’ approach was utilised; here, 4 ml sample aliquots of aquarium water were centrifuged at 30,000 rpm, for 1 h at 18 °C using a Beckman L7 ultracentrifuge before being placed directly onto a wholly carbon TEM grid. The TEM grids were subsequently rinsed using Milli-Q water and dried using absorbent paper before being placed back into the grid holder. Images of the prepared particles were obtained with a Philips (Tecnai Series) TEM, operated at 200 kV with a lens of theoretical resolution 0.19 nm and a Gatan 794 MSC digital camera, with an extension voltage of 4400V. Individual particles were measured using Digital Microraph™ series 3.4.4 Gatan software 1999. All measured particles were verified to be CeO₂ particles by X-ray diffraction (XRD) techniques. Powdered samples were prepared for XRD by loading into a 1 cm² disk holder using scotch tape. This disk was loaded into a Siemens D5000 Kristalloflex X-ray powder diffractometer with nickel-filtered CuKα radiation, and set to run over a range of Bragg angles (25° to 90°) over 50 to 90 minutes at room temperature. Diffraction patterns were obtained and analysed using ETA software.

**Sampling of water, biological tissues and uptake analysis**

pH, temperature and conductivity readings taken for each tank every 48h. Water samples for particle analysis were taken every 48h throughout the exposure and
collected from the centre of the tank. All fish were sampled on day 28 of the exposure in experiment I. Fish were anaesthetised terminally in benzocane and dispatched humanely according to UK HO Animals Procedure Guidelines. Blood samples were collected and stored at -80°C. Organs and tissues were dissected out, washed, weighed and flash frozen before storage at -80°C. Water and tissue Ce concentrations were measured using ICP-MS. All samples for ICP-MS analysis underwent a digestion process, as detailed in Scown et al. Briefly, 3 ml of cold concentrated nitric acid (HNO₃) was added to a known amount of tissue, ranging from over a gram of brain tissue to 50 mg of kidney tissue, for 24 h before addition of 2 ml H₂O₂ and boiling in Gerhardt Kjeldatherm digester. After cooling samples were reconstituted in 5 ml 2% HNO₃ for ICP-MS measurement. All ICP-MS samples were analysed by Steve Baker, University of Birmingham, (UK) and the data adjusted for the specific sample weight.

Statistical analysis

Statistical analyses were run using R (the R project for statistical computing http://www.r-project.org/). To test the significance of the CeO₂ MNPs uptake in the presence of fulvic acids an Analysis of Covariance was run, where CeO₂ was treated as a continuous variable and regression lines were drawn and compared for each fulvic treatment to test the significance of the interaction.

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**Supporting Information Available.** A schematic representation of the experimental set up, pH and temperature readings of each tank throughout the study as well as further DLS readings on non-experimental media and XRD spectrum. This material is available free of charge via the Internet at [http://pubs.acs.org](http://pubs.acs.org).

**List of Figures.**

**Figure 1.** Water concentrations of Ce, as measured by ICP-MS, in treatment tanks with carp exposed to CeO$_2$ in the presence of various concentrations of fulvic acid over 28 days.

**Figure 2.** Concentrations (ppb) of cerium in gills (a), kidney (b) and brain (c) tissues, as measured by ICP-MS, in carp exposed to CeO2 in the presence of various concentrations of fulvic acid over 28 days. Significant enhanced tissue uptake of Ce into fish occurred in tanks with the addition of fulvic acid for gills (p = 0.015), kidney (p = 0.002) and brain (p=0.008).

**Figure 3.** Particle size distribution (PSD) of equivalent circular diameter (a-c) and hydrodynamic diameter measurements (DLS) for particles in each CeO$_2$ treatment regime on day 0 and 10 (d) of experiment II. PSD was measured by image analysis on TEM micrographs of: (A) 50 μg CeO$_2$ NPs /l (mean 138±125 nm, n= 63), (B) 50 μg CeO$_2$ NPs + 50 μg fulvic acids /l (mean 128±107 nm, n = 27) and (C) 50 μg CeO$_2$ NPs + 250 μg fulvic acids /l (mean 212±253 nm, n =40).
Figure 4. TEM micrographs of CeO$_2$ MNPs in the various treatment tanks. (a) uncoated CeO$_2$ nanoparticles (NPs) in aquarium water, (b) 5 μg CeO$_2$ citrate coated NPs /l, (c) 5 μg CeO$_2$ citrate coated NPs + 50 μg fulvic acids /l, (d) 5 μg CeO$_2$ citrate coated NPs + 250 μg fulvic acids /l, (e) 50 μg CeO$_2$ citrate coated NPs /l, (f) 50 μg CeO$_2$ citrate coated NPs + 50 μg fulvic acids /l, (g) 50 μg CeO$_2$ citrate coated NPs + 250 μg fulvic acids /l, (h) 5 μg CeO$_2$ citrate coated bulk particles + 50 μg fulvic acids /l.
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Figure 2.
Figure 3.
Figure 4
SUPPLEMENTAL INFORMATION

Figure S1. Particle Sizes Measured via Dynamic Light Scattering. Hydrodynamic diameter of nanoparticle aggregates in the presence of the citrate and citrate with fulvic acids were consistently smaller than aggregates formed in aquarium water alone. Particles were prepared and measured in identical fashion and in the same media as for the fish exposure studies, described in the methods section. Data indicate a reduction in aggregate formation in the presence of citrate and/or fulvics, similarly to that noted in the fish exposure study. Here however, Ce concentrations were higher than in the fish exposure study to allow for more accurate measurement.

![Figure S1](image.png)

Figure S2. Zeta Potential (ζ) Measurements of CeO₂ Suspensions. The zeta potential (ζ) of the nanoparticles was variable across the different CeO₂ concentrations, however there was a trend for citrate and citrate in combination with fulvics to reduce aggregation at the test concentrations closest to those used in the fish exposure study. The samples were prepared in identical conditions to those used in the main fish exposure study. None of the zeta potential values were greater than ± 30 mV, indicating none of the suspensions were entirely stable.

![Figure S2](image.png)
Figure S3. Transmission Electron Microscopy (TEM) Micrograph. TEM images of CeO$_2$ Alfa Aesar nanoparticles (as a dry powder) used in the exposure study. (nominal size 20 – 30 nm). The size and extent of aggregation of these particles under different treatments is shown in Figure 4.

![TEM Image](image)

Figure S4. X-ray diffraction (XRD) Diffractogram. XRD analysis of the particles collected from the exposure media onto the TEM grids verified that the aggregates we measured were ceria. This diffractogram of nano-ceria was obtained at room temperature and is representative of all CeO$_2$ treatments and identical to the diffractogram obtained from the dry CeO$_2$ Alfa Aesar nanoparticle powder (data not shown).
Figure S5. Schematic of Experimental Set up for Exposing Carp (*Cyprinus carpio*) to CeO$_2$ nanoparticles in the presence of fulvic acid. Fish numbers and exposure regime for both experiment I and II were identical. The study utilised a semi static dosing system over a period of 28 days with a 50% water change every second day.
Figure S6. pH and Temperature in the Different Treatment Tanks for the Carp (*Cyprinus carpio*) Exposures to CeO₂ Nanoparticles in the Presence of Fulvic Acids. The pH and temperature measurements taken throughout the study duration show excellent agreement between treatments and no major fluctuations overall over the entire experimental period. These data would suggest that there would be no major effects of these physicochemical parameters on the particle kinetics over the exposure study.
Chapter 5

Silver nanoparticles show enhanced maternal transfer compared with larger silver particles when dosed in the diet of a live bearing fish species *Poecilia reticulata.*
Silver nanoparticles show enhanced maternal transfer compared with larger silver particles when dosed in the diet of a live bearing fish species *Poecilia reticulata*.

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Abstract

The maternal transfer of manufactured nanoparticles (MNPs) has received relatively little research effort considering the potential vulnerability of developing embryos and early life stages to their biological effects. Few studies other than those considering maternal transfer of nanoparticles in mammals have been reported. In this study, silver nanoparticles (Ag NPs, 7.3 ±0.3 nm) and larger sized (bulk) silver particles (125 ± 0.9 nm) incorporated into the diet were fed to the live bearing, ovoviviparous fish Poecilia reticulata for a full gestation cycle and the amount of Ag uptaken quantified in both the adult females and the larvae they produced using inductively coupled plasma mass spectroscopy (ICP-MS). Effects of the treatments were assessed on indices of body condition in the adult females and the number and of size of the offspring produced. Maternal transfer of Ag was significantly higher for the Ag NP treatment compared with the bulk and control treatments. The larval offspring also had significantly higher Ag burdens per gram of tissue compared with the maternal sires. There was no impact of Ag at the dosing adopted however on larval survival or birth weights, or on indices of body condition in the exposed adults. This study highlights the need for further research into maternal transfer as a route of exposure in the risk assessment for MNPs.
Introduction

The properties of nano form materials that are manipulated and exploited widely for commercial use can also potentially give rise to adverse health effects and there is now concern for exposures in both humans and wildlife (Warheit et al., 2008; O'Brien and Cummins, 2010; Schrand et al., 2010) The current total global market value of nano-enabled products is estimated at around $300 billion and over the last decade this has grown at a rate of approximately 25% per year (Harries and Harries Ltd, 2011). An inventory of products containing nanomaterials published by the European Consumers' Organisation (BEUC) and the European Consumer Voice in Standardisation (ANEC) in 2010 reported 475 compared with 151 the previous year (available from http://www.anec.org/anec.asp).

Of particular interest to both research and industry is nano sized silver. The Project on Emerging Nanotechnologies (www.nanotechproject.org) lists 313 general products globally that contain nano silver (October 2011). Silver nanoparticles (Ag NPs) are one of the most exploited nanoparticles due to their diverse functional properties including high thermal and electrical conductivity, catalytic activity, non-linear optical properties and chemical stability. These characteristics have led to the use of Ag NPs in microelectronics (Wu et al., 2006), inks (Lee et al., 2005; Perelaer et al., 2009) and medical imaging (Jain et al., 2008). It is, however, the antimicrobial activity of Ag NPs for which it is most heavily exploited with incorporation into clinical dressings, textiles, water treatment devices, home appliances, cosmetics and food packaging (Benn and Westerhoff, 2008; Li et al., 2008; Maneerung et al., 2008; Benn et al., 2010; Xu et al., 2010; Farkas et al., 2011). Axiomatically, these uses result in increased human exposure, with the potential associated adverse health risks, as well as increased discharges into the environment. Studies have predicted that the majority of silver NPs from consumer products will enter sewage systems, (Mueller and Nowack, 2008; Gottschalk et al., 2009) and sorb to biosolids. In some cases however, where high levels enter the waste water treatment works (WWTWs) removal efficiency is reduced and up to 70% of the influent Ag NPs can subsequently enter surface waters in the effluent stream (Kaegi et al., 2011). A major source of Ag NPs to the land is via biosolids from WWTW when they are applied as fertiliser.

The toxicity of Ag NPs has been relatively well studied, including in fish models (reviewed in Ahamed et al., 2010 Fabrega et al., 2011) where their effects include a size dependant toxicity in zebrafish (Danio rerio) embryos (with LC$_{50}$ values recorded between 93 and 138 μM for 4 different sized Ag NPs, Bar-Ilan et al., 2009), morphological aberrations and decreased hatching rates in zebrafish embryos (Yeo and Kang, 2008; Asharani et al., 2011), hepatotoxicity
in zebrafish after water borne exposure (Choi et al., 2010), disrupted olfaction in Crucian carp (Carassius carassius) and Eurasian perch (Perca fluviatilis, Bilberg et al., 2011), up-regulation of genes related to metal detoxification, radical scavenging, carcinogenesis and induction of oxidative stress in Japanese medaka (Oryzias latipes, Chae et al., 2009) and uptake and expression of a xenobiotic metabolism marker in rainbow trout (Oncorhynchus mykiss, Scown et al., 2010). However, most of these effects have been shown only for concentrations far exceeding environmentally relevant exposures.

Early life stage organisms are likely to be among the most sensitive to the effects of NPs, as occurs for toxicants generally (McKim, 1977). A sub-population potentially at the highest exposure risk to NP exposure are embryos that develop in the mother due to maternal transfer (Saunders, 2009; Wick et al., 2010). In mammals, maternal transfer to the developing foetus has been studied for a range of nano-drugs (Menezes et al., 2011) and recently also for a range of NPs including titanium dioxide (TiO$_2$) (Shimizu et al., 2009; Takeda et al., 2009; Yamashita et al., 2011), silica (Nagano, 2011; Yamashita et al., 2011), fullerenes (Tsuchiya et al., 1996; Yamashita et al., 2011), carbon nanotubes (Lim et al., 2011), quantum dots (QDs) (Chu et al., 2010) and gold (Au) (Challier et al., 1973; Takahashi and Matsuoka, 1981). These studies have been conducted in both in vivo mammalian models and using an in vitro human placental perfusion technique (Myllynen et al., 2008) and although the findings have been highly variable (reviewed in Ema et al., 2010) many of these materials appear to enter into the developing foetus from the exposed mother.

Guppies (Poecilia sp) are lecithrophic, non-superfetiating ovoviparous fish with internal fertilization and intrafollicular gestation. The gestation period is approximately 21 days and mean inter brood interval around 28 days. It is thought that the egg yolk of the guppy is sufficient to meet nutritive requirements for the term of embryonic development and the ovarian follicular epithelium is a pathway for respiratory exchanges only, supported by the observation of a decrease in embryonic mass from fertilization to parturition (~30 – 40%; Haynes, 1995). However, studies investigating the structure and function of a follicular pseudoplacenta ultrastructure have suggested possible transfer of nutrients from maternal tissue to embryo during the first few weeks of gestation; this evidence includes the localised cycling of glycogen (Jollie and Jollie, 1964), transfer of thyroxine into the embryos from the gestating female (Lam and Loy, 1985) and data showing uptake of nutrients into explanted early stage guppy embryos (Trinkaus and Drake, 1952). This finding is mirrored in lecithotrophic reptiles that exhibit low levels of incipient matrotrophy (Stewart, 1992). Guppies, and other fish species that show matrotrophy, have the potential to provide
powerful models for studies into the susceptibility of early life stages to assess for NP effects, and a single study has shown maternal transfer of 40 nm “fluorescent microspheres” (DeMarais and Oldis, 2005), into embryonic life stages of three types of poeciliid fish, including *Poecilia reticulata*. The principle aim of this study was to investigate maternal transfer of a NP of widespread concern, silver, in females guppies *Poecilia reticulata* exposed over the full gestation period, to inform on possible matrotrophic translocation.

**Materials and Methods**

**Particle synthesis**

Silver nanoparticles (Ag NPs) were synthesised in the manner described in Römer *et al.*, (2011). Briefly, nanoparticles were prepared from a standard reduction of a silver salt (silver nitrate 0.25 mM) in sodium citrate (0.31 mM), by sodium borohydride (NaBH₄) after heating to boiling. Ag NPs were cleaned by ultrafiltration (Amicon, 1 kDa regenerated cellulose membrane, Millipore) to remove the excess reagents before use. The particles were re-dispersed in citrate solution and this process was repeated at least three times. Bulk Ag particles (nominal size 100-600 nm) were acquired from Nanostructured and Amorphous Materials Inc. Houston USA and were stirred with 0.15mM citrate for 24 hours. Details on the size and physicochemical characteristics of the produced particles are given below.

**Experimental set up**

Guppies were maintained in 12 litre tanks under flow through conditions, three quarters filled with mains tap water filtered by reverse osmosis (RO) and reconstituted with Analar grade mineral salts to standardized synthetic freshwater (Na⁺ = 8.27 mg/l, K⁺ = 2.07 mg/l, Mg²⁺ = 4.38 mg/l, Ca²⁺ = 24.50 mg/l). There were 3 exposure treatments each consisting of 4 replicates; 4 control tanks (in which fish were fed on a diet containing citrate at a level equivalent to the coating for the silver particles – see below), 4 bulk particle exposure tanks and 4 nanoparticle exposure tanks. Each replicate tank was split into 3 equal compartments using plastic dividers with fine perforations to allow water flow. Individual female guppies (29.3 ± 0.54 mm) were placed into each tank compartment providing 12 females for each exposure treatment. Isolation of the females allowed us to match the individual offspring produced to a specific female. Single male guppies were introduced to each tank compartment and moved between the compartments every 48h and between tanks within treatments every 12 days. This was conducted to both limit persistent sexual advances of males to the female (that can exhaust her) and to ensure a female was paired with a suitable male to secure effective mating and thus production of offspring.
Dosing regime
Three batches of flaked fish food were prepared containing either Ag nanoparticles, bulk Ag particles or a citrate control (that was used to coat the silver particles). The food was made by grinding 10g of standard flake food (Skretting, UK) in a pestle and mortar to create a very fine powder. One gram of wheat flour was then added to the powder, before the addition of 20 ml of 11ppm bulk or nano-Ag, or a 0.15mM citrate solution. This was then mixed thoroughly. This mixture then formed a paste which was spread very thinly on baking sheets, dried for 24 hours in an oven and then broken up into small flakes (approximately 5x2x1 mm in size). Fish were then fed at a rate of 2% body weight/day of the dosed flake food for the duration of the experiment, this resulted in a dosage of 0.22 μg Ag/fish/day. This dosing was chosen after examining measured Ag levels and modelled Ag release from nanotechnology products. Environmentally measured levels of Ag are in the low ng/l range (Andren et al., 1995; Kramer et al., 2002) but one modelling study showed environmental Ag NP presence had a risk quotient exceeding 1 (Gottschalk et al., 2009) and others predicting surface water concentrations in a high emission scenario between 0.08 and 0.32 μg /l of Ag in surface waters (Mueller and Nowack, 2008; O’Brien and Cummins, 2010). Thus our exposure levels are well within the theoretical realm of an environmentally relevant scenario.

Sampling of water, fish and uptake analysis
The experiment ran for 45 days to ensure each fish underwent an entire term of gestation under the exposure conditions. Water samples of 5 ml were taken from the middle of the tank every 7 days for inductively coupled plasma mass spectroscopy (ICP-MS) analysis to ascertain what level of leaching of Ag occurred from the food and/or faeces into the water. Each chamber containing the individual females was checked daily and any fry produced were removed immediately, counted, assessed for any morphological abberations and then excess water was removed with tissue paper before the fry were weighed, terminated by schedule 1 method in accordance with UK Home Office regulations, and then stored at -80 °C until processing for silver analysis. On day 45, all adult fish were euthanized by terminal anaesthesia and total body weight and length were recorded to determine condition factor (K = (weight X 100)/ length3).Liver and heart were dissected out to determine hepatosomatic index (liver weight/ (total weight - liver weight) x 100), which can provide a measure of toxic stress, and cardiosomatic index (heart weight/body weight x 100) to assess for cardiac hypertrophy. The status of gestation in each female terminated was also assessed at the time of sampling.
To determine content of silver, all fish tissue samples underwent a digestion process prior to ICP-MS, as detailed in Scown et al. (2009). For both larvae and adults silver analysis was conducted on whole body samples. Briefly, 3 ml of cold concentrated nitric acid (HNO$_3$) was added to the body sample (mean 0.007 and 0.5 g for larvae and adult respectively) for 24 hours before addition of 2 ml H$_2$O$_2$ and boiling in Gerhardt Kjeldatherm digester. After cooling, samples were reconstituted in 5 ml 2% HNO$_3$ for ICP-MS measurement. All ICP-MS samples were analysed by Steve Baker, University of Birmingham, (UK) and the silver content expressed relative to the weight of the individual tissues.

Statistical analyses were conducted using the program R 2.12.0 (www.r-project.org) using appropriate model simplification (removal of successive non significant terms to produce minimal adequate models, Crawley 2007). Ag uptake was analysed as a variable of weight, treatment and tank for adult fish and the same parameters plus exposure duration for the larvae using generalised linear models. A simple single factor ANOVA was used for analysis of the food samples.

Results

The synthesized Ag NPs measured 7 ± 0.2 nm as measured by field-flow fractionation (FFF, Figure 1) with dynamic light scattering measurement of hydrodynamic diameter 16 nm and a zeta potential of -40 mV (Römer et al., 2011). Bulk particles measured 125 ± 0.9 nm by FFF (Figure 1). Figure 1 also presents TEM micrographs of the particles with both sizes showing a similar spherical form.

ICP-MS analyses of the dosed food measured Ag concentrations of 35.5 ± 4.31 μg/g for nano and 30.42 ± 3.78 μg/g for bulk treatments, with 8.93 ± 0.23 μg/g measured in the citrate dosed control. Levels of Ag were not significantly different between bulk and nano treatment ($F_{1,6} = 0.731, p = 0.425$).

Mean water concentrations for each treatment for the entirety of the exposure were low at 0.21 ± 0.04 μg/l, 0.2 ± 0.06 μg/l and 0.32 ± 0.1 μg/l for the control, bulk and nano Ag treatments respectively these were not significantly different from one another ($F_{2,33} = 0.4977, p = 0.6124, Figure 2$).

The maternal transfer of Ag NPs as measured by ICP-MS of Ag content in the guppy larvae showed levels of Ag uptake into the fry from the Ag NP exposed female sires significantly
elevated compared to fry from the non dosed citrate or bulk Ag treatments, \( F_{2,117} = 3.579, \ p <0.05, \) Figure 3). There was no significant difference between Ag levels in bulk and citrate control larvae. Although in all treatments Ag burden per g of tissue for fry were above those of the adults, levels of Ag in the larvae of the Ag NP exposed fish were 1.5 times higher than for the larvae in the bulk Ag exposed fish. There was also a significant uptake of Ag into adult females in the Ag NP treatment group \( (F_{2,30} = 15.17, \ p <0.001, \) Figure 3), but this was not the case for the bulk silver that did not differ from the control.

Cumulative Ag levels in the female sires were ~12 and 42% of the food Ag content, per gram, for the bulk and nano treatments respectively. Larval content of Ag was much higher than in the dosed female sires per gram of tissue, with the maternal burden, 2 and 7% of the larval load for the bulk and nano treatments respectively. This corresponded to over a greater assimilation of Ag from the food into the Ag NP treated larvae, compared to the bulk Ag treated larvae.

There was no measurable effect of maternal exposure duration on the Ag burden of the guppy larvae (Figure 4) which was not shown to increase within the exposure period of the first fry to be born (day 5) and 28 days, after which all larvae would have been exposed for the entire gestation period. Only total Ag content was measured for the maternal sires at the end of the experiment, thus exposure duration and Ag burden are unable to be correlated.

There was no significant effect of any of the treatments on the biological endpoints measured. The numbers of fry produced was highly variable both within and between treatments, with between 1 and 11 per female but with little variation between the total fry number produced in each treatment. No larval morphological abnormalities were noted for any of the treatments. Larval weight means were 0.0069 ± 0.0018 g, 0.0063 ± 0.0013 g and 0.0065 ± 0.0026 g for the control, bulk and nano treatments respectively. Exposure duration of the female sires did not affect the subsequent size (weight) of the fry (Figure 5); although there appeared to be a downward trend in fry size over the study period (for all treatments) this was not statistically significant \( (F_{2,117} =0.52 \ p >0.05) \). Indices of condition and toxicological impact (hepatosomatic index, cardiosomatic index and condition factor) taken on adult fish of adult fish did not vary significantly between treatments (Figure 6).
**Discussion**

Assessment of the potential reproductive and developmental effects of nanomaterials has yet to receive much attention, which is surprising given that these life stages tend to exhibit great toxicological sensitivity in organisms generally (e.g. Bryan et al., 2003; Roe et al., 2004; Lacoue-Labarthe et al., 2008; Nyholm et al., 2008). Early life is a critical time of development, during which a greater proportion of the genome is activated compared with any other life stage, and this is recognised in chemical risk assessment with OECD test guidelines that specifically cater for these early life stage exposures. Recent literature has reported on in vivo maternal transfer of NPs in rodent models but these exposures have tended to adopt a non-natural dosing method (such as injection or gavage) and doses that do not reflect environmental relevance reviewed in Ema et al., (2010). The potential for exposure to NPs in early life stages of wildlife species showing ovoviviparity or viviparity has not been researched and here we assessed the potential for maternal transfer of one of the most widely used NPs, silver, in an ovoviviparous fish species.

Despite being a proposed lecithitroph, we show a significant maternal transfer of silver from all treatments into the developing larvae during the 45 day oral dosing study (Figure 3) supporting a previous study in guppies showing transfer of fluorescent microspheres during early stages of oocyte and larval development (DeMarais and Oldis, 2005). We found however, a significantly higher adult body tissue and larval content of Ag for the Ag NP treatment compared with both the control and bulk silver treatments. This would appear to indicate either a greater uptake of Ag NPs from the gut in the gestating female and/or greater subsequent transfer of Ag into the larvae compared with the larger sized silver. It is possible, however, that this enhanced uptake from the gut and subsequently into the developing larvae might relate to a greater dissolution of silver in the gut for the nanoparticle treatment and thus reflect uptake of free silver. Dissociation outside of the fish, however, is not thought to play a significant role as evidenced by the measured water concentrations. Either way, the result is that the gestating larvae received a higher exposure to the Ag administered as nanoparticles in the diet of the adult female. In rodents studies particle size has been shown to be as a critical factor in determining maternal transfer. For silica particles of 3 sizes, 1000, 300 and 70 nm, injected in to the caudal vein, only the 70 nm particles passed through the placenta and transferred into the liver and brain of the unborn pups (Nagano, 2011). Yamashita and co-workers (2011) similarly found found that 70 and 35 nm particles translocated to the placenta, foetal liver and foetal brain after intravenous injection but this was not the case for particles 300 and 1000 nm in size. Further size related placental transfer effects have been found for
gold (Au) NPs, with a faster transfer rate for 5 nm particles compared with that for 30 nm particles (Takahashi and Matsuoka, 1981). Not all studies of this nature however concur and in a study intravenously injecting particles between 2 and 40 nm Au NPs Sadauskas et al. (2007) found no uptake into the placenta or foetus. Both open perfusion and re-circulating perfusion in vitro techniques support this, showing the placenta acts as an effective barrier to gold nanoparticles between 10 and 30 nm (Myllynen et al., 2008). Contrasting with this however, another study reports transfer of fluorescent polystyrene particles (up to 240 nm in size) using the same technique (Wick et al., 2010). As such the size difference between our Ag particles (7 and 125 nm) seems like the most obvious characteristic to explain our observed differences. Our data highlights that for the bulk Ag treatment there was not an enhanced uptake (i.e., did not differ from the citrate control). From this one inference could be drawn is that there is not a large uptake gradient related to Ag size, but that there a tightly regulated physical barrier, although more data would be required to tease apart the size specific relationship.

Levels of Ag detected in fry were significantly higher than the equivalent adult burden when standardised for body weight, with a mean increase of 23 fold (Figure 3). This concentrating effect for contaminants occurs for various pollutants in fish. Examples for this include for polybromodiphenyl ethers (PBDE) where lipid adjusted concentrations in the eggs have been reported to be significantly higher than the concentrations in adult female zebrafish (Danio rerio) (Nyholm et al., 2008) and marine medaka (Oryzias melastigma, van de Merwe et al., 2011). Another example is seen for exposure to perfluorooctane sulfonate (PFOS) where 10% (wt) of the adult PFOS body burden was shown to be transferred to the developing embryos, resulting in a higher total PFOS concentration in eggs (Sharpe et al., 2010). Adult guppies are able to partition and excrete excess dietary levels of pollutants to a far greater extent than embryos or developing fry (evidenced by Hertl and Nagel, 1993). This is potentially a main cause of the observed higher Ag burden in larvae, as lack of Ag excretion by the fry would cause a bioconcentration effect.

Despite common literature citing the lecithotrophic strategy of the guppy, there is evidence for early stage transfer of nutrients to the developing larvae, the pathway of which is likely to be the follicular pseudoplacenta which becomes more elaborate and extensive in obligate matrotrophs (Wourms, 1981). Thus this interface between the embryonic yolk portal system and the maternal follicle, which allows efficient gas exchange and waste disposal but has been deemed surplus to requirements post fertilisation (Turner, 1940), is the most likely pathway for transfer of Ag NPs to the developing larvae/embryos in this study.
DeMarais and Oldis (2005), via histology and confocal microscopy, showed transfer of injected microspheres (injected into the caudal peduncle) at all investigated brood stages, with higher transfer at earlier stages. Our studies on the content of Ag in the developing larvae would indicate transfer of Ag during early life stages only as there was not a relationship between longevity of exposure and body burden in the offspring produced (Figure 4) suggesting that early stage maternal provisioning is the key period for transfer of pollutants, such as NPs. This fits in with data suggesting a transfer of nutrients from maternal tissue to embryos during the first few weeks of guppy gestation (Jollie and Jollie, 1964). Additional in vitro studies have also suggested the ability of embryos to uptake nutrients in early stages, (Trinkaus and Drake, 1952) and even the possible critical provisioning of maternal factors during this time (Martyn et al., 2006). It must be noted however, oocyte production prior to fertilisation is an ongoing process and despite any maternal transfer exchange halting in the early stages of development, exposure to unfertilised eggs maintains potential source of pollutant deposition. This would be best analysed with multiple brood analyses of Ag level, as well as measurement of Ag content in females sires throughout the period of exposure.

Measurements of Ag in rivers, lakes and estuaries indicate background values of total Ag are around the ng /l level (Kramer et al., 2002), thus our background levels of Ag in the tanks are close to natural levels, with modelled concentrations reaching 320 ng/l for European surface waters well within the scope of environmental relevance (Blaise et al., 2008). Exposure to Ag, as both Ag NPs and bulk Ag, had no adverse effects on the female sires or the developing larvae for dosed concentrations. Larval weight and survival (100%, data not shown) were not affected by Ag NP treatment (Figure 5), nor was there presence of morphological aberrations in the fry. This lack of overt toxicity is in good agreement with silver toxicity to guppies, as the 96 h LC₅₀ value for AgNO₃ to guppies has been measured at 17.14 ± 5.43 µg/l. Further data is differential with regards to ionic and Ag NP toxicity as well as fish species; Shahbazzadeh et al. (2009) reported a slightly lower 96 h LC₅₀ concentration of 2.3 µg/l for Ag MNPs to rainbow trout (Oncorhynchus mykiss) whereas a 25 µg/l and 84 µg/l 48 h LC₅₀ for Ag ions and Ag MNPs respectively was observed for zebrafish (Bilberg et al, 2012). Our exposure concentration however, was below these values, and although fish uptake issues did not arise, our delivered dose is likely to have been lower than these studies. Fish condition, cardiovascular fitness (cardiosomatic index) and metabolic load/energy reserve (hepatosomatic index) were not significantly different between the treatments (Figure 6). These measures of chronic toxicity, inform on basic effects only with more detailed measurements beyond the scope of this study. However, these simple effects relate to fundamental toxicity considerations and our data suggests the exposure level did not impart chronic adverse effects. In a mouse spermatogonia
cell line incubation with 10 μg 15 nm Ag NPs /ml for 48 h resulted in increased necrosis and apoptosis and a reduced mitochondrial function and cell viability (Braydich-Stolle et al, 2005) but there are no other studies, to the authors knowledge regarding the reproductive toxicity of Ag NPs in vivo, our study shows no effect on reproductive output for our exposure concentrations. There are, however, other in vivo studies on maternal transfer of NPs, (reviewed in Ema et al., 2010) with subsequent assessment of toxicity. Examples include, subcutaneous injection of TiO₂ in mice where there was toxicity to the gametes of pups (Shimizu et al., 2009; Takeda et al., 2009). Size related biological effects were also noted after intravenous injection of 0.8 mg of TiO₂ per mouse resulting in smaller uteri and smaller foetuses. Silica particles injected in to the caudal vein also caused a reduction in body weight of unborn pups in a size dependant manner (Nagano, 2011). Fullerene molecules were not shown to cause complications at 0.8 mg per mouse, a finding similar to an earlier study (Tsuchiya et al., 1996) where at 25 mg/kg (~0.625 mg per mouse) only one abnormal embryos was present, but showed 50 and 138 mg/kg (~1.25 and ~ 3.45 mg per mouse) resulted in 50% and 100% embryo mortality. For other carbon based nanomaterials no morphological aberrations or decreased in survival was found in rats at 1000 mg/kg/day for repeated exposure by gavage of carbon nanotubes to rats (Lim et al., 2011), although reaggregation and uptake were not assessed. Finally, low levels of intravenously injected QDs were detected in the foetus of mice, with a concentration dependant toxicological effect measuring less than 50% and 34% survival of the pups after a QD challenge containing 86 and 125 μg of cadmium (Cd) respectively (Chu et al., 2010). All of these studies, highlight the toxicity potential of nanomaterials to unborn progeny and the diverse nature of their effects from lethality to a reduction in growth. No studies are known to have primarily assessed the reproductive toxicity of nanoparticles in fish, using these studies on known toxicity mechanisms might inform on sensitive stages of development

**Conclusion**

It is essential that adequate risk assessment of nanomaterials takes place concurrently with the development in this industry. We have shown that nanoparticulate transfer of Ag occurs into developing offspring but this did not occur for larger sized bulk Ag particles. This study realises the potential scope for maternal transfer of nano-sized particles at environmentally relevant levels and although no toxicity indications were found in this study, assessment was not extensive. Using a natural dosing mechanism we highlight the importance of exposure to nanomaterials to a vulnerable and sensitive subpopulation. The range of effects in rodent models as well as the size related differences in this study, suggest that particle characteristics
and size will dictate much in terms of uptake and toxicity. Our study showed no effect of exposure duration on larval burden, however, it is a limitation of a study organism that is predominantly lecithotrophic, that maternal provisioning capabilities are not as advanced or comprehensive as more matrotrophic species. This study and supporting literature emphasises the need for further assessment of maternal transfer of nanoparticles, with emphasis on environmentally pertinent exposure routes and concentrations.

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**Figure 1.** TEM images and size distribution results obtained with FFF for the synthesised Ag NPs (A, B) and stabilised commercial bulk particles (C, D) both in citrate. TEM images and fractogram data show good conformity for the measured particle sizes.

**Figure 2.** Water Ag concentrations (ppb) for the 3 treatments, citrate control, bulk silver, and nano silver over the duration of the study. Samples were taken every 7 days from each tank and measured with ICP-MS. Silver concentrations were not significantly different between the three treatments (vertical bars represent standard error.

**Figure 3.** Weight adjusted concentrations (ppb) of silver in guppy larvae as measured by ICP-MS. Significant enhanced uptake of silver occurred for larvae (p <0.05) in tanks where nano silver was incorporated into the diet. Bars represent standard error.

**Figure 4.** Weight adjusted concentrations (ppb) of silver in adult guppies as measured by ICP-MS. Significant enhanced uptake of silver occurred for female sires (p <0.001) in tanks where nano silver was incorporated into the diet. Bars represent standard error.

**Figure 5.** Weight adjusted larval concentrations of Ag as a function of experiment duration. There was no significant impact of maternal exposure duration on Ag burden of the offspring.

**Figure 6.** Larval weights throughout the course of the experiment. There was no significant interaction of exposure duration or treatment on larval weights.
Figure 7. Condition indices of condition factor (K, right axis), hepatosomatic index (HSI, left axis) and cardiosomatic index (CSI, left axis). No significant effect of treatment was found for any indices, bars represent standard error.

References


**Figure 3**

**Larval Burden**

![Bar graph showing larval burden with values for Citrate, Bulk Ag, and Nano Ag. The graph indicates a statistically significant difference between Nano Ag and the other two groups, marked with an asterisk (*)]
Adult Burden

Ag ppb

Citrate |Bulk Ag| Nano Ag

Figure 4
Figure 5
Figure 6
Figure 7
Chapter 6

General Discussion
6 - GENERAL DISCUSSION

The field of nanotoxicology has grown rapidly since the commencement of this PhD, but the science is still in its infancy compared with other areas of (eco)toxicology. Given the importance of the nanotechnology industry and the likely role it will play in the future for global economies, understanding its impacts on both human and environmental health are crucial.

Despite the vulnerability of the aquatic ecosystem to anthropogenic pollutants, the number of studies currently assessing environmentally relevant exposures of MNPs to aquatic organisms, in particular fish, are both sporadic and limited. Many statements are made within the scientific literature that fate and behaviour assessment of MNPs are drastically needed, yet this field of nanotoxicology has received little attention, possibly due to its extremely complex nature. This basic knowledge is required to underpin almost all pertinent MNP exposure experiments. To increase our understanding on the potential impacts for a wide range of MNPs, the fundamental characteristics and interactions that drive MNP behaviour demand attention. This is a considerable challenge however requiring a multifaceted approach to inform on uptake routes and mechanisms, target organs and potential toxicity pathways as a rudimentary foundation in this diverse field of research. By building an understanding on the fate and behaviour of MNPs in the aquatic environment in addition to how and where they penetrate into exposed animals, we will be better able to undertake more targeted health effects assessments.

This chapter provides a critical synopsis of the work undertaken, placing the studies and findings into the wider context of the expanding field of nanotoxicology. The shortfalls and limitations of the studies are also discussed with regards to the experimental approaches and technical challenges that working with nanoparticles presents. Future requirements in this area of research are also highlighted, especially with regards to developing methods and techniques to better enable studies tracing the uptake and biodistribution of MNPs uptake in aquatic organisms.

6.1 Critical Analysis on major findings in this thesis

The planning and execution of most of the experiments and investigations within this thesis occurred when the study of MNP toxicity to fish was in its infancy, and there were an extremely limited number of reports on all aspects of their (eco)toxicity. During my thesis
studies, available background knowledge has grown, furnishing us with an enhanced understanding of concepts and mechanisms involved. These studies have provided information on the impact of exposure conditions on nanoparticle dynamics and the effect of particle characteristics and interactions events within an environment (such as the presence of natural organic matter) on size, uptake and even toxicity. These data have been established through a significant amount of scientific trial and error and an embracing of interdisciplinary collaboration. The lack of any substantial understanding on how to approach exposure studies and at the same time the rapid emergence in new data has meant that my thesis studies have had to be adapted constantly to try to ensure the best experimental approaches throughout. As such, the many failings in aspects of the thesis work that allowed me to adopt the best possible experimental scenario are equally as contributory to this field of research as the final results attained.

The predominant objective for this thesis was to examine and undertake experiments that would inform on the bioavailability of MNPs to fish. In order to do this, it was first necessary to investigate whether uptake of MNPs occurred and, if it did, what their biodistribution was within an organism. Imperative to these studies was the establishment of whether there were size related differences between nano-scale and micron-sized particles.

In the first thesis experimental chapter, an attempt was made to develop a high throughput \textit{in vitro} screening assay for nanoparticles. Previous studies had shown that the liver was a potential target site for MNPs and therefore the suitability of primary isolated rainbow trout hepatocytes was assessed for investigating the features of MNPs reported to induce adverse effects. Isolated fish hepatocytes have been used extensively for a screening system for xenobiotic pollutants, such as endocrine disruptors, and were applied here to a range of metal and metal oxide nanoparticles. Membrane integrity was not found to be affected by exposure to any of the MNPs tested, irrespective of the nature of the particle or size. However, increased lipid peroxidation was observed in cells exposed to 500 μg/ml ZnO nanoparticles and increased GST activity was observed in cells exposed to 500 μg/ml ZnO bulk particles. Isolated hepatocytes were visibly normal and showed evidence of intact membranes as assessed by lactate dehydrogenase (LDH) release but they did not appear to be responsive to hydrogen peroxide and silver nitrate (positive controls for lipid peroxidation and GST). Why this was the case, was not resolved. However, it is known that hepatocytes retain functional abilities found \textit{in vivo} (for example vitellogenin induction on stimulation by oestrogen (Bickley et al., 2009). There are a number of possible reasons for the lack of toxicological responses from MNP exposure, firstly it should be considered that the MNPs under examination were not toxic in
their exposed form. Secondly, only two biological responses were measured, although commonly noted responses of MNP exposure, these biological responses may not be the most applicable for detecting potential effects. Thirdly, the amount and the form of MNPs internalised into cells was not established. The aggregation of MNPs in solution is a well known occurrence, thus the effective dosing level of nano-sized particles may be far below the nominal concentration. The hepatocytes, therefore, did not appear to be a good model for investigation into the subtleties of toxic effects that may be elicited as a result of nanoparticle exposure. One factor that needed to be established to confirm the ineffectiveness of this system, given the lack of any obvious toxicological responses, was whether the MNPs penetrated the isolated hepatocytes in culture. This was investigated by performing coherent anti-Stokes Raman scattering (CARS) microscopy on cultured cells. This provided evidence of internalisation of MNPs by hepatocytes, combining z-stack image slices into a 3D reconstruction, identifying the origin of MNP CARS signal within the confines of the cell. This finding suggests that this in vitro model may have use in uptake assessment of MNPs into cells, however CARS lacks the ability to determine particle size, thus it is unknown if uptaken particles were micron sized aggregates or individual nanoparticles.

The work applying CARS to the isolated hepatocytes in chapter 1 illustrated some of the potential power and utility of the technique for application to the exposure of MNPs to aquatic organisms more widely. In this chapter, therefore, CARS was investigated as a technique to trace uptake of MNPs into a wide range of biological, particularly fish, samples after experimental exposures to MNPs. Initial investigations were required to analyse the contrast potential of different type of MNPs under the CARS system, to see what MNPs could be effectively detected. Analyses were then undertaken to investigate the range of cells, tissues and organisms (primarily from aquatic organisms), to establish the resolution and contrasting capabilities of the CARS system were, including for fresh tissue samples and live cells and organisms. Finally CARS was applied in combination to establish the ability to localise MNPs within various biological matrices. Uptake, biodistribution and the applicability of CARS in cell cultures, excised tissue and whole organisms was assessed for various metal and metal oxide particles. The contrast derived from MNPs provided a strong and easily detectable signal that facilitated particle localisation. Particle uptake was determined on a cellular level for copper (Cu) nanoparticles into hepatocytes and gold (Au) MNPs into macrophage cells. Uptake and distribution of titanium dioxide (TiO$_2$) MNPs into trout gills and kidney tissue was also determined after in vivo exposure. Imaging at the organism level showed cerium dioxide (CeO$_2$) and silver (Ag) MNPs differentially partitioned after microinjection into zebrafish (Danio rerio) and that ZnO particles entered into the gut of Daphnia magna, Corophium volutator but
did not spread further. Of particular benefit was the ability to image live samples, where, biological processes and responses that are stimulated by MNP presence are ongoing in situ. This makes the resulting image a real time snapshot of MNP interactions within a sample, which can also be used to generate three-dimensional reconstructions of the sample that enabled precise MNP signal origin. Of added environmental relevance is the lack of necessity to employ contrast agents such as stains, dyes, or fluorescent labels, which may modify the behaviour, bioavailability or toxicity of the MNPs examined. These findings, combined with the expansion of this technique to other nanomaterial types and tissues demonstrates CARS as an effect MNP tracing and uptake tool, that has many advantages over other imaging and uptake techniques. However, there are shortfalls to the CARS system and these are discussed in the below section on technical limitations. Thus, the data generated for this chapter led to the development and further understanding of what the capabilities of CARS are and its utility in nanotoxicology studies, critically however, the study also informs upon where the technique is not appropriate. Generally these limitations revolve around the spatial resolution available using CARS and the lack of quantification data produced from the imaging process.

With a significant deficit of studies looking at the fate and behaviour of MNPs within a natural and environmentally relevant system, the third study set out to inform on the consequences of the presence of natural organic matter (NOM) on the uptake of MNPs. To test this, a high (50 μg/l) and low (5 μg/l) concentration of CeO₂ MNPs and a high (250 μg/l) and low (50 μg/l) concentration of NOM were assessed in combination in a chronic, 28 day exposure to carp. Analysis of brain, gill and kidney tissue by ICP-MS showed significant presence of cerium after exposure to the highest concentrations of CeO₂ MNPs and natural organic matter, but not to other combinations of the two variables or bulk sized CeO₂ particles with NOM. As environmental conditions will play a significant part in the toxicokinetics of MNPs in aquatic ecosystems, this chronic study to carp contributes valuable data into an area where data are scarce. However, a firmer knowledge base in this area is still needed 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 impacts. A large part of the chronic exposure study to CeO₂ MNPs was geared toward understanding particle dynamics and the effect that the state of MNP dispersion, particle size and association with NOM has on uptake. Similar experiments to inform on this, that are within in an ecologically relevant system at a realistically low concentration, are extremely limited in their number due to the complexity in undertaking, measuring and concluding the study. This is mainly due to the techniques employed to measure particle size and dispersion behaviour (dynamic light scattering (DLS), transmission electron microscopy (TEM) and zeta
potential) being less sensitive when analysing environmentally pertinent samples, *i.e.* low concentrations of a poly dispersed solution, see section below. This leads to disparity between the techniques which make assessing particle kinetics and their effect on bioavailability to fish difficult to undertake. Despite this, these are some of the most predominant and readily available tools that collaborators on nanotoxicology studies possess. The lack of conformity within my results regarding the size and dynamics of MNP behaviour in the presence of NOM is of significant importance. In the field of nanotoxicology although there are frequent arguments that a multifaceted approach is required to better understand particle fate and dynamics, the available methods for doing so can produce different outcomes and this adds further complexity to the analyses and their interpretation. Of course multiple techniques are likely to have variations in agreement, but this study shows that the interpretation can have significant effects upon the data. Therefore these data are a key contribution that emphasise the importance of considering the most appropriate suite of techniques, and understanding the limitations that each possesses (see later section). Here, my study highlights the complex nature of measuring environmental samples, yet this is the goal when considering ecological impacts and risk assessment of MNPs and further development of analytical tools and methodologies for both imaging and measuring nanoparticle parameters are needed. Information on the behaviour of MNPs in varying environmental conditions 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.

Few experiments report on the effect of NOM on bioavailability of MNPs and the data here reinforces the call for natural systems to be considered when evaluating toxicity potential of novel materials. This study informs solely on the bioavailability of MNPs in the presence of NOM, with measuring biological effects beyond the scope of the experiment. However, due to potentially altered pathways of uptake, extrapolation of acute or even chronic effects measured for a specific MNP may not be suitable to studies in the presence of NOM. As the data here presents evidence that NOM may enhance MNP uptake, this is critical issue in relation to the risk assessment process. Although the underlying mechanisms of enhanced uptake are not yet fully understood, it serves to highlight an important process that is not yet substantially considered when analysing the potential for MNP toxicity.

Considering uptake pathways and life stage sensitivity to MNPs led to the consideration of the maternal-embryo relationship within organisms as a possible route of MNP exposure that has been little studied and not at all outside of mammals. A study was undertaken on a live bearing, ovoviviparous fish, the guppy. To investigate the potential of maternal transfer,
guppies were exposed to Ag nano- and bulk-sized particles dosed via the diet for a full gestation cycle and their larvae assessed for Ag content. In this study, nanoparticles were synthesized to be 7 nm in diameter and capped with a citrate coating to reduce aggregation and stability issues. Bulk particles were measured to be 125 nm and also citrate capped. The bulk particles were chosen to be only slightly larger than the conventional nano-size range to determine how significant a factor MNP size is. It is possible that different effects may have occurred for micron sized particles, and this should be investigated further. Maternal transfer of Ag was significantly higher for the nanoparticulate treatment compared with the bulk and control treatments but there was no effect of exposure duration on larval burden. Maternal burden was not assessed throughout the exposure, as all female sires were sampled at the end of the experiment. These data would have informed upon the bioaccumulation, or rapid clearance of Ag from the maternal system, and thus allow a more thorough understanding of when maternal transfer of Ag may have been most pronounced. To inform upon which developmental stage greatest uptake occurs, a study analysing Ag content in the female gametes and regular stages of fertilised embryos up until parturition could be run. This would be key to assessing the development stage at greatest potential risk and thus inform further targeted studies. A difficulty, however, in working on guppy species is their ability to store sperm after mating events, leading to different fertilisation times within a group of fish. This can impact on exposure duration of developing embryos or fry. To compensate for this, guppies can be separated from the opposite sex for over 2 months, or employ naïve females that haven’t had contact with a male. Unfortunately, this places a large demand on time and resources and wasn’t undertaken in this study. To ensure, that all guppies gave birth to young who had been exposed for a significant amount of time, the exposure ran for double the guppies’ gestation period. This study also showed no impact of Ag on larval survival or birth weights or on indices of body condition in the exposed adults. Given the extremely low level of exposure, acute effects were not expected, although early life stage sensitivity could make larvae more susceptible to nano-pollutants. The markers of toxicity, however, were not particularly sensitive, nor measured highly sensitive sub-lethal end-points. To fully inform on the impact of maternal transfer, more detailed assessment for a variety of molecular and physical markers should be employed. This study assessing maternal transfer of Ag MNPs in guppies is the first of its kind regarding natural exposure methods and in vivo assessment of Ag MNPs transfer capability to offspring. Despite guppies not being highly matrotrophic, this study suggests this species to be a good model for future assessment of MNP exposure to early-life stage organisms. This area, especially in fish, has had minimal research undertaken and represents a difficult to monitor, yet highly sensitive sub-population at risk of exposure to MNPs.
6.2 Limitations and shortfalls of the analytical techniques employed

Of the many challenges facing nanotoxicologists, one of the biggest is ascertaining both the transport pathways and final destination of MNPs in organisms subsequent to exposure. This has been an important consideration within this work, equally for confirmation of exposure or dosing success to fish and for experimental assessment of biodistribution. Although previous studies have added imaging labels or radio tracing techniques to particles, often these modifications have their own significant limitations. Imaging labels lack quantitative assessment qualities and usually require surface modification which can have potential impacts on the behaviour of the particles. Isotopically labelled MNPs, however, are by nature difficult to work with, costly and challenging to make in the nano-form, although they provide accurate measurement capabilities. The techniques below were all employed at some point in this study to measure or image MNPs and their relative limitations are discussed.

ICP-MS measurement of samples was chosen as the most appropriate method for two of the studies undertaken, determining tissue localisation and burden after MNP exposure. Its choice was determined by its increased sensitivity compared to inductively coupled plasma optical emission spectrometry (ICP-OES), where for the two elements I measured (Ce and Ag) detection limits of 50 and 5 μg/l respectively were available for ICP-OES but this decreased to 0.5 ng/l for both elements with ICP-MS. ICP-MS is not without its limitations, however, particularly with regards sample preparation. Firstly, all samples for ICP-MS processing must undergo acid digestion, and although variation in sample weight can be accounted for, accuracy of the technique with regards to tissue burdening of MNPs can give variable results if the entire tissue, organ or organism is not being measured due to differing partition of MNPs. Furthermore, the sample must be of sufficient size to record a measurement, with approximately 50 – 10 mg of tissue required. This may be regarded as a small amount, but this places a limitation on the measurement of certain organs in small fish i.e. eliminating the possibility of larval organ/tissue MNP distribution in the maternal assessment study. Additionally, samples collected for ICP-MS cannot be used for other analyses such as histological examination or toxicity testing, which is preferable to link MNP content with biological effects. This can be partially avoided if the tissue is large enough to divide, however, the earlier mentioned problems regarding MNP distribution within a tissue must be considered. Furthermore, ICP-MS measures the elemental metal content solely and the form of the metal is unclassified; thus the distinction between ionic dissociation from MNPs and particle presence is not possible and in these instances is it important to consider incorporating adequate size related controls into experiments, whereby larger particles need
to be utilised to help distinguish for effects that relate to nano-sized properties. For each experiment that utilised ICP-MS, a bulk particle control was included, although ionic controls were not. This was predominantly due to space and logistical restrictions. Consequently, the form of the metal that was uptaken in the chronic CeO$_2$ MNP exposure and maternal transfer assessment of Ag MNPs is unknown.

The employment of CARS for visualising MNP uptake has many advantages. It provides contrast based on the intrinsic molecular vibrations of a specimen, circumventing the need for chemical perturbation by exogenous labels. CARS uses near-infrared excitation wavelengths which allow microscopy at depths of several hundred microns into intact tissues and minimises photo-damage. Finally, the label-free nature of the technique requires no modification of MNPs that could alter the transport kinetics and cellular uptake of particles and eliminates the potential for false positives from MNP fluorescent labels (from leaching). However, the limitation of CARS ensures its application is a carefully considered solution and that the image interpretation is accurate. One of the restrictions on CARS is that its quantitative capabilities are limited. Despite many of the MNPs imaged are being aggregates even then it is not possible to determine the size of the smallest resolvable aggregate due limitations within the system where particles appearing to be of similar size give different signal intensities. This means size determination of a particle is not possible; unless a single nanoparticle has been imaged to provide signal comparison, however, this is not without its difficulties. The result of this is that bulk- and nano-sized particles are not discernable with CARS. Another key limitation of CARS is its spatial resolution, whilst being able to image single cells, small organelles are unable to be resolved. This limits the utility of CARS on uptake mechanism and cellular fate of MNPs. To gain more detailed resolution would require longer imaging time which exposes the sample to greater laser energy, potentially causing damage. On a practical note, although no fixative is necessary for imaging live samples, small heating events in a media solution from the laser energy creates movement within the system and consequently image capture requires a stabilised sample. This also applies to imaging zebrafish at 24 hpf, where a mild anaesthetic is required to keep the embryo motionless. Signals arising from non-resonant contributions can complicate image interpretation and limit detection sensitivity. These include the signal from solvents, such as water, that make the image not background-free. Further non-resonant signals include those from natural chromatophore pigment cells in zebrafish, these appear very similar to signals arising from MNPs. With pigmentation beginning in certain regions from 24 hpf this significantly inhibits accurate MNP detection. To counter this I experimented with propylthiouracil (PTU) to inhibit melanisation (Karlsson et al., 2001), at various concentrations and exposure duration. This facilitated imaging with successful inhibition of melanophores,
however, PTU has known toxic effects (van der Ven et al., 2006) and should not be used for anything other than imaging purposes. There are also other non-linear label-free imaging techniques based upon Raman scattering that have since improved upon certain aspects of CARS, mainly dealing with the removal of the non-resonant background. These have included stimulated Raman scattering (SRS), where if Raman shift does not match any vibrational resonance no signal is generated. However, as previously mentioned the non-resonant spectral background of CARS provides the contrast for imaging metal nanoparticles and is thus required in either the forwards or backwards detection of a sample. Further critique of the CARS process with illustrative examples is offered in chapter 3.

TEM provides some of the most powerful resolving capabilities available to nanotoxicologists allowing both single particle characterisation as well as information on particle agglomerates. Therefore, TEM offers a well established option to qualify and quantify information about particle state of agglomeration and dispersion, size and shape. With regards its usage in determining particle dynamics for the study involving NOM a critical shortfall of this technique was its extensive sample preparation. In order to have sufficient material to image on a TEM grid, a certain amount of sample concentration is required, this can involve the evaporating processes which can cause MNP movement and agglomeration or the ultracentrifuge preparation technique employed in this instance, although in both cases the increase in MNP concentration promotes the formation of agglomerates due to the higher probability of particle collisions. In summary, despite the accurate measuring capabilities of TEM, its utility for assessing particle behaviour in low concentration environmentally relevant samples is limited, due to the artefacts created by the preparation method.

The limitation of TEM is the strength of DLS measurements, where hydrodynamic diameter of MNPs can be calculated in an aqueous suspension. A minimal disruption of the sample provides more accurate information on the actual dynamics occurring within a system. However, environmentally pertinent samples tend to be polydispersed, potentially containing both nanoparticles and larger aggregates. Due to its method of size calculation DLS skews particle measurement towards larger aggregates and has a significant error in size measurements for non-spherical particles and at low concentrations. Consequently, DLS is not an accurate methodology for sizing of primary particles although can inform on agglomerates within a suspension.
6.3 Considerations for studies on MNP exposure to aquatic organisms

A significant issue for consideration when exposing MNPs to test systems is the dispersion state of the particles and the parameters that can be adjusted to alter this. The issue of whether or not to employ chemical dispersants or solvents in order to achieve monodispersed suspensions of nanoparticles in the delivery system was also given much thought and developed throughout the PhD. Initial investigations were carried out in the hepatocyte cultures as to whether nanoparticles, in their 'raw' state, could elicit toxic responses. In the chronic waterborne exposure, particles were incorporated with a type of organic matter common in the aquatic environment that has been shown to associate and aide MNP dispersion. In the final dietary study, a ‘typical’ particle coating was employed on synthesised MNPs, one that is commonly used as a dispersant in industry to maintain the nano form of a particle and thus likely to be pertinent in terms of environmental exposure. These various dispersion and exposure methods alter the aggregation and bioavailability of the particles, their nano-sized behaviours and consequently their target tissues. As can be seen in the results of this thesis, the initial results with raw particles revealed limited toxicity, yet a discernable nano-sized effect is present for the exposure with NOM and the particles coated in citrate. These studies represent the developed understanding of particle dynamics gained from previous experiments and their design reflected the current state of knowledge regarding MNP form and levels in the environment.

6.4 Future studies

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, significant efforts should be directed towards developing standardised aquatic test methodologies for examining MNP availability and toxicity to organisms. This includes variation in the parameters of similar nanoparticles including those of different size ranges as well as corresponding bulk particles in order to elucidate the relationship between particle size and toxicity. Once these standard methods are in place, similar toxicity characteristics between certain classes of MNPs (e.g. metal, metal oxides or carbon based nanoparticles) can begin to be identified, so that more predictive interpretive approaches can be developed. Development of in vitro systems offer the potential of initial high throughput screening systems for MNP toxicity, although the data presented here highlights that the biological endpoints and in vitro system used must be thoroughly assessed.
As there is currently very little known about the actual levels of MNPs within the environment this invites further study. The difficulty reached here is that, current techniques require further development to fully analyse and measure environmental levels of MNP pollutants. For nanotoxicologists this means that conducting environmentally relevant studies is not possible, and data on both classical exposure responses to MNP and environmental prevalence, are desperately required for more accurate modelling studies. Additionally, further In vivo exposures are needed to provide further detail of particle dynamics with the corresponding array of appropriate detection methods to tease apart the influence of natural exposure conditions on MNPs. Furthermore, effects data in the face of evidence for increased MNP bioavailability in the presence of NOM is urgently required, including species sensitivity to increased uptake. Studies informing upon potential differences in uptake and toxicity due to NOM interaction with MNPs are vital for informing risk assessment criteria, especially for environmentally relevant MNPs.

Due to the paucity of data with regards maternal transfer of MNPs in fish, significant scope for development is apparent. Potential future assessment includes, but is by no means limited to; multiple generation impacts, different particles types, specific developmental stage sensitivity, exposure levels and exposure methods. The study in this thesis used a fish species that is known to have a lecithotrophic strategy and it is important to consider that over half the chondrichthysans are viviparous with all the elasmobranchs showing some form of ovoviviparity and that over 500 species of teleost give birth to live young. Such is the extent of the ‘unknown’ in this field, that all the basic knowledge gathered from the development of nanotoxicology as a discipline should now be applied to intelligently assess this fascinating exposure pathway from a very basic beginning.

Initial developments from the maternal transfer study in this thesis include assessing ionic levels of maternal transfer in relation to MNPs and measuring more sensitive end points of MNP exposure, such as expression of molecular markers for metal toxicity or xenobiotic detoxification processes. The caveat is that early life stages may not have developed such response yet and as previously mentioned, each developmental stage should be assessed separately both for MNP uptake and toxicity.

On a human level, the involvement of multi-disciplinary groups is a fundamental aspect of the progression of nanotoxicology and effective communication, such as an establishment of standard terminology between groups, is essential. This is of particular importance when considering communication between different scientific fields.
6.5 Conclusion.

At this time the environmental impact of nanotechnology is unknown. Future impacts are also unclear and difficult to predict with novel applications of nanomaterials regularly and rapidly emerging. Although the depth of science has increased significantly during this period of study, many studies do not address key issues where there is a dearth on current information. Additionally uninformed research can potentially lead to stagnation in risk assessment criteria, with few well thought-out studies and many acute, environmentally irrelevant studies that present conflicting data and restrict definitive results.

The field of nanotoxicology has required many scientists to develop their knowledge into research areas normally not associated with their own. In aquatic exposures, for example, it is clear that MNPs will elicit a different behaviour to conventional xenobiotic compounds, and as such a different approach to experiments is required. What is of considerable importance is sufficient understanding of the scientific question to use the correct tools to answer it. It is likely that many studies in nanotoxicology which fail to demonstrate a ‘nano response’ are not reported. Unfortunately this means other researchers elsewhere are not aware of this and risk repeating science already undertaken.

Imperative to the development of nanotechnology and its subsequent toxicity assessment is the integrative approach of multi-disciplinary research to further the science and maximise its potential. Fortunately this is occurring, with a growing number of relevant articles appearing in a broad spectrum of journals. Yet basic parameters of MNP exposure are still often not measured, resulting in ambiguous interpretation of data. Knowing what conditions affect MNP characteristics is just one of the areas which is vitally important to assessment, yet data is sparse for real-world situations. It is likely that in 10 years time, we will have a much better understanding of the potential impact of MNPs on the environment, yet the fascinating aspect of this research area is the need for its concurrent development and the urgent need to keep up with the increasing production and evolution of nanotechnology. Axiomatically, working at the forefront of technology means the specific problems raised will be constantly changing with the advances in nano-applications. In order to confidently face this growth of nanotechnology an appropriate framework for aquatic toxicity testing must be put in place, drawing on data from reliable feeder studies.
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Chapter 8

Appendix
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 throughput 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 µg 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. 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, bronchial epithelial cells and pneumocytes and dermal fibroblast cell lines. Uptake of nanoparticles by cells has been demonstrated as well as a range of cellular responses such as inflammation, generation of reactive oxygen species, lipid peroxidation and alterations to membrane integrity.

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...
The characteristics of ENPs under such conditions are there-}

cytotoxicity of ENPs. In terms of assessing uptake into organisms or cells there are fewer standard imaging methods, although transmission electron microscopy (TEM) is sometimes used.\(^1\)\(^2\)\(^3\) 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.\(^4\)\(^5\) 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.\(^6\)\(^7\) 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\(^8\); 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\(^9\);\(^10\)\(^11\) 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.,\(^12\) 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 nonlinear optical responses, and hence large CARS signals that are independent of the vibrational frequency being probed.\(^13\)\(^14\) 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 nanoparticles 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.\(^15\)\(^16\) 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.\(^17\)\(^18\) 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 µg mL\(^{-1}\) H\(_2\)O\(_2\) 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

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<td>Particle type</td>
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Fish hepatocytes for screening nanoparticle toxicity

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 µg 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 µg mL$^{-1}$ H$_2$O$_2$, 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.
Fish hepatocytes for screening nanoparticle toxicity

**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, $P < 0.001$. 
**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...
Fish hepatocytes for screening nanoparticle toxicity

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 µg mL$^{-1}$ [30,31]. TiO$_2$ nanoparticles caused LDH leakage in mouse neuroblastoma cells and BRL 3A rat liver cells at concentrations of between 10 and 250 µg mL$^{-1}$[26,30] and Ag nanoparticles were found to cause LDH leakage in BRL 3A cells at concentrations of between 10 and 50 µg 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, a recent review 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. 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. 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$^{-1}$ and ROS-mediated toxicity in exposures to zebrafish embryos at concentrations of 1–10 mg L$^{-1}$. 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. It was surprising therefore to find that neither silver nanoparticles nor 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. Silver nanoparticles have previously been shown to cause lipid peroxidation in human carcinoma and sarcoma cells at a concentration of 6.25 µg mL$^{-1}$ and toxicity and apoptosis mediated by generation of ROS in alveolar macrophages and mouse embryonic fibroblasts at a concentration of 50 µg mL$^{-1}$. Both TiO$_2$ and CeO$_2$ nanoparticles have also been found to induce ROS in human bronchial epithelial cells at concentrations of 5–40 µg mL$^{-1}$. 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$^{-1}$. 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$^{-1}$. Interestingly, in our studies, exposure of isolated trout hepatocytes to 500 µg mL$^{-1}$ hydrogen peroxide failed to elicit an oxidative response as has been demonstrated in mammalian cell culture exposures, suggesting a low responsiveness for oxidative damage. The glutathione-S-transferase enzyme family play an important role in the defence of cells against xenobiotics. They are involved in detoxification both through catalysing the conjugation of reduced glutathione, via a sulphhydryl group to electrophilic centres on a wide variety of substrates, and through direct binding of compounds including free radicals and peroxides. Few studies have so far investigated the effect of nanoparticle exposure on GST activity. Park et al. 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 but in the other by the same research group, no effect was seen. Increased GST activity has been associated with metal exposure in several fish species. 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$_2$O$_2$, 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, but others have shown lower activity in hepatocytes than whole liver samples and both liver cell mono-oxygenase activity and conjugating activities can be rapidly lost in hepatocyte cultures. 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$^{+}$ ions into the culture media, arguably, GST activity would be expected to be elevated since GST catalyses the conjugation of zinc ions to glutathione sulphhydryl 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$^{+}$ ions with glutathione. The inability of zinc ions to cause oxidative stress in the livers of exposed fish, however, is well known 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 and in inducing oxidative stress in the mouse brain following intraperitoneal injection. 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. in our laboratory where the isolated fish hepatocytes are responsive to both cytochrome P450 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,\textsuperscript{[64]} it has also been shown that some liver functions may be lost upon culturing of the cells.\textsuperscript{[24,58]} Even if a certain level of responsiveness is maintained, subtle responses may not be measurable 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 (\textit{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, Hatchlands Trout Farm, Devon, UK) unless otherwise stated. Nanoparticle and bulk particle preparations of TiO$\textsubscript{2}$, ZnO and CeO$\textsubscript{2}$ 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.

Materials

All chemicals were purchased from Sigma-Aldrich (Gillingham, UK) unless otherwise stated. Nanoparticle and bulk particle preparations of TiO$\textsubscript{2}$, ZnO and CeO$\textsubscript{2}$ 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.\textsuperscript{[63]} All solutions were sterile filtered and all instruments and glassware were autoclaved before use to maintain aseptic conditions. Perfusion solutions based on HEPES-buffered Hank's salts\textsuperscript{[65]} were prepared as described in Bickley et al.\textsuperscript{[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$_2$O) and then 100 mL perfusion solution (Hank's solution containing 358 mg L$^{-1}$ CaCl$_2$ × 2H$_2$O 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$_2$O. 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 75g 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. Sero-well 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\textsuperscript{6} 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$^5$ 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.

Nanostructure exposures

Tryptic 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\textsuperscript{6} 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 250g 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
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.\textsuperscript{[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.\textsuperscript{[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\textsuperscript{−1} H\textsubscript{2}O\textsubscript{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 protein concentrations were determined accordingly.

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.\textsuperscript{[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 l-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 (Sonicsprep 150 MSE Scientific Instruments, UK) over ice for 3 min. The resulting homogenate was centrifuged at 2300 g for 4°C at 10 000g. 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\textsuperscript{−1} H\textsubscript{2}O\textsubscript{2} and 10 ng mL\textsuperscript{−1} and 500 ng mL\textsuperscript{−1} AgNO\textsubscript{3} were set up as positive controls due to the ability of H\textsubscript{2}O\textsubscript{2} to induce oxidative stress in cell cultures in vitro\textsuperscript{[48]} and the known toxicity of AgNO\textsubscript{3} in fish tissues.\textsuperscript{[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\textsuperscript{−1} mg\textsuperscript{−1} protein (extinction coefficient, \( \varepsilon_{340} = 9600 \frac{L \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}}{} \)).

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\textsuperscript{−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.\textsuperscript{[19]} Laser excitation was achieved using an optical parametric oscillator (Levante Emerald, APE Berlin) pumped with a frequency doubled Nd:Vanium picoseconds oscillator (OPO LEVANTE\textsuperscript{TM}, 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.\textsuperscript{[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.\textsuperscript{[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 beams by a long-wave pass dichroic mirror (2855 nm, 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\textsubscript{2} stretching vibration (2855 cm\textsuperscript{−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\textsuperscript{−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 Osiris 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 a set at 0.05. All data were checked.
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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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References


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