

**Biological effects of selected metal nanoparticles
in zebrafish (*Danio rerio*)**

Submitted by

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I certify that all the 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.

.....(Olivia Joanna Osborne)

ABSTRACT

As the nanotechnology industry grows rapidly, the release of nanoparticles (NPs) will increase in the environment. The special functional properties of NPs bring uncertainty on how they will affect exposed organisms. Aquatic organisms may be particularly at risk because this environmental compartment acts as a sink for most contaminants. Two major NPs used in the nano industry are silver (Ag) and titanium dioxide (TiO₂). Ag is being exploited principally for its antimicrobial properties and TiO₂ for its photocatalytic properties. There is evidence that both of these particles can induce harmful effects in exposed organisms but our understanding on the mechanisms for their effects, uptake and fate of these NPs is still very limited, especially for environmentally relevant exposure regimes. The studies conducted during this thesis investigated the potential for biological effects to Ag and TiO₂ in the aquatic environment using the zebrafish (*Danio rerio*).

Uptake and effects of a range of NPs of different sizes (Ag -10nm, 35nm and a bulk counterpart 600-1600nm, and TiO₂ -7nm, 10nm, 35nm and a bulk counterpart 134nm) were assessed in zebrafish embryos exposed via the water column. It was established that TiO₂NPs had no adverse effects on zebrafish embryos even at an exposure concentration of 25 000 µg/L. In contrast, AgNPs induced dose dependent lethality and the AgNP (35nm) was most toxic. At lower exposure concentrations Ag induced a number of morphological defects in embryos and apoptosis was seen to occur around 7 hours post fertilisation (hpf) in the extended yolk sac region of the embryo. Coating the AgNP materials with citrate and fulvic acid significantly reduced toxicity. Coherent Anti Raman Scattering (CARS) microscopy was applied to the exposed embryos and indicated that there was little, if any uptake of Ag/TiO₂ NPs as particles into the embryo. CARS however showed very significant aggregation of both NPs at the chorion surface. It was concluded from this work that the toxicity observed was most likely mediated via silver ions dissociating from the AgNPs. In this work, the metallothionein (*mt2*) gene was activated (detected using whole mount *in-situ* hybridisation-*WISH*) at sublethal exposure concentrations (500 µg AgNP/L and 12 µg AgNO₃/L) in the extended yolk sac region of the embryo (24hpf), which is known to be a site of metal detoxification.

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In a second series of studies to explore the mechanisms and target tissues for AgNPs, *WISH* was adopted in early life stages of zebrafish using various genes that were markers of stress responses. These genes were metallothionein (*mt2*), glutathione S-Transferase Pi (*gstp*), glutathione S-Transferase Mu 1 (*gstm1*), hemoxygenase 1 (*hmox1*) and ferritin heavy chain 1 (*fth1*). In the first instance the ontogeny of expression of these genes was established for up to 12 days post fertilisation (dpf) to determine the optimal time point to test for responses of these genes for the particle exposures. Early life stages of the zebrafish were exposed to citrate coated AgNP (10nm, 500 µg/L), Ag bulk (160nm, 500 µg/L) and silver ions (20 µg/L). The Ag materials induced upregulation in *mt2*, *gstp* and *gstm1* in various target tissues including the yolk sac, olfactory bulbs, lateral line neuromasts, ionocytes in the skin and in regions of the head. Silver ions affected the same target tissues and induced the same gene responses as AgNPs, albeit there were differences in the levels of these gene responses between these two treatments. In contrast, both *hmox1* and *fth1* were downregulated as a result of Ag exposure. To further explore the molecular mechanism by which AgNP toxicity occurs, an Nrf2 (a transcription pathway involved in oxidative stress) mutant zebrafish was included in exposures to Ag. These studies found that *mt2* and *gstp* were both expressed at lower levels in the Nrf2 mutant zebrafish exposed to Ag materials compared with in the wild type zebrafish. This suggests that the Nrf2-Keap pathway plays a key role in controlling the expression of these genes that are responding to the AgNPs exposures. This work demonstrated that *WISH* provided a highly effective integrative approach for identifying target tissues exposure to the different silver materials and for exploring functional pathways of effects.

In the final study of this thesis, adult breeding zebrafish were fed AgNP via the diet to investigate uptake and accumulation into target organs, the possibility of maternal transfer and the potential for subsequent effects in exposed offspring. Adult zebrafish were fed at a rate of 5µg Ag/g at 3% of their body weight to Ag materials (AgNP 10nm and AgB 600-1600nm) over a period of 26 days. No effects were seen in the adult fish on any of the measures taken of fitness (condition factor index, gonadosomatic index, hepatosomatic index, haematocrit index). Ag did not significantly affect fecundity (numbers of eggs spawned) or fertility (numbers of eggs fertilised). A detectable level of uptake of Ag in target organs occurred only in the AgNP treatments: liver (up to 2.1 µg/g in males) and gonads (up

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to 0.5 µg/g in males), suggesting particle size plays a role in the uptake and translocation through membranes into target organs and thus an enhanced bioavailability for AgNP compared with AgB. Maternal transfer was only detectable in the AgNP treatment (up to 0.43 ng Ag/per embryo). Gene responses of *mt2* and *gstp* measured in the subsequent offspring via *WISH* analysis at 24 hpf indicated significant *mt2* upregulation occurred after 2 weeks of adult exposure in both AgNP and AgB treatments. This demonstrates the potential for maternal exposure effects for exposure to AgNPs. Furthermore, challenging the subsequent embryos to the same Ag materials and measuring responses of *mt2* via *WISH* indicated de-sensitisation to Ag in offspring where adults were treated with both nano and bulk forms of Ag. This finding has important considerations for risk assessments for silver base materials.

Overall, the findings presented in this thesis have provided a body of evidence to show that silver material on a nanoscale may be of a greater hazard to fish and this warrants further investigation to consider more appropriately the potential risks associated with discharge of AgNPs into the aquatic environment.

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I dedicate this letter as an expression of gratitude to you.

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To the TK lab group; *Probably the best lab group in the world*. For their support in the lab, and their banter outside of it. Website: over 5,400 views #worldwide

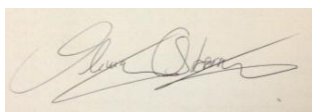
To my comrades past/present in no particular order: General~~, Corporal, Private, Captain, Lieutenant...etc. A massive thank you goes to you all for the support and love you have given me. You have ridden this wonderful PhD journey with me, kept me sane (questionable) and provided me with friendships and memories that I will cherish forever. Shout out goes to my second in command: Commander- for sharing my vision, concept and *avant garde* life style.

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Lots of Love Olivia (Master)



“Water is the driving force of all nature”

(Leonardo da Vinci 1452-1519)

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RESEARCH PAPERS AND AUTHOR'S DECLARATION

Research Paper 1. Osborne, O. J., Johnston, B. D., Moger, J., Balousha, M., Lead, J. R., Kudoh, T. & Tyler, C. R. (2013). Effects of particle size and coating on nanoscale Ag and TiO₂ exposure in zebrafish (*Danio rerio*) embryos. *Nanotoxicology* 7 (8) 1315-1324.

Research Paper 2. Osborne, O.J., Kobayashi, M., Nakajima, H., Mukaigasa, K., Stolpe, B., Romer, I., Hirose, S., Lead, J.R., Kudoh, T. & Tyler, C. R. (2013) Sensory systems and ionocytes are the potential target tissues for silver nanoparticles in fish. (To be submitted to *Nanotoxicology*)

Research Paper 3. Osborne, O.J., Kudoh, T. & Tyler, C. R. (2013) Altered toxicological responsiveness in larval zebrafish after parental dietary exposure to silver nanoparticles. (To be submitted to *Nanotoxicology* or *ACS Nano*)

Statement: I, Olivia Osborne, was involved in the following parts of the presented papers:

Paper 1: I planned and carried out the exposures, dissolution experiment, video analysis, imaging, apoptosis assay as well as writing the paper (with valuable input from co-authors). I designed the *mt2* plasmid for probe synthesis for *in-situ* hybridisation with guidance from Tetsuhiro Kudoh. I also undertook CARS microscopy with assistance from Julian Moger and Blair Johnston. Nanoparticle characterisation work was carried out by Mohamed Baalousha and ICP-MS dissolution analysis was done by the University of Birmingham FENAC team led by Jamie Lead.

Paper 2: I planned and carried out the ontogeny work via *in-situ* hybridisation on 5 stress response genes. The *mt2* plasmid made in Paper 1 was used for this paper as well. The *gstp*, *hmox1* and *fth1* plasmids for probe synthesis were acquired from the University of Tsukuba. I designed the *gstm1* plasmid for probe synthesis for *in-situ* hybridisation with guidance from Tetsuhiro Kudoh. I carried *in-situ* hybridisation and imaging on all exposure work. The antibody staining for ionocytes was done by me using the antibody obtained from Shigehisa Hirose from the University of Tokyo. I performed all exposure work, except the Nrf2 mutant work which was carried out by Katsuki Mukaigasa from the University of Tsukuba. I carried out the *in-situ* hybridisation analysis and imaging on the Nrf2 mutant analysis samples. All imaging analysis was performed by me. Characterisation work and dissolution was done by

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Bjorn Stolpe from the University of Birmingham and me. I wrote the paper with editing help from Charles Tyler and Tetsuhiro Kudoh.

Paper 3: I planned and carried out the breeding exposure work including making the synthetic flake. Gregory Paull set up the aquarium facilities and raised the fish for the experiment. I digested the tissue samples/embryos. ICP-MS analysis on tissue samples/embryos was done by the FENAC facility and University of Plymouth. I performed the *in-situ* hybridisation analysis, exposure work and imaging. Characterisation work and dissolution was done by Bjorn Stolpe from the University of Birmingham. I wrote the paper with editing help from Charles Tyler and Tetsuhiro Kudoh.

Charles Tyler and Tetsuhiro Kudoh contributed to study design on all research papers.

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LIST OF ABBREVIATIONS

Al	Aluminium
AFM	Atomic Force Spectroscopy
Ag	Silver
AgNO ₃	Silver nitrate
Ag ⁺	Silver ion
AgB	Ag Bulk
AgNP	Ag nanoparticles
AuNPs	Gold nanoparticles
CARS	Coherent Anti Raman Scattering
CuNPs	Copper nanoparticles
CNTs	Carbon Nanotubes
DLS	Dynamic Light Scattering
hpf	Hours post fertilisation
dpf	Days post fertilisation
Ce	Cerium
Cu	Copper
Cr	Chromium
C ₆₀	Fullerene
Fe	Iron
Fth1	Ferritin heavy chain 1
GSH	Glutathione
GST	Glutathione S Transferase
Gstp	Glutathione S Transferase pi
Gstm1	Glutathione S Transferase mu 1
Hmox1	Hemeoxygenase 1
ICP-MS	Inductively coupled plasma –mass spectroscopy
LC ₅₀	Median lethal concentrations
Mg	Magnesium
Mt	Metallothionein
Mt2	Metallothionein 2
NOEC	No observable effect concentration
Nrf2	Nuclear factor erythroid 2-related factor 2
NP	Nanoparticle
QD	Quantum Dots
QSAR	Quantative structure-activity relationship
QNTR	Quantitative nanostructure-toxicity relationship
PAH	Polycyclic Aromatic Carbons
PEC	Predicted Environmental Concentrations
ROS	Reactive Oxygen Species

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TEM	Transmission electron microscopy
TiO ₂	Titanium Dioxide
STM	Scanning Tunneling Microscopy
UFP	Ultrafine Particles
UO ₂	Uranium dioxide
WISH	Whole mount in-situ hybridisation
UV	Ultraviolet
Zn	Zinc
ZnONPs	Zinc oxide nanoparticles
ZnO ²⁺	Zinc Oxide ions
ZnS	Zinc Sulphide
Zr	Zirconium

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LIST OF SPECIES

Algae

Chlamydomonas reinhardtii

Pseudokirchneriella subcapitata

Thalassiosira weissflogi

Plant

Nicotine plant

Nicotiana tabacum

Bacteria

Escherichia coli

Crustaceans

Corophium volutator

Fish

Eurasian perch

Perca fluviatilis

Largemouth bass

Micropterus salmoides

Rainbow trout

Oncorhynchus mykiss

Medaka

Oryzias latipes

Zebrafish

Danio rerio

Invertebrates

Freshwater Flea

Daphnia magna

Nematode

Caenorhabditis elegans

Molluscs

Asian clam

Potamocorbula amurensis

Baltic clam

Macoma balthica

Icelandic Scallop

Chlamys islandica

Moth

Tobacco horn worm

Manduca sexta

CHAPTER 1

INTRODUCTION

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Foreword:

Nanotechnology is considered one of the most pioneering areas of technology in the 21st century. It will not only influence the way in which technology advances but will have a significant impact economically, socially and environmentally.

1. INTRODUCTION

1.1 THE 'ORIGINS' OF NANO

A nanoparticle is defined as an ultrafine material between 1-100nm. The term "nano", means dwarf in Greek and as a unit measure (n) is one billionth. The history of nanotechnology dates back to the 1950s when physicist Richard Feynman described the possibility of altering atoms to a smaller magnitude and as a result changing their physical properties. In 1974 the term nanotechnology was derived as "the processing of separation, consolidation, and deformation of materials by one atom or one molecule" (Taniguchi, 1974). In the late 1980s Dr Eric Drexler wrote the book *Engines of Creation: The Coming Era of Nanotechnology*; and this spurred scientists to explore the possibility of self assembly of particles on a nanoscale (Drexler, 1981). This is the process of particle self replication and assembly mediated with chemicals. Technological advances such as Scanning Tunneling Microscopy (STM) were fundamental in the advent of nanoparticle creation and nanotechnology itself. Scientists subsequently helped grow public awareness in this field and public funds were used to aid the establishment of the associated industries exploiting nanomaterials.

1.2 PROPERTIES OF NANO

Exploring the surface and interface of bulk materials (a material at a macro scale that has constant physical properties due to its size) led to an understanding on surface effects and in turn studies investigating the conduction of electrons. Nanoparticles (NPs) can be highly mobile and exert quantum effects which possess size dependent properties including; their reactivity, conductivity and optical properties. An example of this is gold; in bulk form it has a yellow/gold colour, however, at the nano scale it is exhibited as a red/purple colour. The structure of the nano establishes their properties. Encompassing the fact that there is a

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size difference between the molecular and solid state structures creates new advantages to these particles. Some of the properties that can be achieved are: altered thermo physical properties (He et al., 2007), lower melting temperature (Goldstein et al., 1992) and a higher self diffusion coefficient, meaning a faster diffusion of particles by greater chemical potential (Horvath et al., 1987). The properties such as the interactions between the nanos themselves, also determine how the particle behaves (Kelly et al., 2003). These are usually dominated by weak Van der Waals forces or covalent interactions.

NPs can be found in different states: in a suspension (solids in a liquid form), aerosol (liquid phase in air) and emulsion (two liquid phases). NPs can be a wide range of different structures and compositions, illustrated in the figure below (Figure 1; taken from Buzea et al., (2007)). They are usually classified according to their morphology, dimensionality, whether they are coated or non-coated and the agglomeration state. NPs can be composed of one or various chemicals. Based on their agglomeration state (which can change due to electromagnetic or chemical properties) they can be made into one, two or three dimensional (D). (1D) means the NPs can be thin films or surface coatings, (2D) fixed long structures that contain nanostructures in two dimensions at the nanoscale or (3D) materials that are all at the nanoscale in three dimensions usually fixed in small structures or membranes with nanopores that contain free small NPs. Their morphology determines whether they have high-aspect ratio or low-aspect ratio. The high-aspect ratio ones include nanowires, nanozigzags and nanotubes; the low-aspect ratio ones include nanospherical, nanocubes and nanopyramids.

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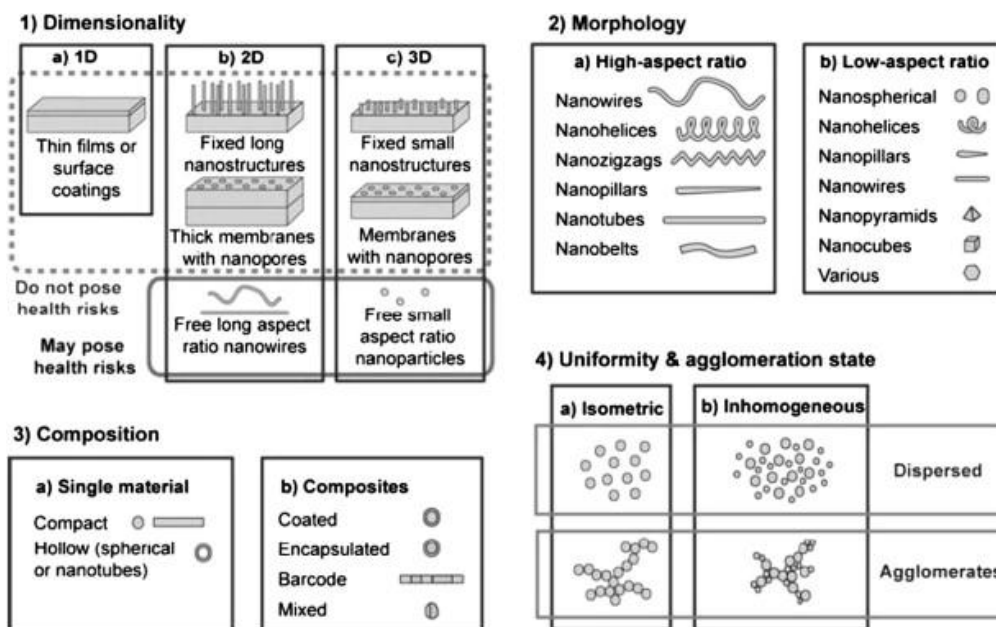


Figure 1. Classification of nanostructured materials considering their nanostructure dimensions, morphology, composition, uniformity and agglomeration state.

1.3 NATURAL NANOPARTICLES

NPs can also derive from natural sources (Banfield and Zhang, 2001), and exist in the atmosphere, ocean and subsurfaces. These natural NPs can be formed by atmospheric, geogenic, pyrogenic or biogenic. Environmental NPs are usually by-products, arising as a consequence of combustion in cars with defective catalysts (Zhiqiang et al., 2000), or from diesel engines (Schneider et al., 2005). They can be inorganic or carbon containing NPs. For example; some fullerenes usually considered to be man-made, have derived from polycyclic aromatic carbons (PAH) formed from algal matter at temperatures between 300-500°C in the presence of sulphur or during a natural combustion procedure. Some of these natural fullerenes are also believed to have arrived on earth via comets or asteroids (Becker and Bunch, 1997).

Atmospheric Ultrafine Particles (UFP) form in atmosphere via nucleation processes, chemical reactions in atmosphere that lead to chemical species with low saturation vapour pressure (Covert et al., 1992). The formation of the NPs can vary due to geographical location, season and types of reactants in the atmosphere. Routes by which atmospheric

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UFPs are produced, can include volcano eruption (Gislason et al., 2011), pyroclastic cloud from an active volcano and forest fires (Ribeiro et al., 2010). Other natural NPs include soot NPs, also known as Black Carbon (BC) continuum; these are a product of incomplete combustion of fossil fuels and vegetation.

It has been discussed that dust storms are the largest single source of environmental NPs. A case example of this, is dust storms that occur in the Gobbi dessert strongly affect the air in Asia and North America (Husar et al., 2001). It has been calculated that 90% of aerosol NPs are natural origin, and the other 10% are anthropogenic (Taylor, 2002). Weathering of oxides, minerals and silica can produce NPs such as amorphous silica, halloysite and magnate oxide. Other weathering examples include; sulphide rich rocks form NPs such as Ferrihydrate when mixed in natural water due to changes in pH, temperature and higher oxygen concentrations (Nowack, 2009). As a result of weathering, natural NPs can exist in the aquatic environment (Wigginton et al., 2007). An interesting natural NP recently discovered is Uranium dioxide (UO₂) NPs; which is formed from a uranyl reaction with green rust (O'Loughlin et al., 2003). It is known that microorganisms can also produce NPs through metabolic energy pathways involving ions that take part in redox reactions. Some examples include sulphate-reducing bacteria producing sulphides such as Zinc sulphide (ZnS).

1.4 NANO INDUSTRY

The nanotechnology industry has boomed in recent years and assured us high performance products for a range of applications in the modern consumer world. It is likely to be one of the most dominating technologies of the 21st century. It is currently considered to be a multibillion \$ industry in the US market and by 2015 it is expected to grow to 1 trillion US\$ (Aitken et al., 2006). Most NP manufacturing takes place in the United States (49%), followed by the European Union (30%) and then the rest of the world (21%) (Chaudhry et al., 2005).

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1.5 NANO PRODUCTS

According to the Project for Emerging Nanotechnologies (PEN) inventory, there are currently over 1000 consumer products on the market that contain NPs. The nanotechnology industry is continually presenting us with new refined products with novel functions that are impacting the consumer market. It is interesting to note that a new range of nano-foods are becoming increasingly available (Chaudhry et al., 2008), promoting health benefits and enhancing taste. PEN stated there are 3 to 4 nano containing manufactured goods entering the market per week. The following are areas and products in the market that are considered to have high market value:

- **Human health:** Cancer therapeutics and drug delivery
- **Consumer products:** cosmetics, sports equipment and food storage
- **Defence:** Lightweight armour and energetic materials
- **Energy:** Improved efficiency and catalysis
- **Agriculture:** Increase crop yields and secure packing
- **Environment:** Remediation, water filtration and reduced air emissions

The consequence of nanotechnology growth and its potential worldwide means that it is estimated to be worth \$2.6 trillion in manufactured goods by 2014 (Hullmann, 2007).

As a result of nanotechnology industry growing, aquatic organisms may be at risk as their environmental compartment act as a sink for many contaminants including NPs. In this thesis work, the two nanomaterials studied were silver and titanium dioxide; both of which are heavily used in the nano industry. This thesis looks at the potential biological effects of these two nanomaterials in zebrafish (*Danio rerio*).

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1.6 NANO PRODUCTION

There are a range of chemical and physical methods used to synthesise NPs but all use toxic chemicals to do so (Krutuyakov et al., 2008). There are two classified chemical and physical methods in the production of NPs. The first one is classified as the “top-down” method, which involves breaking up bulk materials and then stabilizing the particles by addition of a colloid. The second method is “bottom-up” and requires the reductions of metals, electrochemical methods or sonodecomposition that uses ultrasonic waves to generate cavitations. As a result the solution produces microscopic bubbles that eventually enlarge and burst. As a favourable alternative for producing NPs there are also biological methods which use microbes and plants (Gilaki, 2010). There is a growing need for environmentally friendly (and economically favourable) technologies to help avoid the use of toxic reducing and stabilising agents in the NP industry.

1.7 TITANIUM DIOXIDE NANOPARTICLES

Titanium dioxide nanoparticles (TiO_2NPs) are particles of titanium dioxide (TiO_2) that have photo catalytic properties making them anticorrosive. Traditionally TiO_2NPs were used as a white pigment in products such as paints and toothpastes; however, with the expansion of the nano industry their use has been broadened extensively. Uses of TiO_2NPs now include in self-cleaning textiles and anti-fogging windows. They are also being included in cosmetic products to reduce the visibility of wrinkles via light diffusion (Anselmann et al., 1998), enhance penetration of certain vitamins and antioxidants in the epidermis (Mu and Sprando, 2010) and in sunscreens for their high UV absorbing capability (Popov et al., 2005). Current research is trying to increase their solar absorption capability through new production methods, such as doping them with non-metallic elements creating new optical absorption through electronic transitions (Chen et al., 2011b). Other uses of TiO_2NPs include, as photocatalytic agents in the treatment of water to aid organic chemical removal. TiO_2NPs are also being used to develop new ways or detecting disease and combating antibiotic resistant strains such as *Staphylococcus aureus* by exploiting their photocatalytic properties which produce action oxygen species (Shiraishi et al., 2009) that destroy the outer cell membrane that leads to death (Wiesenthal et al., 2011).

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TiO₂ is a natural occurring mineral that can be found in three crystalline forms: rutile, anatase and brookite. Rutile is the most common form found in nature. There are numerous techniques to create TiO₂NPs; the most common way is through hydrolysis of titanium salts in an acidic solution (Mahshid et al., 2007). With further treatment, for example a chemical vapour, the shape, structure and size can be altered, as required (Wu et al., 2005). Anatase is the most favourable form used in consumer products that require photocatalytic properties, such as self cleaning products or sunscreens (Sadrieh et al., 2010). The rutile form provides white pigment and is used in consumer products such as toothpaste and paints (Weir et al., 2012).

TiO₂NPs also have biocidal action. It is thought that under UV light conditions the NPs catalyse lipid peroxidation (Maness et al., 1999) and reactive oxygen species (ROS) subsequently play an important role in antibacterial properties (Blake et al., 1999). As with other compounds that are antimicrobial, it is thought that the inner cell membrane of the microbe is disrupted and this leads to cell death (Saito et al., 1992). Within the cell, leakage of K⁺ ions occurs causing changes in the cell permeability (Lu et al., 2003). There is also evidence that the outer membrane can be disrupted by photocatalytic action, and this also can induce cell death (Sunada et al., 1998). Increasingly TiO₂, and silver NPs are being used because of their photocatalytic properties as antimicrobial agents. The increasing use and exploitation of TiO₂NPs in this regard will inevitably mean increases in their environmental discharge and the greater potential for impacts on ecological systems.

1.8 SILVER NANOPARTICLES

Although silver (Ag) has been used for over 2 000 years, it is only recently that it has been exploited widely in biological, chemical, physical and pharmaceutical applications. Silver nanoparticles (AgNPs) are being applied to increase thermal and electrical conductivity; enhance optical spectroscopies to efficiently harvest light and as a biosensor for quantitative detection (e.g. pathogens, toxins). The major commercial use of AgNPs is in consumer products for their antimicrobial properties. They are being used in food containers-to keep food fresh for up to three times longer than occurs for conventional

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storage methods, in clothing equipment for promoting anti-bacterial, anti-fungal, anti-odour resistant and in epilators for inhibiting the growth of microorganisms on the head.

The mechanism of AgNPs antimicrobial action is not fully understood. It is believed that AgNPs are able to accumulate on the outside of the surface forming 'pits' (Sondi and Salopek-Sondi, 2004) which in turn cause disruption of the permeability of the membrane subsequently leading to cell death. Another theory is that the AgNPs penetrate the cell membrane and interact with DNA bases (Hatchett and White, 1996) that then disrupts DNA replication and therefore cell death ensues. Another possibility is that AgNPs accumulate on the surface of the cells and release the Ag ions that interact with the thiol group in enzymes and inactivates them (Liau et al., 1997). It is also known that that Ag generates ROS causing inhibition of a respiratory enzyme which induces the cell to induce an autodestruct system, again leading to cell death (Stohs and Bagchi, 1995). In eukaryotic cells, it is thought that the Ag inhibits the uptake of phosphate and causes an efflux of intracellular phosphate. It has also been suggested, that AgNPs can inhibit the antioxidant defense by interacting directly with Glutathione (GSH) binding, GSH reductase or other GSH maintenance enzymes (Carlson et al., 2008).

Ag is now one of the most commonly engineered nanomaterials. It has been stated that of over the 1 000 nano consumer products on the market, 25% contain AgNPs (Marambio-Jones and Hoek, 2010). AgNPs are mostly comprised of silver oxide. There are two main methods to synthesise AgNPs, one physical and one chemical. Physical processes use evaporation/condensation or laser ablation of the bulk metal and then adding colloid stabilising agent. The chemical method is reduction of the metal by a chemical such as sodium borohydride and in the presence of a colloid stabiliser. When synthesised, AgNPs usually exhibit a yellowish brown colour in an aqueous solution due to excitation of surface plasma vibration.

AgNPs can also be synthesised biologically. The three main biological ways to synthesise AgNPs are by using silver synthesising organisms. The first of these uses a silver-synthesising bacteria e.g. *Pseudomonas stutzeri* a strain of bacteria isolated from a silver mine (Haefeli et

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al., 1984), that has adapted to tolerate metal ion through a number of ways such as alteration in solubility (i.e. controlling how much metals enters cells) and lack of metal transport systems. The silver-synthesising bacteria convert nitrate into nitrite through nitrate reductase enzyme. The second method utilises silver-synthesising fungi such as *Fusarium oxysporum* (Mandal et al., 2006). Here, the surfaces of fungal cells are used to trap Ag^+ ions and then α -NADPH dependent nitrate reductase enzyme converts silver ion (AgNO_3) to AgNPs. The major benefit of this process is it produces larger amounts of NPs. The third method uses silver-synthesising plants. Here, reduction of ions uses phytochemicals such as flavones, ketones and aldehydes. A recent example of this has been the use of a carob leaf extract to synthesise AgNPs ranging in size between 5-40nm, conducted in under 2 minutes at ambient temperature (Awwad et al., 2013). This method is especially safe and lacks toxic by products (Prabhu and Poulouse, 2012).

Ag particles can be produced and supplied as uncoated or as coated materials. Uncoated materials tend to aggregate which is not desirable. Coating of particles with peptides, sugars, citrate and polymers prevents aggregation and allows for more even dispersions. Due to their small particle size and high surface area to volume ratio, dissolution of the silver ions from AgNPs can occur at a faster rate compared with bulk counterpart Ag materials. AgNPs are produced and supplied in a variety of shapes, including as triangles, rods and spheres and they are usually in the size range of 1-40nm, but they range in size up to 100nm depending what their usage or purpose they will be used for.

1.9 OTHER METAL/METAL OXIDE NANOPARTICLES AND THEIR APPLICATIONS

Other metal oxides that are being used widely in industry include Zinc (Zn), Cerium (Ce), Copper (Cu), Iron (Fe), Aluminium (Al), Magnesium (Mg), Zirconium (Zr) and chromium (Cr).

Zinc oxide nanoparticles (ZnONPs) have UV light absorbing properties and are used in a wide range of applications spanning topical sunscreens (Becheri et al., 2008) to hybrid solar cells (Beek et al., 2004) for producing “green electricity”. ZnONPs are also used in garments to

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improve breathability and reduce friction (Yadav et al., 2006). In addition, ZnONPs are used to produce antimicrobial materials because they are stable and generally considered safe for humans (Liu et al., 2009).

Cerium oxide nanoparticles (CeO_2NPs) have been used in a number of applications that include sunscreens- for their UV absorbing properties (Wu et al., 2010a), fuel additive to promote combustion, and in polishing agents. However, most recently their main use has been in biomedical applications for their antioxidants properties; this includes radiation protection to cells. As with other metal oxides, CeO_2NPs are also known to be antimicrobial and therefore research is ongoing to see how these properties can be exploited.

Copper oxide nanoparticles (CuONPs) possess magnetic, catalytic and optical properties amongst others that are used in a diverse number of sectors in industry. CuONPs are being applied to plastics, coating and textiles for their anti-fungal/anti-microbial properties (Ren et al., 2009) and to produce high strength metals and alloys (Karlsson et al., 2008). They can be an efficient catalyst enhancing the rate of chemical reactions in manufacturing processes. In the medical sector, CuONPs are being introduced in diet supplements to increase delivery of vitamins and minerals more efficiently.

Zirconium oxide nanoparticles (ZrO_2NPs) are mainly used for their thermal, optical and physical properties. They are applied in the production of ceramic pigments, glazes and fire-retarding materials due to heat insulating properties (Mueller et al., 2004). Interestingly ZrO_2NPs (single crystals) are used as a diamond stimulant in jewellery. Research is currently underway into applying ZrO_2NPs (single crystals) into concrete to increase quality and tolerance in structures as zirconium crystals are known to display mechanical properties of high strength and flexibility (Negahdary et al., 2012).

Gold nanoparticles (AuNPs), also named gold colloids, are the most stable metal NP (Daniel and Astruc, 2004) and are being used in biomedical applications for their optical, catalytic and magnetic properties. They are specifically being developed to provide drug delivery methods systems as they can be uptaken by human cells but do not induce cytotoxicity

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(Connor et al., 2005). This non-toxic method of drug delivery is under current research for transporting and releasing pharmaceuticals and for applications spanning cancer therapy to condition diagnosis (Coelho et al., 2013). The targeted delivery of the drug is achieved by an internal factor such as pH (Polizzi et al., 2007) or glutathione (Hong et al., 2006) that triggers drug release.

1.10 ENVIRONMENTAL NANOTECHNOLOGY

There is a branch of nanotechnology dedicated to researching products that can be used to enhance environmental quality, referred to as Green Nanotechnology. These areas include researching into the prospect of producing cleaner energy. Some examples of this include; reducing energy consumption through the use of lighter insulation systems, more efficient heating and cooling system using nano polymers that avoid the production of fluorocarbons, longer lasting batteries produced by copper hexacyanoferrate NPs that allow faster charging and discharging of electricity which would create a low cost battery with increased longevity. Other areas using nanotechnology to improve the environment include in remediation of pollutants. For water, this includes the nanomaterials applied to water filtration methods. Nanotechnology therefore offers exciting opportunities to reduce environmental wastes and reduce the adverse impacts man is placing on the environment due to consumerism. The schematic below (Figure 2) from Bystrzejewska-Piotrowska et al., (2009) shows the pathways where nanotechnology can help to improve sustainability.

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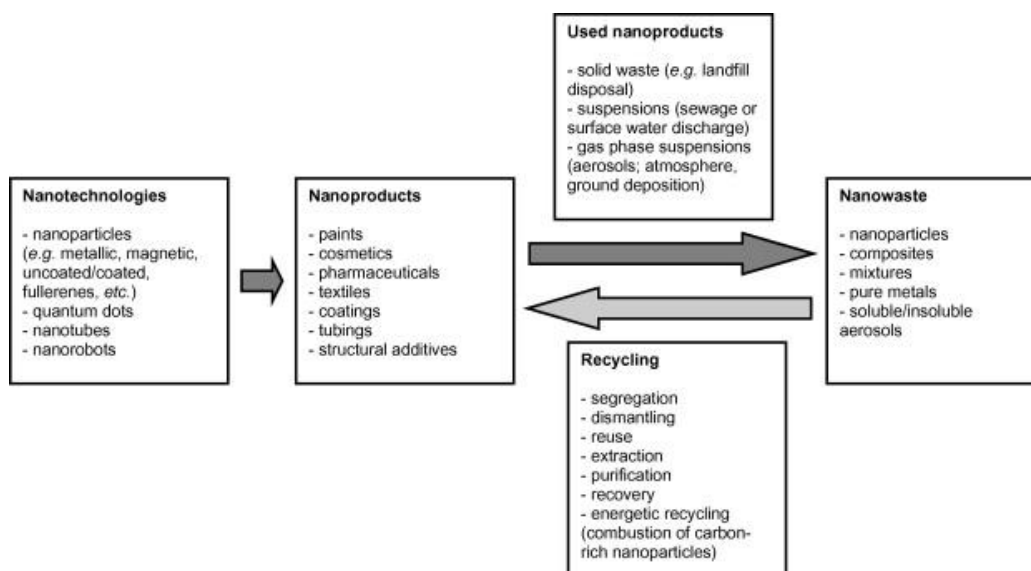


Figure 2. Towards 'green nanoscience': a schematic showing pathway from nanotechnology to nanowaste (Bystrzejewska-Piotrowska et al., 2009).

It is being increasingly recognised that in the future with the development of novel NPs the issue of environmental effects and impact needs to be part of the thinking in their production. The fact that eco friendly methods can actually lower the cost of manufacturing should attract industries in some of the greener nanotechnologies.

1.11 ENVIRONMENTAL IMPACT OF NANOTECHNOLOGY

Nanotechnology is advancing rapidly (Piccinno et al., 2012) with the development of and application of many more products. As nanotechnology becomes increasingly part of our everyday lives more commercially available anthropogenic NPs will inevitably enter the environment through numerous routes. In the first instance, the manufacturing process generates toxic waste (nanopollution) that inevitably will disseminate into the aquatic environment. Once the substance is manufactured, there is concern over waste disposal or recycling. In addition, after synthesis of NPs the chamber where they are made also has to be cleaned. As a result, the water from the cleaning brushes and sponges will enter into waste stream. A schematic diagram outlining some of the routes of entry into the environment is shown in the schematic below (Figure 3).

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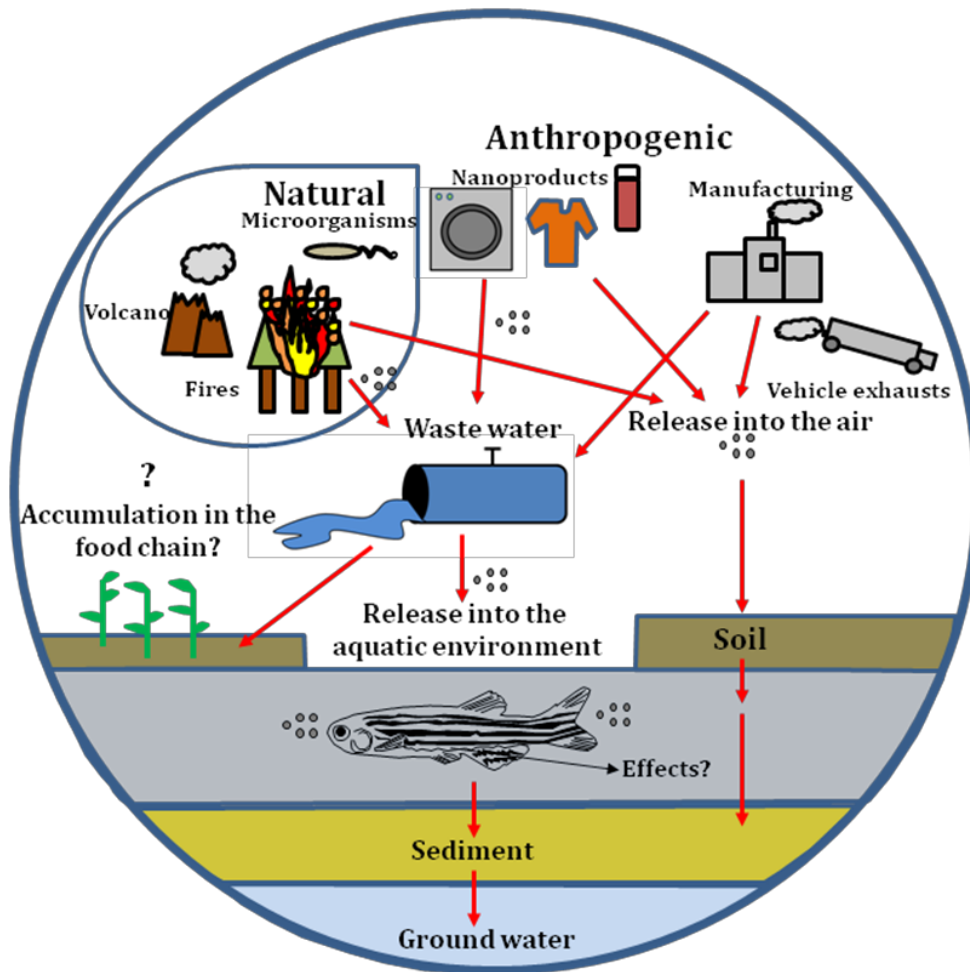


Figure 3. Schematic diagram showing how nanoparticles will enter the environment.

There are also natural occurring NPs as discussed earlier, which would also have to be taken into consideration. Recent research suggests that carbon monoxide and other combustion gases that contain NPs produced during a forest fire remain in the earth's stratosphere. Some anthropogenic NPs are released into the air via aerosols through industrial combustion and vehicle exhausts. Aerosol NPs <100nm levels can range between 5 000 and 10 000 particles/ml and can reach to 3 000 000 particles/ml in heavily pollution events (Donaldson and Stone, 2003). Car emissions provide a major source of NPs into the environment (Palmgren et al., 2003) and inhaling such particles has been shown to pose health risks (Donaldson et al., 2001). Road traffic density has a major bearing on the associated particle numbers and size; in some urban areas emission numbers have been

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estimated to range from $(2.8 \pm 0.5) \times 10^{14}$ particles/km, (1.3 ± 0.2) g NO_x/(veh km) and (11 ± 2) g CO/(veh km) per average vehicle (Ketzler et al., 2003). They can also be released in the air through cigarette smoke (Zhiqiang et al., 2000). Another route of release is through the products of incomplete combustion of fossil fuels and vegetation which produces soot NPs. It has been estimated that 0.05 to 0.27 Gt/year are produced via biomass burning (Kuhlbusch and Crutzen, 1995) and 0.012 to 0.024 Gt/year from fossil combustion (Penner et al., 1993).

Major discharges of NPs into water also occur via domestic discharges. Inevitably NPs will pass through sewage treatment plants and they will end up in our freshwater and eventually marine ecosystems. Countries such as the USA use a large proportion of sewage sludge as a agricultural fertiliser (Nicholson et al., 2003) and this will in turn deposit nanomaterials directly onto soils with the potential for uptake into human crops and or leaching into water systems. TiO₂NPs derived from anthropogenic use have been reported in sewage treatment plants (Westerhoff et al., 2011). For predicted environmental concentrations (PEC) see modelling section. Some NPs leach very slowly from the consumer products. As an example, it takes up 3 years for Ag in plastic products in continuous contact with water to be completely released (Blaser et al., 2008).

There is an uncertainty as to whether ultra fine nano materials will cause environmental impact on ecosystems and the organisms they contain. Concerns include the potential for bioaccumulation and the potential also for them to transport other contaminants into the bodies of exposed animals. There is some evidence that some NPs can trophically transfer from organism to organism. Examples of this include for the transfer of TiO₂NPs (21nm) to daphnia into zebrafish (Zhu et al., 2010b), and for AuNPs (15nm) from the *Nicotiana tabacum* (nicotine plant) to *Manduca sexta* (tobacco horn worm) (Judy et al., 2010). This may also be of concern for human health for NPs applied via fertilisers to soils could end up in the food chain (Rico et al., 2011).

Release and Environmental Impacts of TiO₂NPs

It has been predicted that by 2025 2.5 million metric tons of TiO₂ will be produced commercially (Robichaud et al., 2009). A modelling study predicted that as a result of urban

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applications and weathering a significant amount of TiO₂NPs (0.7-16 µg/L) end up in natural waters (Mueller and Nowack, 2008). A study estimated 47 300 kg/y in Switzerland surface waters (Battin et al., 2009). As these TiO₂NPs end our aquatic systems, these NPs will also be exposed to environmental factors such as light. As TiO₂ is known to have high photocatalytic activity, this could induce ROS and in turn oxidative stress in microbial communities including planktonic microorganisms.

Release and Environmental Impacts of AgNPs

Different NPs will cause different toxicological effects and their impacts on the environment will also depend on their fate in the environment. A major area of concern is for AgNPs as they are produced in high volumes for commerce. It has been estimated that 620 kg/y will enter surface waters of Switzerland (Battin et al., 2009). When Ag nano products are washed or discarded, they enter the environment and with resulting dissociation of silver ions which are toxic to aquatic life (Hogstrand and Wood, 2009) (see nanotoxicology section). The amount of Ag dispersed into the environment could be substantial enough to pose a risk to aquatic systems (Luoma and Nanotechnologies, 2008). A case example of this is how the release of silver ions (from 300-600 µg/g in the winter season) during the 1980s in the San Francisco Bay area led to sterility of *Macoma balthica* clams which were found to contain 15 µg/g (Cain and Luoma, 1985, Boisson et al., 1998). Another study in 2003, showed *Potamocorbula amurensis* clams had 60% reduction in their reproductive output when Ag exceeded >2 µg/g (Brown et al., 2003). Another concern with AgNPs is the possibility of altering bacterial communities in the environment (Marambio-Jones and Hoek, 2010). The exploitation of AgNPs at current rates to tackle bacteria (Sondi and Salopek-Sondi, 2004) and viruses, has the likelihood of the development of resistance. The majority of Ag released into wastewater is incorporated into sewage sludge and then spread onto agricultural fields. Although there is some past research into the ecotoxicology of Ag, most of it precedes the nanotechnology era and therefore does not foresee the possible new consequences and risks of it in NP form in these environments and settings.

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1.12 NANOTOXICOLOGY

The consequence of a booming nanotechnology industry and concerns expressed on the lack of safety information associated with NP use and release into the environment has driven governments to start to invest in evaluations of the potential effects that these nanomaterials have on organisms, including humans. The term nanotoxicology refers to the study of the toxicity of nanomaterials. It is important to establish the potential effects of these novel materials for planning safety measures and whether some nanomaterials should be allowed to have continued use.

Some of the exposure routes for NPs include: atmosphere through car emissions (Kittelson et al., 2004), through water by means of using NPs for filtration methods (Jain and Pradeep, 2005) and the potential of ending up in our food chain through applying NPs as fungicides to the soil (He et al., 2011). As a result it is perhaps inevitable that organisms, including humans, will come into environmental contact with many NPs.

Research on the effects of UFP in humans, principally via air pollution has been active for a long time. Studies since have shown that UFP entering lung tissue from the air can induce oxidative stress (Donaldson et al., 1998), proinflammatory activity (Brown et al., 2001), as well as mitochondrial damage through penetration of the cell causing structural damage (Li et al., 2003). Furthermore, these studies have shown that small sized materials have the potential to induce greater adverse health effects than their bulk counterpart (Oberdörster et al., 1994). There have been many studies (Peters et al., 1997, Heinrich et al., 1995) into how these ultrafine materials cause their effects and on their clearance from the body (Ferin et al., 1992).

Some of the first research on the potential for toxicological effects of NPs in non-humans, was conducted in 2004 when environmental toxicologist Eva Oberdöester exposed large mouth bass to fullerenes and discovered they induced oxidative stress in the brain (Oberdörster, 2004). Since then, research into the biological and ecological threats posed by NPs has expanded dramatically. This is illustrated in the Figure 4 below through the numbers of peer review research papers on nanoparticle toxicology that has grown

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dramatically in the last 10 years, with more than 9500 publications in 2013 (using the key words <nanotoxicology> in Google Scholar search for the period of 2003-2013). In a recent report, it was established that nanotoxicology had grown exponentially 600 percent since the year 2000 (Ostrowski et al., 2009).

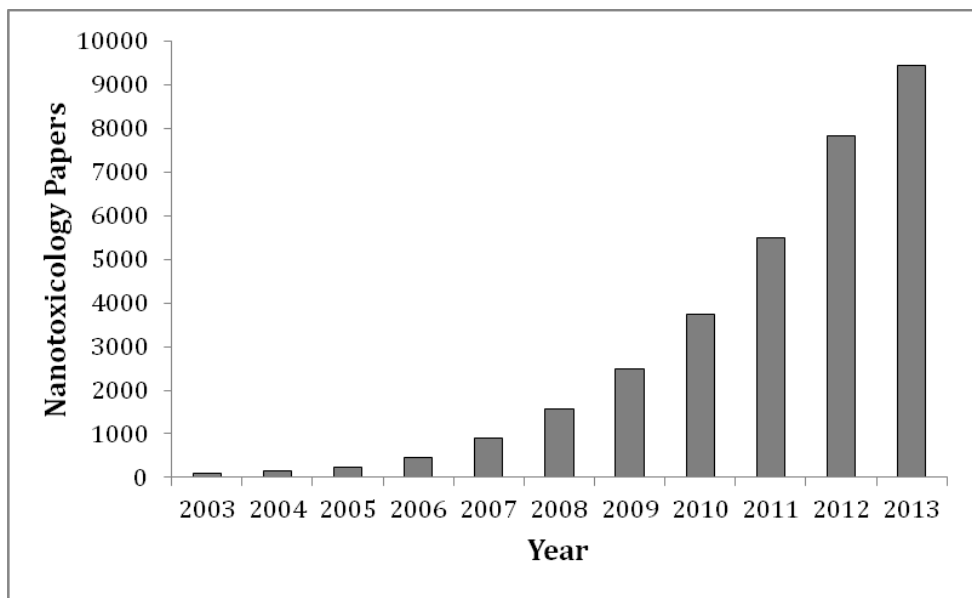


Figure 4. Graph to show the exponential increase of nanotoxicology research in the last 10 years. Number of peer reviewed papers (numbers taken from a Google Scholar search with key word “nanotoxicology” from the period of 2003-2013).

As nanomaterials are released in the environment, there are many parameters that determine the likelihood for toxicological effects and therefore, impact, which include features of the particle itself, notably the material, the particle shape, size, and surface reactivity with the surrounding tissue. There are also factors influencing uptake and the bioavailability of the particles including its fate in the environmental compartment, that is water is especially affected by the presence of organic compounds (Fabrega et al., 2011a) and the level and degree of aggregation that takes place (Navarro et al., 2008a). Aquatic animals that live in sediment areas or filter large quantities of water may be more at risk via ingestion (Fabrega et al., 2011b). The transport of these materials will be dominated by the different diffusion forces, which ultimately will determine mobility.

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The following section describes some of the key research and findings for the nanotoxicology of TiO₂ and Ag NPs.

TiO₂NP toxicology

In the past, research into TiO₂NPs on airborne exposure has shown that TiO₂ can cause toxicity through respiratory systems (Baggs et al., 1997). Oberdörster et al., (2000) used rats and mice to establish the effects of TiO₂; and concluded pulmonary toxicity and shown that translocation of the TiO₂NPs (20nm) occurred across the respiratory epithelium. TiO₂NPs (10nm and 20nm) cause an increased inflammatory response after inhalation and in turn caused oxidative damage (Gurr et al., 2005). TiO₂NPs (21nm) also cause DNA damage at 500mg/kg in mammals in mice exposed via the drinking water (Trouiller et al., 2009).

TiO₂ has now been shown to have effects in a wide range of organisms. Bacteria play key roles in the environment, including in aquatic systems, and in processes spanning carbon cycling to nitrogen fixation (Paerl and Pinckney, 1996), thus understanding the effects of NPs, especially those that can act as anti-microbial and/or antifungal agents is a fundamental requirement in considering nanoparticle sustainability. It has recently been established that commercially available TiO₂NPs (15nm) at 2.7 mg/L in the anatase form caused dose dependent cytotoxicity under simulated solar radiation in *Escherichia coli* (Tong et al., 2013). TiO₂ in anatase form is 100 times more toxic than in the rutile form due to the rutile form being less photoactive (Kawahara et al., 2002). Given the above, there is the concern of the possibility that release of significant quantities of TiO₂ could alter the balance in microbial communities (Battin et al., 2009) by a number of ways including: directly penetrating cell membrane and through ROS production. By changing microbial communities, this in turn would have consequences for aquatic systems as microbial communities are the founders for some of our food webs.

The findings for effects of TiO₂NPs in bacteria correlate with the recent report on the toxicity effects on phytoplankton, another key organism in ecosystem functioning (Miller et al., 2012). Freshwater algae species: *Pseudokirchneriella subcapitata* and *Chlamydomonas*

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reinhardtii both show growth reduction due to lipid peroxidation as a result of exposure to TiO₂NPs (21nm) and caused upregulation in stress response genes at 1 mg/L (Wang et al., 2008, Blaise and Vasseur, 2005). These exposure concentrations however, bear little environmental realism (see section on *predicting the impacts of nanoparticles using modelling*).

Invertebrates too have been shown to be affected by exposure to TiO₂NPs. Tests with *Daphnia magna* show lethality concentrations for TiO₂ ranging from 5.5 mg/L (Lovern et al., 2007) up to 143 mg/L (Zhu et al., 2009b). The reasons for this wide range of toxicities are not clear. Differences in testing conditions, preparation of solutions (Velzeboer et al., 2008), stability, coatings, size and the bioavailability of the TiO₂NPs all change the toxicity of the particle. A wide range of toxicity thresholds can also be observed in other studies using other invertebrate organisms, such as crustaceans (Velzeboer et al., 2008). Chronic exposures TiO₂NPs (66nm) 0.1 mg/L have shown to cause higher toxicity and in some studies bioaccumulation has been observed in *Daphnia magna* (Zhu et al., 2010a). Further evidence supporting this, was a study that established TiO₂ at 20 mg/L for 8 consecutive days caused 40% mortality (Adams et al., 2006). Thus the duration of exposure may be critical when considering TiO₂ particle toxicity. Predicted concentrations of TiO₂NPs in surface waters however, are 0.7- 16 µg/L (Mueller and Nowack, 2008) therefore, some of the high range toxicity studies are not environmentally relevant.

Research into fish has provided some evidence also for biological effects of exposure to TiO₂. Studies on early life stages of zebrafish (*Danio rerio*) showed no toxicity for exposures up to 500 mg/L (Zhu et al., 2008). Until recently, TiO₂ was not thought to be especially toxic to aquatic organisms, however a recent report (Bar-Ilan et al., 2012) has identified that when zebrafish embryos are exposed to TiO₂ under light, this led to death and malformations through ROS. Due to its photocatalytic properties, TiO₂ is known to create ROS in the presence of UV light (Armelao et al., 2007). Additional to this, research has shown that illumination played an important part in the toxicity process of TiO₂ with induced toxicity and oxidative stress (Xiong et al., 2011). Further studies on the zebrafish, have shown how TiO₂NPs (15nm) at 10 mg/L can alter the biochemical constituents in the tissue cells of the gills of this fish (Palaniappan and Pramod, 2010). In other fish too, oxidative stress has also

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been observed in the gills which led to gill injury (Federici et al., 2007). In that study, it was stated that TiO₂ caused a decreased Na⁺/K⁺ ATPase activity. In another study on rainbow trout (*Oncorhynchus mykiss*), 100µg of TiO₂ intravenously injected was found to accumulate in the kidneys (22.81 µg/g tissue) of the trout (Scown et al., 2009). In a dietary exposure study of rainbow trout to TiO₂NPs (21nm), the fish were reported as being unable to eliminate TiO₂NPs from target organs such as liver and especially from the brain (Ramsden et al., 2009). Accumulation of TiO₂ has also been observed in the gills and viscera of carp for exposures via the water using TiO₂NPs (21nm) (Zhang et al., 2007). Handy et al., (2008) discussed that the mechanisms involved in some of the toxicity and bioaccumulation observed for TiO₂. They deduced that the anatase form (10nm/20nm) is more toxic than rutile (200nm) because it generates ROS which leads to lipid peroxidation and DNA damage.

AgNP toxicology

AgNPs and in turn silver ions that dissociated from them, will increase very significantly in the environment due to their widespread and increasing use in consumer products, notably as antimicrobials (Benn et al., 2010). It is known that AgNPs cause toxicity through a number of ways and this may differ in different organisms. Generally it is thought that Ag causes cell damage via mitochondrial damage and increases the production of ROS (Hwang et al., 2008). Some studies support the theory that AgNP toxicity is derived directly from the release of silver ions (Fabrega et al., 2011a), however, others have concluded an AgNP effect relating to the particle itself (Choi and Hu, 2008). What is established, is that toxicity is influenced by how well the particle is dispersed within the medium (Kim et al., 2013). It has been shown how truncated triangular nanoplates exert stronger antibacterial activity than spherical and rod shaped ones because they contain more reactive facets.

Mammalian studies have illustrated AgNPs have toxic effects through a number of biological effects. *In vitro* tests using mammalian keratinocytes demonstrated AgNPs induced cytotoxicity (Paddle-Ledinek et al., 2006). It is thought that these cytotoxic effects occur through apoptosis (Miura and Shinohara, 2009). Additional evidence has been provided by another study using human glioblastoma cells, which determined that AgNPs (6-20nm)

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induced genotoxicity at 100 µg/ml speculating that through ROS DNA damage occurred (AshaRani et al., 2008a).

Furthermore; there is evidence that these materials can penetrate into a wide range of tissues. For humans, a major exposure route is through the skin as AgNPs through the treatment of wounds/skin lesions. Due to their small size, AgNPs applied to the skin could be taken up into the blood and may then migrate to major organs (Singh and Ramarao, 2012). A recent report interestingly revealed that the quantity of Ag released from AgNPs in textiles would also be mediated by sweat (Kulthong et al., 2010), which could potentially in turn induce adverse (as well as beneficial – as an anti-microbial) effects. It has already been shown that AgNPs can move between parts of the body through skin exposure and induce inflammatory and cytotoxic responses (Tiwari et al., 2011). When mice were fed AgNPs (22nm and 42nm) at 1 mg/kg the AgNPs were found in various organ tissues: lung, kidney, liver, brain and testis. It was reported that organ toxicity had occurred with inflammatory responses measured by blood chemistry (Park et al., 2010). Smaller particles appear to induce greater ROS than larger AgNPs. As an example, at the same concentration AgNPs of 15nm generated a higher level of ROS in macrophages than 30nm particles (Carlson et al., 2008).

There has been a range of studies conducted on AgNPs toxicity in plants, fungi (Kim et al., 2009) and aquatic organisms. Studies on the algae *Chlamydomonas reinhardtii* (Navarro et al., 2008b) have shown that AgNP toxicity was mediated by silver ions. It also revealed that uptake of the silver ions occurs and the efficacy of uptake depended on the nature of the surrounding media. Thiosulphate for example enhances Ag uptake, as the silver ions and thiosulphate form hydrophilic complexes (Smith et al., 2002) which make the complex more stable and easier to uptake into membranes through anion transporters. The unicellular microalgae *Thalassiosira weissflogi* was found to have reduced cell growth, related to the reduced rate of photosynthesis and chlorophyll production due to the release of silver ions from AgNPs (10nm) at 1.08×10^{-5} M (Miao et al., 2009). Toxicological effects AgNPs in aquatic invertebrates such as daphnia (*Daphnia magna*) have been shown for chronic exposure (Zhao and Wang, 2011). It was concluded from other daphnia studies that silver

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ions again were the components of the AgNPs responsible for the effects seen (Griffitt et al., 2008). Other invertebrates studies using oyster (*Crassostrea virginica*) embryos showed significant increases in MT mRNA expression at 0.16 µg/L and harmful effects on embryonic development with AgNPs (15nm) (Ringwood et al., 2010). Oxidative stress in response to AgNPs has been reported in other organisms, including the nematode *Caenorhabditis elegans* for exposure to AgNPs (20nm) at 0.1 mg/L (Roh et al., 2009). Other nematode studies have established a growth inhibition for exposures to AgNPs (7nm) at 5 mg/L (Meyer et al., 2010). Recent studies using earthworms also found toxicological effects were mediated by silver ions (Shoults-Wilson et al., 2011).

The toxicity of Ag to fish has been well studied (Wood et al., 1999) with LC₁₀ values at 0.8 µg/L (Birge and Zuiderveen, 1995). In nanoparticulate form these effects from Ag might differ and there has been extensive research recently to investigate this. *In-vitro* tests using medaka (*Oryzias latipes*) fish cells OLHNI2-medaka cell line established from adult fin tissue have shown that AgNPs (30nm) induced cytotoxicity at 0.05 g/cm² (Wise et al., 2010). In another study using medaka, acute toxicity tests established a AgNPs (20nm-37nm) LC₅₀ in adult medaka of 1.03 mg/L (48h) with 100% mortality at 2.0 mg/L (Wu et al., 2010b). In other studies, exposure of zebrafish (*Danio rerio*) embryos have shown morphological abnormalities such as spinal deformities for exposure to AgNP (5-20nm) BSA (bovine starch albumin capping agent) at 5 µg/L and an LC₅₀ 50 µg/L (Asharani et al., 2008b). Some recent studies have shown oxidative stress in medaka (*Oryzias latipes*) embryos as a consequence of exposure to 250 µg AgNPs (35nm)/L (Wu and Zhou, 2012). Other studies using zebrafish, have suggested physiological responses to AgNPs (81nm) such as respiratory toxicity as determined by increase in operculum movement (Bilberg et al., 2011). These findings have also been reported in other fish species, such as the Eurasian perch (*Perca fluviatilis*) (Bilberg et al., 2010).

Ag may induce toxicological effects through a number of pathways, including inflammation (Eom and Choi, 2010), free radical generation, apoptosis and membrane damage. The pathways involved are still under research but thus far it seems AgNP toxicity results principally from the release of silver ions and damage to mitochondria within the exposed cells. AgNO₃ release can increase DNA mutation frequencies during DNA replication and/or

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repair (Yang et al., 2009). Adopting a sequencing approach on zebrafish embryos to Ag materials, it was recently identified in van Aerle et al., (2013) that up/down regulation of 13 genes was specific to AgNPs compared to that of their bulk counterpart and silver ions. Therefore it reported that these specific differences illustrate that toxicity observed from AgNPs is not only as a result of the silver ions.

Considering the data as a whole on the toxicity of AgNPs, it would appear that there are numerous factors that affect the toxicity of this material, which include shape, particle size, stability, pH and coatings. Aside from their direct toxicological effects, another uncertainty is whether AgNPs will be retained within organisms and their potential for bioaccumulation. Some evidence has already shown how AgNPs can concentrate in the bodies of organism for aqueous exposures. Research using *Paracentrotus lividus* (sea urchins) illustrated how AgNPs (5-35nm) were found to be internalized after a 51hr exposure at 0.3 mg/L. An *in vivo* study on the icelandic scallop (*Chlamys islandica*) revealed AgNPs (10-20nm) were readily uptaken and concentrated in the hepatopancreas (Maya et al., 2013). In studies on fish, it has been established that AgNPs (10nm) at 100 µg/L accumulates in the liver tissue (Scown et al., 2010a), with prevalence of particle size uptake at 10nm (Scown et al., 2010a). Furthermore; there has been some evidence showing AgNPs (5-46nm) can pass through the chorion membrane and being restricted in the chorionic space of zebrafish embryos (Lee et al., 2007) however; there was no indication of AgNPs present in the developing organs of the embryo.

1.13 CHALLENGES IN ASSESSING THE TOXICITY OF NANOPARTICLES

There are many challenges for assessing the toxicity of nanomaterials (Nel et al., 2006). As discussed previously, due their particle size they possess special chemical properties of which includes quantum effects. This in turn brings uncertainty as to how they will behave in different environments and how these particles interact with different surfaces. The small size of these particles means that they can be readily uptaken by organisms, but their fate in the body of the organisms for the most part is still uncertain for most nanomaterials.

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Measurements of NPs in the environment

As discussed previously, there are both natural and anthropogenic NPs to consider; which arise from incomplete combustion of fossil fuels to commercial products. Therefore, distinguishing the natural NPs from anthropogenic NPs in the environment is hard (Lead and Wilkinson, 2006). In the past, no actual measurements in the environment were determined due to the lack of analytic methods to able to quantify trace concentrations in nanogram per litre (Mueller and Nowack, 2008). However; new characterization methods using high resolution techniques are aiding in achieving this (Simonet and Valcárcel, 2009b). New imaging methods include; near infrared fluorescence spectroscopy (NIFR) which uses molecular fluorescence microscopy making this technique highly sensitive with low background signal. Additionally, there is environmental scanning electron microscopy (ESEM), this has minimal disturbance to the samples and allows imaging of wet specimens. For quantification of NPs we usually rely on inductively coupled plasma membrane (ICP-MS), however, single particle identification cannot be achieved this way and this measures total amount of metal but cannot distinguish between micro and NP metals. However; new techniques by combining methods (ICP-MS, hydrodynamic chromatography (HDC) and field flow fraction (FFF)) are already being developed (Farkas et al., 2011, Lorenz et al., 2012) to achieve this.

A recent review by Von der Kammer et al., (2012) established some analytic concentrations in surface waters. A study by Neal et al., (2011) and colleagues took measurements for TiO₂NPs (<0.45 µm filtered fraction) in UK rural, agricultural and urban rivers; they established an average of 2.1 µg/L ranging between 0.55 up to 6.48 µg/L. It also demonstrated that 79% was TiO₂ colloidal/nanoparticulate (1-2nm) in rural rivers and only 28% was found in urban/industrial rivers.

Predicting the concentrations of the nanomaterials is challenging as there are many factors that need to be considered and which are addressed in detail in the modelling section.

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Provision of standardised (and well characterised materials)

Characterisation of the NPs is fundamentally important when trying to evaluate the possible risks and comparing results across different studies (Stone et al., 2010). When trying to compare toxicity across different research it is often the case that results are not comparable. This is due to the lack of appropriate characterisation in both raw and the exposure medium that the NPs were tested in. We have now seen how thresholds and effects are different for same studies conducted and this in part may be due to differences in the way the particles are treated.

In the past, it wasn't known that the behaviour of these particles would change dramatically in the different experimental mediums, and in turn the toxicological effects as well (Powers et al., 2009). After a Workshop run in Florida in November 2004, it was advocated that the NPs should be characterised in the experimental medium (Stone et al., 2010, Bucher et al., 2004). From then on, more research revealed that dispersion would also be affected and also aggregation would lead to different sized nanomaterials that would create non-nano sized materials (Powers et al., 2006). This approach was fundamental when attempting to assess and pin point the reasoning behind the effects that were being observed. The Joint Research Centre (JRC) is involved in the support for European Policy making. One of its important objectives is for reliably sizing NPs.

There is constant debate as to what type of characterisation universally should be applied (Klaine et al., 2008). Therefore, in order to explore such characteristics, it has been deemed necessary to carry out a number of techniques on the NPs with a range of media as it is known that this influences behaviour changes or their potential to induce toxicity (Colvin, 2003). In addition to toxicological studies, it has been advised that characterization of nanomaterials should also be applied in the life cycle assessment of NPs to predict fate (Fischer and Chan, 2007).

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Dynamic nature of NPs and how the environment can fundamentally affect their nature and bioavailability

Since water chemistry dramatically changes the toxicity of NPs, it may be difficult to predict their environmental impacts without considerably greater research efforts studying the materials of concern in relevant environmental compartments (Nowack and Bucheli, 2007). The toxicity and behaviour of NPs is known to change by a wide range of factors which range from humics to pH.

Bioavailability will change as these particles come into contact with different elements in the marine environment (Jenne and Luoma, 1977). It is known for example; that NPs in a high salt solution generally causes the NPs to form aggregates (Hartmann et al., 2010) and in turn generally lead to a decrease in toxicity (Jiang et al., 2009). This result can be misleading and a challenge in toxicological studies, whereby the NPs tested agglomerate in the media tested and therefore possibly deeming the NPs less toxic than they are. Other concerns include; that aggregation may introduce these NPs into benthic organisms via the adsorption of the surface of such organism (Handy and Eddy, 1991). Furthermore, recent research has shown that marine aggregates facilitate ingestion of NPs in bi-valves (Ward and Kach, 2009) also proving a cause for worry.

Another factor in the environment can be organic constituents/natural humics, such as fulvic acid found in surface waters through biodegradation of dead matter. Evidence has illustrated that humics can form complexes with metals (Mantoura et al., 1978) and that organic matter can improve dispersion (Hyung et al., 2007). It is also known that the distribution of metal ions between solution and colloids has a heavy influence on metal availability (Lead and Wilkinson, 2006). Therefore; once these nanomaterials enter the environment, natural colloids and even other NPs will interact with them and possible change the behaviour/effects seen in nanotoxicology studies. Evidence has already shown that natural organic matter (NOM) enhances NP stabilisation and as a result could mean the NPs entering aquatic systems will be stable enough to persist for a long period of time (Chinnapongse et al., 2011).

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It has been established that pH can affect NPs size (Solomatin et al., 2003); evidence has shown that at a pH <4 particle size increases due to aggregation which in turn would decrease toxicity and at a high pH, particle size decreases, suggesting higher toxicity through size dependent differences. Other factors can also include illumination which plays an important role in the toxicity effects for photoactive NPs (Chen et al., 2009).

Target organs for NPs

Major questions that arise in trying to get a better understanding on the toxicity of NPs are where they partition within the body and what the main target tissues and whether they bioconcentrate. As NPs as being used for drug delivery in human therapies and diagnostic uses (Pissuwan et al., 2011), there is concerns for NPs to reach target organs by translocation through membrane barriers due to their small size (Oberdorster et al., 2009). It has been established that the main target organs in murine models is the brain and liver through the blood system (Wu et al., 2009). Interestingly, in another study using rats, it was demonstrated that 20-30nm carbon nanotubes (injected intravenously) were excreted in the urine suggesting that the nanomaterials can pass through glomerula pores (Lacerda et al., 2008). There is environmental concern for the NPs to potentially accumulate in organism (as discussed previously).

In fish studies, it has been demonstrated that NPs target organs include liver (Johnston et al., 2010), gills (Chen et al., 2011a), gut (Johnston et al., 2010) and brain (Zhu et al., 2010c). Evidence for accumulation of metal based NPs in these target organs in different aquatic studies is as follows:

- Liver: 10 day exposure to AgNPs (10nm, 35nm) via the water column (100 µg/L) to rainbow trout showed Ag uptake in the liver at 1.50 ± 0.30 µg/g and 0.92 ± 0.16 µg/g respectively (Scown et al., 2010a).

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- Gills: 20 days exposure to TiO₂NPs (50nm) at 10 mg/L to carp via the water column demonstrated TiO₂ concentrated in their gills by a bioconcentration factor (BCF) of 0.74 (Zhang et al., 2007).
- Brain: A 36 day dietary exposure to gold (Au) in zebrafish illustrated 4.6±2.3 µg Au/g in the brain (Geffroy et al., 2012).

From this information it raises the possibility of NPs crossing membrane barriers from the gut into the blood system and translocation to target organs. However; this data only confirms trace metal measurement found in the target organs but not individual NPs themselves. In the past, it was hard to quantify uptake of NPs in organism as analytical methods were limited but new methodologies have helped tackle this problem. In 2006, it was suggested that improved methodologies were needed and stable isotopic tracing was suggested as the way forward to aid research in tracing the uptake and fate of these NPs in organisms (Gulson and Wong, 2006). For example for Zn, which is a natural occurring element, it is essential to be able to distinguish background metal concentration found in the environment from the new accumulated metals from the NP exposure. Another advantage to this method is that it enables us to use low environmentally relevant concentrations. A recent study has explained a relative cost effective way of using isotopically labelled ZnONPs to be able to differentiate between these (Larner and Rehkemper, 2012). An exposure study using this technique on *Corophium volutator*; established the majority of toxicity was due to ionic Zn with nano effects seen (Larner et al., 2012).

In vitro vs in vivo

Consequently there is a need to determine a systematic approach for testing NPs and standardising methods is essential. Both *in vitro* and *in vivo* test methods have their place in assessing the toxicity of nanomaterials; and below summaries some of the advantages and disadvantages of using them.

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In vitro

As with other chemicals *in vitro* toxicity assays have been able to screen and provide mechanistic information (Hayashi et al 2005). These assays include apoptosis, DNA damage and oxidative stress. New techniques using video enhanced differential interference contrast (VEDIC) on cells *in vitro*, allow for NP observations on uptake and internal localisation are which easy to observe without the need to add fluorescent label (Connor et al., 2005). However; there are new imaging methods using fluorescently labelled particles which are currently being used to monitor the movement of NPs within cells (Takahashi et al., 2008). Controversy arises from using fluorescent techniques, as coating the particles can change behaviour and toxicity, therefore not representing the NP in its true state. Another advantage of using *in vitro* is a fast, cost effective approach for toxicity testing which minimises ethical issues on animals (Marquis et al., 2009). Furthermore; new *in vitro* toxicity methods combined with high-content screening assay (HSA) allow for RNA expression analysis determining whether RNA expression was up/down regulated in cells exposed to NPs (Zhang et al., 2006). In contrast to this, *in vitro* is hard to compare with *in-vivo* work and concentrations used in *in vitro* testing are somewhat higher than those found at a cellular level *in vivo*. Additionally, *in vitro* lacks the complexity of using an animal model which is why the research needs to be combined and complement one another to become a whole piece of research that we can put forward into measuring the possible effects of NPs.

In vivo

Studying the effects of NPs on living organisms allows for long term effects, localization, biodistribution and retention/excretion. Typical organisms used in *in vivo* studies are mice (Kim et al., 2001) and rats (Baker et al., 2008), however; aquatic species are also being used to assess the ecotoxicological effects of NPs (Menard et al., 2011). Typically, *in vivo* exposures determine the LC₅₀ of the organism tested. To monitor biodistribution; fluorescent NPs can be detected within live or sacrificed animals. In addition to this, *in vivo* also allows for any morphological changes on the organism and target organs to be observed. Furthermore, histology can be performed on organs to explore presence of NPs.

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An assay test used in *in vivo work* is the blood chemistry of the organism that can be used to analyze blood composition as well as blood cell population during/after an NP exposure. One of the controversies faced with *in vivo* tests is the handling and preparation of samples could alter effects seen.

Lack of standardised test systems

Even after outlining some of the benefits of *in vitro* and *in vivo*; there is still a lack of analytical and *in-situ* techniques which can detect these NPs in a complex or biological medium (Fabrega et al., 2011a), and in turn making it harder to predict the effects these particles will have within different mediums. Most of these issues lie within testing aquatic organisms in water systems. There is still a need for a standardised approach to ecotoxicological testing these NPs (Handy et al., 2012a); because, as discussed previously, water chemistry can change NP behaviour and effects dramatically.

Preparation of the NPs is an important part as it can determine toxicity. It is known that better stability and dispersion of the NP usually induces increased toxicity. Sonication methods have been favoured as there is no need to add extra chemicals to NPs. However; these methods have been quite different i.e. different times, temperature and machinery - and therefore, could account for some differences seen in the results. In addition, sonication methods have been criticised as they can overestimate toxicity of NP in an environmental scenario. Another factor to consider is the container of testing medium; it is known that some NPs adhere to glass and for that reason appropriate cleaning methods using e.g. nitric acid need to be established. Due to detection limitation capabilities; there is still a knowledge gap in biodistribution studies involving NPs which needs to be addressed and new testing methods need to be established. After a recent SETAC workshop; it has been discussed that the OECD are considering new methods of NP toxicity testing (Handy et al., 2012b).

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1.14 PREDICTING THE IMPACTS OF NANOPARTICLES ON THE ENVIRONMENT USING MODELLING

Given that quantifying man-made NPs in the environment is extremely challenging, modelling approaches have been adopted to help predict amounts of NPs in the environment, now and in the future (Nowack et al., 2012)

It is hard to estimate how many NPs will be released in the ecosystem as there is still no full inventory of these products (Gottschalk et al., 2010b). However; various studies have started to estimate PEC in air, soil and water. These PEC values will vary for different materials, different countries and different states in which the NPs are in. Depending on the nature of the NP, different scenarios have to be taken into account and different emission flows. The first modelling studies were reported in 2008; it took into account the whole life cycle of AgNPs, TiO₂NPs and carbon nanotubes (Mueller and Nowack, 2008). The calculated PEC values they reported ranged from a realistic scenario to a high emission scenario: TiO₂NPs (0.7- 16 µg/L); AgNPs (30-80 ng/L) and carbon nanotubes (0.5-0.8 ng/L).

Gottschalk et al., (2009) used the probabilistic method of taking into account a number of consumer products that contain AgNPs to predict the environmental concentrations in Europe. They estimated concentrations of AgNPs at 0.5-2 ng/L in surface waters, 32-111 ng/L in sewage treatment plants effluent and 1.3-4.4 mg/kg in sludge (Gottschalk et al., 2009). This was supported by further data reporting that NPs will most likely accumulate in surface waters (Benn and Westerhoff, 2008).

Later Gottschalk et al., (2010a) came up with another model they called the probabilistic material flow analysis (PMFA). It again, took into account a whole life cycle of the NP and considered application quantities, emission factors and the fate of the compounds in various environments. The simulation was used to establish PEC values for TiO₂ in Switzerland and their results showed higher concentrations of TiO₂ in surface water (~0.3 µg/L) than for air; and that there would be an annual increase in soil and sludge (Gottschalk et al., 2010a). The highest TiO₂NP emission flow was in waste water (30.7-33.8 t/a) at a 95% confidence interval.

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Nevertheless, these models are clearly limited and how well they fit with real world scenarios has not been forthcoming due to a lack of empirical studies. Where there have been empirical studies on the amounts of NPs in the environment, these have compared favourably to some studies with those in the models described above. In a recent review (Gottschalk et al., 2013), it revealed TiO₂ concentration in river (using combined modelling predictions) at 3 ng/L -1.6 µg/L correlated well with a modelling prediction study (Gottschalk et al., 2009). The measurements established versus modelling prediction are higher but still give an approximate indication at NP concentrations in the environment. The differences can be possibly explained by size ranges of found in the environment (<0.45-0.7µm) to the modelled NP size (<0.1µm). Nonetheless, the models available presently for predicting NPs in the environment, lack data needed for many parameters to ensure robustness of the predictions. These factors include geographical location and varying water levels.

The need for legislation on nanomaterials

There have been numerous debates as to whether nanotechnology requires government regulation and whether there should be handling, labelling and risk assessments specifically for into these new materials. Research in technology has thrived in comparison to research into the possible effects on the environment and humans. As an example of this, in 2002, the U.S. government spent \$710 million on nanotechnology research and \$500 000 was spent on ecological assessments although the recent years the balance has improved significantly.

In 2004, The Royal Academy of Engineering produced a report indicating the strong likelihood for nanomaterials highlighting their likely threats to the environment (Royal, 2004). No specific legislation exists, or even a framework to set this out, for how to handle or dispose of these novel nanomaterials and there is no provision of any safety measures. The UK government Department for Food, Agriculture and Rural Affairs (DEFRA) released a report outlining in detail the research that was needed to identify the gaps in our knowledge relating to these new substances and the need for a better understanding on their possible associated health effects (DEFRA, 2005).

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In 2007, the forty civil societies coalition (The International Centre for Technology Assessment) released a report *Principles for the Oversight of Nanotechnologies and Nanomaterials* (Kimbrell, 2009) declaring the need for these new substances to be regulated and properly investigated before their placement into the marketplace.

There are a number of European Union (EU) framework projects that have started to now undertake risk assessment of nanomaterials in the environment. These projects include:

- (2009) Engineered Nanoparticle Impact on Aquatic Environment: Structure, Activity and Toxicology (ENNSATOX)-€2.816 million
- (2010) Nanoparticle Assessment and Toxicity in the Environment (NanoFATE)-€2.5 million.
- (2011) Modelling nanoparticles toxicity: principles, methods, novel approaches to toxicology (ModNanoTox)-€1 million
- (2011) Managing Risks of Nanomaterials (MARINA)-€9 million
- (2011) A pan-European infrastructure for quality in nanomaterials safety testing (QNano)-€7 million.

It is obvious from the listing presented above that the scale of the projects funding nanomaterials research for investigating their potential hazards and risk to the environment and human health has increased dramatically in the last 4 years, reflecting the need for full assessments on new nanomaterials as they enter commercial use.

In an EU statement reflecting this concern for the use and exposure to NPs, it was reported that “Full risk assessments should be performed on new nanomaterials that present a real risk of exposure during manufacture or use. Such assessments should take into consideration the toxicological hazard, the probability of exposure and the environmental and biological fate, transport, persistence, transformation into the finished product and recycling” (Rickerby and Morrison, 2007).

Belgium has recently provided a lead on how we might handle the future implications of nanotechnology. In July 2013, it released a draft on Nano Legislation to the European commission that will ‘implement a register of substances manufactured at a nanoscale

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based on declarations of products containing such substances by the parties placing these products on the market'. This law <substances manufactured at the nanoscale, and preparations containing them, be declared if more than 100 grams of these substances are placed on the market per year> would take place from January 2015. EU and Canada too have taken the first steps into limiting the use of NPs in food (Paull, 2010).

As with any new developments in technology, the research on the technology outweighs the research in the associated health risks to society and the environment. However, in Europe we are beginning to see a change in labelling on products and codes of conduct using nano including safety for workers. It has been seen that regulation has been slow to emerge. Therefore, this advocates the need for continuing research in this field and the need for funding in this sector. There are still a lot of gaps in our knowledge to fully understand the impact of these nanomaterials on the environment.

1.15 AIMS OF PHD

The main aims of this PhD were to investigate the potential health effects of two selected NPs, TiO₂ and Ag, in fish. These NPs were chosen for investigation because of their wide scale commercial use and literature evidence for the potential for biological and adverse health effects.

Both TiO₂ and AgNPs end up in aquatic systems and modelling flow charts have predicated significant discharges via sewage treatment plants and a potential also for them to enter the human food chain from the environment.

This PhD set out to investigate the ecotoxicology and biological effects of commercially available Ag and TiO₂ NPs, exploring their possible uptake and fate within exposed fish for aqueous exposures. The purpose of this work was also to explore their possible effect mechanisms and target tissues (most notably for Ag). Another major goal was to investigate the potential impact of AgNPs on reproduction and for possible transgenerational effects for a dietary exposure to AgNPs. The work overall was to establish

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toxicity effects from fish early life stages to adulthood and verify/compare the toxicity results with other scientific research to gain a better understanding into the fate, behaviour and biological effects of the selected metal based NPs. Fundamentally, throughout the work conducted, careful physical characterisations on the particles in their raw state and as part of the exposure matrices were conducted to help in understanding of the exposures and aid in interpretation of the biological effects seen. Throughout, also effects for NPs were compared with larger (bulk) particle counter parts to help discriminate effects that related to 'nano effects'. A wide range of techniques were applied in the thesis to better establish exposure, target tissues and biological effects of the selected NPs, including, embryology, whole mount *in situ* hybridisation for target gene expression analysis, ICP-MS, in life *in vivo* exposures and advanced imaging with Coherent Antistokes Raman Scattering (see Moger et al., (2008)).

Throughout this thesis work, the zebrafish (*Danio rerio*) was the study organism used. The zebrafish is an excellent model organism well established as a platform for toxicity studies. The zebrafish also has a sequenced genome and is widely adopted in the field of developmental biology to examine gene pathways and create mutant lines. The egg of the zebrafish has a transparent chorion membrane which means physiological observations can be made very easily on the developing embryo. It also has short completion time for embryogenesis (96hr hatch), which allows for high throughput for toxicity studies. The zebrafish is small (adults 4-5cm in length) and easily maintained in the laboratory, facilitating studies on reproduction. Furthermore, Exeter has extensive experience in conducting studies on embryo development and reproduction with this species.

The key specific objectives of my research were:

Objective 1: To establish the effects of particle size and coating of different sized Ag and TiO₂ NPs on zebrafish embryogenesis.

Objective 2: To identify target tissues and some of the effect mechanisms of AgNPs in zebrafish using the application of whole mount *in-situ* hybridisation.

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Objective 3: Establish whether Ag particles impact on breeding of zebrafish and/or can undergo maternal transfer with biological consequences for their offspring?

The following studies were carried out to address these objectives and are presented in the form of scientific papers that have either been accepted or are being submitted for publication in peer-reviewed journals.

Study 1 (Chapter 2): Here zebrafish (*Danio rerio*) embryos were exposed via the water to different sizes of TiO₂ and AgNPs, and for Ag, particles with different coatings. The endpoints measured were acute toxicity, alterations in morphology, apoptosis, and expression of metallothionein 2, analysed via whole mount *in-situ* hybridisation. Supporting the biological analyses, Coherent Anti Raman Spectroscopy was conducted on the exposed embryos to explore evidence for particle uptake across the chorionic membrane. The Null hypothesis tested was *particle size and coatings have no effect on the toxicity to zebrafish embryos*.

Study 2 (Chapter 3): In the second study, zebrafish (*Danio rerio*) embryos/larvae were exposed to citrate coated AgNPs and bulk counterparts via the water column and whole *in-situ* hybridisation analysis was carried for a number of genes that represent key biological processes thought to be affected by NP exposure, principally related to oxidative stress and metal handling. These genes were: metallothionein (*mt2*), glutathione S-transferase Pi (*gstp*), glutathione S-transferase Mu 1 (*gstm1*), hemeoxygenase 1 (*hmox1*) and ferritin heavy chain 1 (*fth1*). In this work, the ontogeny of expression of these genes was conducted in early life stage embryos and larvae to establish the most appropriate time point for the exposure assessment studies. Exposure studies were then conducted to determine whether AgNPs/bulk counterpart stimulate the different stress response genes in the identified target tissues at the most appropriate life stages established in the ontogeny work (for embryos/larvae up to 12 days post fertilisation). Further investigations into the mechanistic pathways for the effects of AgNP were investigated using a mutant transgenic fish line for Nrf2. Nrf2 plays a role as a transcription factor in oxidative stress. This study tested the Null

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Hypothesis that *there were no differences for stress responses (as determined by induction of key stress response genes) for AgNPs compared with a bulk counterpart.*

Study 3 (Chapter 4): Here, zebrafish (*Danio rerio*) were exposed for three weeks to citrate coated AgNPs and bulk counterparts via the diet to investigate whether AgNPs could a) impact the breeding of zebrafish b) establish where they uptaken in the body – i.e. target tissues c) investigate for maternal transfer and the possibility for biological effects in the offspring. For this, fish were exposed via the diet and fecundity was measured over the course of the study. To test for Ag uptake into target organs in adults, liver and gonad tissues were acid digested and then analysed via ICP-MS to determine Ag concentrations. Embryos from treated adults were collected at two time points per week for measuring Ag uptake (via ICP-MS). Embryos were also analysed at 24 hour post fertilisation via whole mount *in-situ* hybridisation to investigate the response of *gstp* and *mt2*. Embryos on the last week of exposure were challenged to Ag materials for further *in-situ* analysis on the expression of *mt2* to assess whether adult exposures to the different Ag materials affected subsequent responses. This chapter set out to test the Null hypothesis that *AgNPs do not impact on breeding of zebrafish or undergo maternal transfer with biological consequences to their offspring for a dietary exposure.*

The final chapter of this thesis (**Chapter 5**), provides a critical overview on the main findings of my thesis studies, the challenges faced and discusses future prospects for nanotoxicology.

CHAPTER 2

**EFFECTS OF PARTICLE SIZE AND COATING ON NANOSCALE AG AND TIO₂ EXPOSURE
IN ZEBRAFISH (*DANIO RERIO*)**

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BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

Effects of particle size and coating on nanoscale Ag and TiO₂ exposure in zebrafish (*Danio rerio*) embryos

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Abstract:

Manufactured metal (oxide) nanoparticles are entering the aquatic environment with little understanding on their potential health impacts for exposed organisms. Adopting an integrative approach, we investigated effects of particle size and coating on biological responses for two of the most commonly used metal (oxide) nanoscale particles, silver (Ag) and titanium dioxide (TiO₂) in zebrafish embryos. Titanium dioxide nanoparticles (nominally, 4nm, 10nm, 30nm and 134 nm) had little or no toxicity on the endpoints measured. Ag both in nano form (10nm and 35nm) and its larger counterpart (600-1600 nm) induced dose dependent lethality and morphological defects, occurring predominantly during gastrula stage. Of the silver material tested 10nm nanoparticles appeared to be the most toxic. Coating Ag nanoparticles with citrate or fulvic acid decreased toxicity significantly. *In-situ* hybridisation analysis identified the yolk syncytial layer (YSL) as a target tissue for Ag-nano toxicity where there was a significant induction of the heavy metal stress response gene, metallothionein 2 (Mt2) at sub-lethal exposures. Coherent Anti-stroke Raman Scattering (CARS) microscopy provided no evidence for silver particles crossing the chorionic membrane in exposed embryos. Collectively, our data suggest that silver ions play a major role in the toxicity of Ag nanoparticles.

Keywords: nanoparticle, ecotoxicology; *Danio rerio*; embryo; titanium dioxide, silver.

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Introduction

Nanoparticles are being introduced rapidly into the consumer market but there is still little understanding on their potential consequences for human and environmental health. Two of the first metal based nanoparticles to gain widespread use are titanium dioxide (TiO₂) and silver (Ag). TiO₂ is of global importance with in excess of 4.3 million tonnes produced annually, with extensive use in sunscreen and in the pigmentation of paints. The surface reactivity and general properties of TiO₂ are well documented (Long et al., 2007). Nano-TiO₂ has been reported to cause oxidative stress effects in mammals and in fish, inducing inflammation, cell damage and genetic damage, both with and without exposure to ultraviolet A (UVA) radiation. Available data suggest sub-lethal toxicity in the concentration range of 5-50 µg L⁻¹ for exposures via the water in both invertebrates (Lovern et al., 2007, Heinlaan et al., 2008, Zhao et al., 2009) and fish (Lee et al., 2007, Scown et al., 2009). Modelled environmental concentrations indicate TiO₂ concentrations may in some circumstances reach between 0.7 and 16 µg L⁻¹ (Nowack and Bucheli, 2007) and this could present a risk to aquatic organisms.

In the 1970s, 2.5 million kg of Ag was discharged into the environment in the United States of America (Luoma and Rainbow, 2008) and its high toxicity to aquatic animals subsequently led to stringent environmental regulations by the 1980s under the Clean Water Act in the US (Purcell and Peters, 1999). Nano Ag is now used extensively in consumer products, predominantly for its effective antimicrobial properties and low production cost. In wastewater treatment works receiving influents from industries using silver nanoparticles (AgNPs), levels of Ag have been shown to reach 100 µg L⁻¹ (Hu, 2010) and this exceeds tolerable limits for some bacteria and which may therefore impact adversely on bacterial communities (Marambio-Jones and Hoek, 2010). Of particular concern is the potential for nano Ag to concentrate in sewage sludge as in some countries (including the UK) this can be subsequently applied to land as fertilizer. Several studies have indicated that AgNPs have the potential to induce toxic effects in a range of species, including in fish (Skebo et al., 2007, Braydich-Stolle et al., 2005, Hussain et al., 2005, Scown et al., 2009, AshaRani et al., 2009). One study exposing zebrafish embryos to an extremely high level of AgNPs (100 mg L⁻¹), that were stabilised with citrate or fulvic acid, showed Ag penetrated into various body

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tissues, including brain, heart, and skin (AshaRani et al., 2009). This toxicity for exposure to AgNPs in fish may, in part, relate to an enhanced dissociation in the exposure water, and thus bioavailability, of free silver ions (Jin et al. 2010).

The purpose of this study was to adopt an integrative approach to determine potential toxicity to zebrafish embryos of well characterised Ag and TiO₂, of various sizes both as unmodified nanoparticles and dispersed with citrate or fulvic acid, and across a range of exposure concentrations. Zebrafish embryos offer a wide range of experimental conveniences including the ease for observing developmental effects through a transparent chorion. Mortality rates, developmental abnormalities, apoptosis, and targeted (*in situ*) gene expression were used as effects assessment endpoints. Advanced imaging techniques, including Coherent Anti-stokes Raman Scattering (CARS) were employed to investigate for uptake and distribution of nanoparticles in the tissues of the exposed embryos.

Materials and methods

Fish source, culture, and husbandry

Wild-type WIK (Wild-Type India Calcutta) strain zebrafish embryos were obtained from the Max Planck Institute, Tubingen, Germany and maintained at University of Exeter as described in the supplementary material (S1).

Nanoparticle source and characterisation

AgNPs (nominal sizes 10nm and 35nm) and Ag bulk (nominal size 600-1600 nm) were acquired from Nanostructured and Amorphous Materials Inc. Houston USA. Titanium dioxide nanoparticles (TiO₂NPs) (nominal sizes 3nm, 10nm and 35nm) and 134 nm particles were acquired from Alfa Aesar- A Johnson Matthey Company, Shore Road, Port of Heysham Industrial Park, Heysham, Lancashire, LA3 2XY, United Kingdom

Physicochemical characterisation

A number of techniques were carried out to characterize and quantify the particles. The techniques applied included: nanoparticle tracking analysis (NTA), Braun Emmett Teller

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(BET) method of specific surface area analysis, X-ray diffraction (XRD), atomic force microscopy (AFM), and (high resolution-transmission electron microscopy (HR-TEM) with associated spectroscopy - X-ray electron dispersive spectroscopy (X-EDS). A full detailing of the methods applied to Ag and TiO₂ particles can be found in Scown et al., (2009); and details of data analysis in Ju-Nam et al., (2012).

Silver dissolution

Samples of silver nitrate (Perkin Elmer) were made up in milli Q water and embryo culture water (0.60 mg of marine salts [Tropic Marin] per litre of de-ionised water) as test standards for analysis by the ICP-MS. Sample concentrations were 0, 15, 30, 60, 120 and 260 µg L⁻¹. Dissolution rates were determined for 35nm Ag and bulk Ag particles. For this, duplicate 1 litre solutions containing 50 µg L⁻¹ AgNPs were made up in embryo culture water (0.60 mg of marine salts–Tropic Marin per litre of de-ionised water) and mixed constantly using magnetic stirrers at a temperature of 21°C. For each treatment, 8 Spectra/por dialysis membranes MWCO 1 000 (1KDa) (pre-washed in 0.05% sodium azide in Milli Q water) were set up containing 10 ml of Milli Q water, that were then clip sealed at each end before being submerged into the AgNP or Ag bulk solutions. At different time points; 4hrs, 24hrs, 48hrs and 72hrs, 1 sample for each treatment vessel (2 per treatment) was taken, pipetted into a 15ml falcon tube and the silver ions stabilised through the addition of 1% of HNO₃ added before analyses using ICP-MS.

Ag/ TiO₂ nanoparticle exposure and effects assessments

Particles were made up in a dilution series of 6 stock solutions (50 µg L⁻¹, 500 µg L⁻¹, 5 000 µg L⁻¹, 50 000 µg L⁻¹, 250 000 µg L⁻¹) for each particle size. Solutions were sonicated in a water bath for 30 minutes and placed into glass, amber, Boston round 125ml tubes fitted with a Teflon lined cap and kept at 4°C until required. When the solutions were required for the exposure studies they were sonicated in a water bath for 30 minutes and pipetted into the exposure wells. To investigate for effects of particle coating on biological effects a further dilution series of 10nm Ag particles was mixed with either 0.0075% sodium citrate or a 2% fulvic acid suspension prior to the exposures. For the exposures to silver ions, a stock

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solution of silver nitrate (Perkin Elmer 2% AgNO₃) was made and the required amount for each exposure concentration was added into the embryo culture water.

For the embryos exposures, 500 µl of the stock solutions were added to 450ml embryo culture medium to give final exposure concentrations of 5 µg L⁻¹; 50 µg L⁻¹; 500 µg L⁻¹; 5 000 µg L⁻¹; 25 000 µg L⁻¹. Controls received 5ml of embryo culture water only. Eggs/embryos were collected from breeding colonies transferred into a Petri dish and washed twice with embryo culture water (0.60 mg of marine salts Tropic Marin per litre of de-ionised water) with the addition of 15 µl of methylene blue to prevent fungal and bacteria growth. For all exposures there were 20 embryos (at the 1-2 cell stage, 1-1.5hpf) per treatment well, and the studies were replicated at least 3 times. The embryos were incubated at 28± 1 °C up to 48hr. After 2h in culture, the numbers of unfertilized embryos were recorded and these were removed. At 48hpf (hours post fertilization) survival rates and any phenotypic deformities were recorded. Any physical deformities observed were recorded and converted to percentages for each treatment. Embryos were observed and photographed using Nikon SMZ1500 microscope equipped with a digital camera. To gain an insight into the timing of mortality and developmental effects induced by the exposures to Ag, time lapse video analysis was used, as described in the supplementary material (S2).

Cell necrosis

To investigate further for silver particle toxicity, cell necrosis was recorded in embryos exposed during early life to either 35nm Ag or 35nm TiO₂ at concentrations of 500 µg L⁻¹ and 25 000 µg L⁻¹. Twenty embryos for each exposure concentration were incubated at 28 ± 1°C from the 1 to 2-cell stage and subsequently removed from the exposure at 7hpf and stained with a Propidium Iodide (PI) (Sigma P-4170) at 1mg/1ml mixed in distilled water; Fluorescein Diacetate (DAF) (Sigma F-378) at 1.5mg/1ml mixed in DMSO; Hoechst (HO) (Sigma B2261) at 1mg/1ml mixed in distilled water and PBS (Pinero et al., 1997). The final concentrations of materials in the necrotic staining solution were; PI, 250 mg L⁻¹; DAF, 750 µg L⁻¹; HO, 200 µg L⁻¹. Embryos were incubated in the dark for 10 minutes in a 24-well plate and photographed using Leica DMI 4000 B Compound Microscope equipped with a digital camera.

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Metallothionein gene expression assessed via whole mount in-situ hybridization

In situ hybridization on exposed zebrafish larvae was undertaken to investigate for differential activation of gene expression for metallothionein 2 (Mt2), known to play key roles in toxicological responses to metals, including Ag (Choi et al., 2009). Mt2 cDNA was obtained from Imagen/RZPD (clone No IMAGp998C0115598Q). To prepare the RNA probe, Mt2 cDNA was amplified by PCR using two primers, Mt2_F: ATC AAC TCA TTC ACA AGC TGA; T3_Mt2_R: GGA TCC ATT AAC CCT CAC TAA AGG AAA TAC CAC CAT TTA TTT TAG, and *in vitro* transcribed with using digoxigenin labeling mixture (Roche) and T3 RNA polymerase (Promega). Using a G50 column the RNA was purified and precipitated using Lithium Chloride. The probe was then diluted with hybridisation buffer at 1/200. The *in situ* hybridisations were conducted as described in the supplementary material (S3). For these studies embryos were exposed to 35 nm Ag and 35 nm TiO₂ particles at 500 µg L⁻¹, and AgNO₃ at 12 µg L⁻¹ (to represent the maximal rate of dissolution for the silver particle exposures – see results) from 1-2 cell stage to 24hpf, fixed with 4% PFA (S3). Embryos were observed and photographed using Nikon SMZ1500 microscope equipped with a digital camera. The expression level for Mt2 expression (localized in the YSL) was quantified using Image-J 1.44 P. The levels of expression were determined from the mean of 15-20 embryos for each treatment subtracting for the background measurement. Responses to silver treatment were then given as fold-increase above controls.

CARS microscopy

To investigate for uptake of nanoparticles into the exposed embryos Coherent Anti-Stokes Raman Scattering (CARS) microscopy was used to provide label-free imaging with sub-cellular resolution (S5). For this imaging work, embryos were exposed at the 1 cell stage to 500 µg L⁻¹ and 5 000µg L⁻¹ 35nm Ag and 35nm TiO₂ and their bulk counterparts. Ten embryos were taken at random for each exposure concentration at 24hpf, 5 of which were manually dechorionated and the other 5 the chorion left intact. Embryos were embedded in 1% low melting agarose with 0.05% of tricaine (to anaesthetise the fish) in a glass bottomed petri dish photographed using IX71 and FV300, Olympus UK.

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Statistics

Unless stated otherwise, all data are presented as means \pm S.E.M. The co-efficient of variation (CV) statistic was calculated for comparisons of variation, as $CV = (\text{standard deviation}/\text{mean}) * 100$. All statistical analyses were performed using Sigma Stat version 12.0 (Jandel Scientific Software, USA). Differences among groups were analyzed by one-way/two way ANOVA, followed by Holm-Sidak method comparison post-hoc test, where data were not normally distributed. All data were considered statistically significant at $p < 0.05$.

Results

Particle characterisation

A summary of the characterisation and physicochemical properties of the silver particles is provided in the supplementary material Table S4 and further details are reported elsewhere (in Scown et al., (2009)). All particles had purity >99% based on trace metal analysis. The measured sizes by TEM were found to be different from those reported by the manufacturer (the nominals) and were 49.0 ± 18.5 nm and 114.0 ± 65 nm for the 10 nm and 35 nm particles, respectively. In our assessments, 10nm Ag particles had a specific surface area of $9\text{-}11\text{m}^2 \text{g}^{-1}$, bulk density of 2.05g cm^{-3} and a true density of 10.5g cm^{-3} . 35nm Ag particles had a density of 10.5g cm^{-3} , a specific surface area of $30\text{-}50 \text{m}^2 \text{g}^{-1}$, and a bulk density of $0.30\text{-}0.60 \text{g cm}^{-3}$. Ag bulk particles had a range in particle size of $0.6\text{-}1.6 \mu\text{m}$ and purity of 99.95%.

The measured physicochemical properties of TiO_2 and TEM images of the different sized materials are shown in the supplementary material Table S1 and Figure S1, respectively. When in suspension, particles formed large aggregates of several hundreds of nanometres (Supplementary material Figure S1, Table S1). The high resolution TEM micrographs show that both the 3nm and 35 nm were comprised of very small particles likely to be less than 10 nm, but their precise dimensions were not resolved due to the dense aggregation resulting in the formation of sheet like structures. The 10 nm particles formed fractal (*i.e.* porous) aggregates of about $19.1 \pm 13.8\text{nm}$, as measured by TEM. It is worth noting the relatively

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high polydispersity of this sample (Supplementary material Figure 1B, inset). The TEM measurement for 10nm particles were in good agreement with the sizes calculated from the SSA measured via the BET data and crystallite size measured by XRD (Supplementary material Table S1). The crystallite sizes were slightly larger than those measured by TEM or calculated from SSA, most likely due to the high polydispersity and aggregation observed. Measured sizes of the TiO₂ were thus again different from the data supplied by the manufacturer.

Dissolution of silver

Mean recoveries of silver for the silver nitrate control standards (in Milli Q water) were between 78% and 103%, with greater recoveries at the higher concentrations (Supplementary material Table S2). In contrast, quantification of silver in embryo culture water gave measured concentrations at between 9.9% and 64.2% of nominals (Supplementary material Table S3). Dissolution of silver ions over the 72h period for 50 µg AgNP L⁻¹, ranged between 0.1 and 2%; and for 50 µg Ag bulk L⁻¹ between 0.21-0.83%.

Lethality

Overall TiO₂ had an extremely low level of toxicity: 3nm and 35nm TiO₂ particles showed no toxicity and the lowest effect concentration for 10nm TiO₂ was 5 000 µg L⁻¹ (p=0.029) and for 134 nm TiO₂, 25 000 µg L⁻¹ (p=0.004; Figure 1B). In contrast, there was a clear dose dependent toxicity for the different sized Ag NPs and the bulk counterpart (Figure 1A). There was a statistically significant interaction between concentration and Ag particle size (Two Way ANOVA p=0.001 DF=2 F=172.161) with the following lowest effect concentrations; 5 µg L⁻¹ for 35nm Ag (p=0.002) and 50 µg L⁻¹ for both 10nm Ag (p=0.001) and Ag bulk (p=0.003). 10nm Ag was significantly more toxic than 35nm Ag across all concentrations and it was also significantly more toxic than Ag bulk for almost all concentrations tested (the exception was for 5 µg L⁻¹). Exposure to silver ions showed a dose dependent toxicity with a no effect concentration (NOEC) of 30 µg L⁻¹ and a lowest effect

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concentration (LOEC) of $60 \mu\text{g L}^{-1}$ (Two Way ANOVA $p=0.001$). At $500 \mu\text{g L}^{-1}$ there was 85% embryo mortality (Figure 1E).

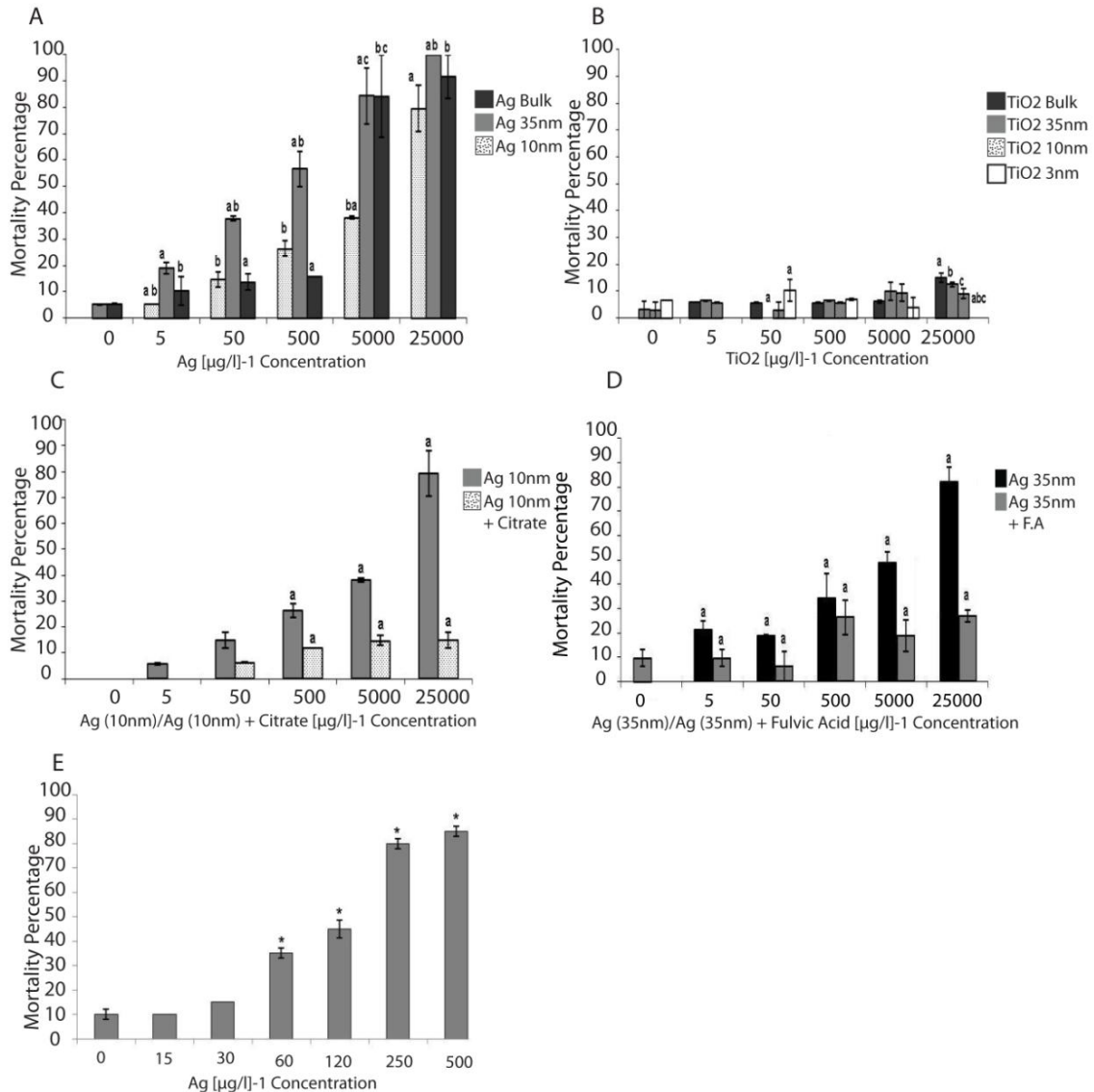


Figure 1. Mortality and morphological abnormalities at 48hpf in zebrafish embryos exposed to Ag nanoparticles (nominal sizes), TiO₂ nanoparticles (nominal sizes), Ag coated with citrate, Ag coated with fulvic and AgNO₃. Mortality rates are shown for exposure to silver particles (A), TiO₂ particles (B), Ag (10nm) and Ag (10nm) citrate coated particles (C), Ag (35nm) and (D) Ag (35nm) with fulvic acid (F), silver nitrate (E). For the different exposures there were statistically significant interactions between concentration and particle size (A, B, E), citrate coating (C) or fulvic acid (D) (Two way ANOVAs $p<0.001$). The letters (a, b, c) indicate statistical difference ($p<0.05$) between particles/coating for each concentration

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tested (All Pair-wise Multiple Comparison Procedures, Holm-Sidak method). There was no statistically significant interaction between concentration and particle size for effects on heartbeat rate (Two Way Anova $p=0.817$). Significant differences in mortality between the treatment groups for exposure to silver nitrate were assessed by One Way ANOVA ($p<0.0001$) to that of the control group.

Effects of coating on silver nanoparticle toxicity

Coating the 10nm Ag with citrate significantly reduced their toxicity across the concentration range tested (Two Way ANOVA $DF=1$ $H=6.456$ $p=0.001$) (Figure 1C). The maximum mortality rate (exposure to $25\ 000\ \mu\text{g L}^{-1}$) in the citrate coated Ag particles was 14% compared with 79% for non coated Ag particles of the same size. The lowest effect concentration for 10nm Ag was $50\ \mu\text{g L}^{-1}$ ($p<0.001$) and for Ag 10nm+citrate, $500\ \mu\text{g L}^{-1}$ ($p=0.05$, Two way ANOVA). 10nm Ag was more toxic than 10nm Ag coated with citrate for all concentrations above $50\ \mu\text{g L}^{-1}$.

Similarly, the addition of fulvic acid significantly reduced the toxicity of the 35nm Ag (Two Way ANOVA $DF=1$ $H=8.610$ $p<0.001$) (Figure 1D). The lowest effect concentration for 35nm Ag was $5\ \mu\text{g L}^{-1}$ ($p<0.001$) and for 35nm Ag with fulvic acid, $500\ \mu\text{g L}^{-1}$ ($p<0.001$). 35nm Ag was significantly more toxic than 35nm Ag + fulvic acid for all adopted exposure concentrations.

Ag nano predominantly induces embryonic lethality at gastrula stage

Video analysis on the developing embryos in the controls established that at 8hpf half had reached gastrulation stage, which is in accordance with the normal progression of expected development. In contrast, half of the embryos exposed to $25\ 000\ \mu\text{g Ag L}^{-1}$, had died by this stage. Time lapse analysis showed that for embryos exposed to 35nm Ag the yolk sac membrane of the embryo became damaged, leading to the leakage of yolk and subsequently mortality (Figure 2). It was observed that the surface of the blastoderm became rough (Fig. 2E, F) and epiboly, the process where cells move and spread out into

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sheets of tissue that overlie or surround other groups of cells, was delayed in comparison with control embryos (normally occurring at approximately 4hpf). The blastoderm in the surviving embryos treated with 35nm Ag did not cover the yolk and had only reached approximately 40% epiboly in comparison with the control embryos where there was nearly 70% epiboly. Embryos that survived the exposure to Ag at the high exposure concentrations subsequently had morphological abnormalities including bent tails, small head and a reduced yolk sac size (Figure 2).

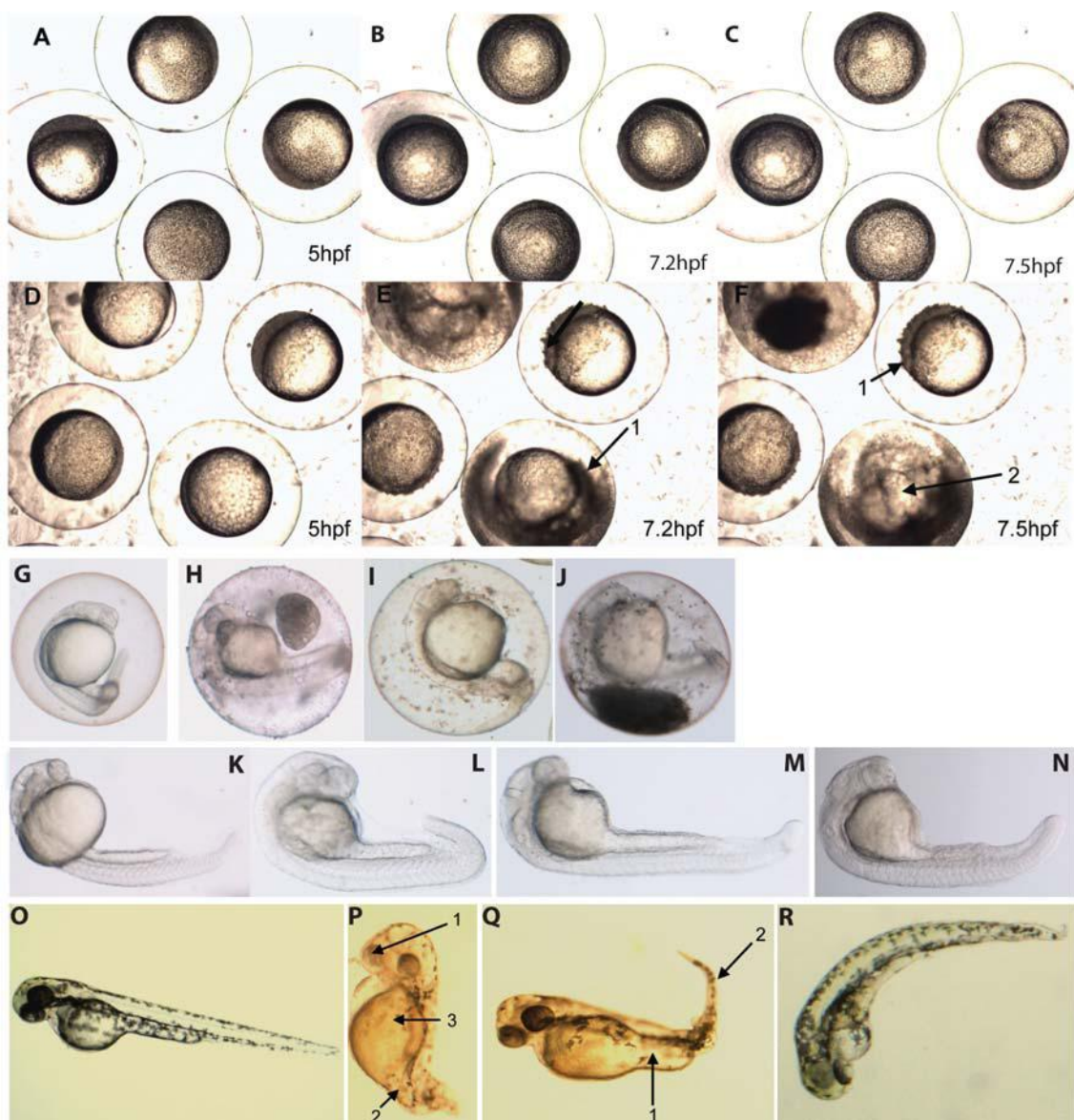


Figure 2. Images of embryos exposed to silver particles at various developmental stages.

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Video captured images of zebrafish embryos in controls (**A-C**, 5hpf, 7.2hpf and 7.5hpf, respectively) and exposed to silver particles (35nmAg at 25000 µg/l) from 1-2 cell stage (**D-F**, 5hpf, 7.2hpf showing - 1.Yolk leaking out from the cells, 7.5hpf showing -1. Uneven surface of dividing cells, 2. Embryo bursting within the chorion membrane).

G-R, microscope images of control/ exposed embryos at different developmental stages: **G**. 24hpf control embryo, **H**.24pf Ag (10nm 5 000 µg/l) **I**. 24pf Ag (Bulk 5 000 µg/l) **J**. 24hpf AgNO₃ (120 µg/l), **K**. 24hpf control embryo **L**. 24hpf Ag (35nm 500 µg/l) **M**. 24hpf Ag (Bulk 500 µg/l) **N**. AgNO₃ (120µg/l) **O**. 48hpf control embryo, **P**. 48hpf Ag (35nm, 5 000 µg/l) showing -1. eyes spaced more widely on the head compared with controls, 2. absence of a tail, and 3. deformed yolk sac, **Q**. 24hpf Ag (Bulk, 5 000 µg/l) showing -1.bent tail, 2. reduced yolk sac, **R**. AgNO₃ (120µg/l).

Cell necrosis in early life stage embryos

Staining for cell necrosis during the gastrula stage (7hpf) identified a high prevalence cell death in the exposures to 35nm Ag (500 µg L⁻¹ and 25 000 µg L⁻¹; Figure 3H, L). In the controls and embryos exposed to TiO₂ there was a very low/no incidence of necrotic cells (Figure 3B, E). Based on a qualitative assessment only, there appeared to be similar numbers of live cells in all embryos examined in controls, TiO₂ exposures and for Ag at 500 µg L⁻¹ (Figure 3 I, H). There was a high level of necrotic nuclei in the yolk syncytial layer (YSL), which forms during blastula stages of larval development (Figure 3 H).

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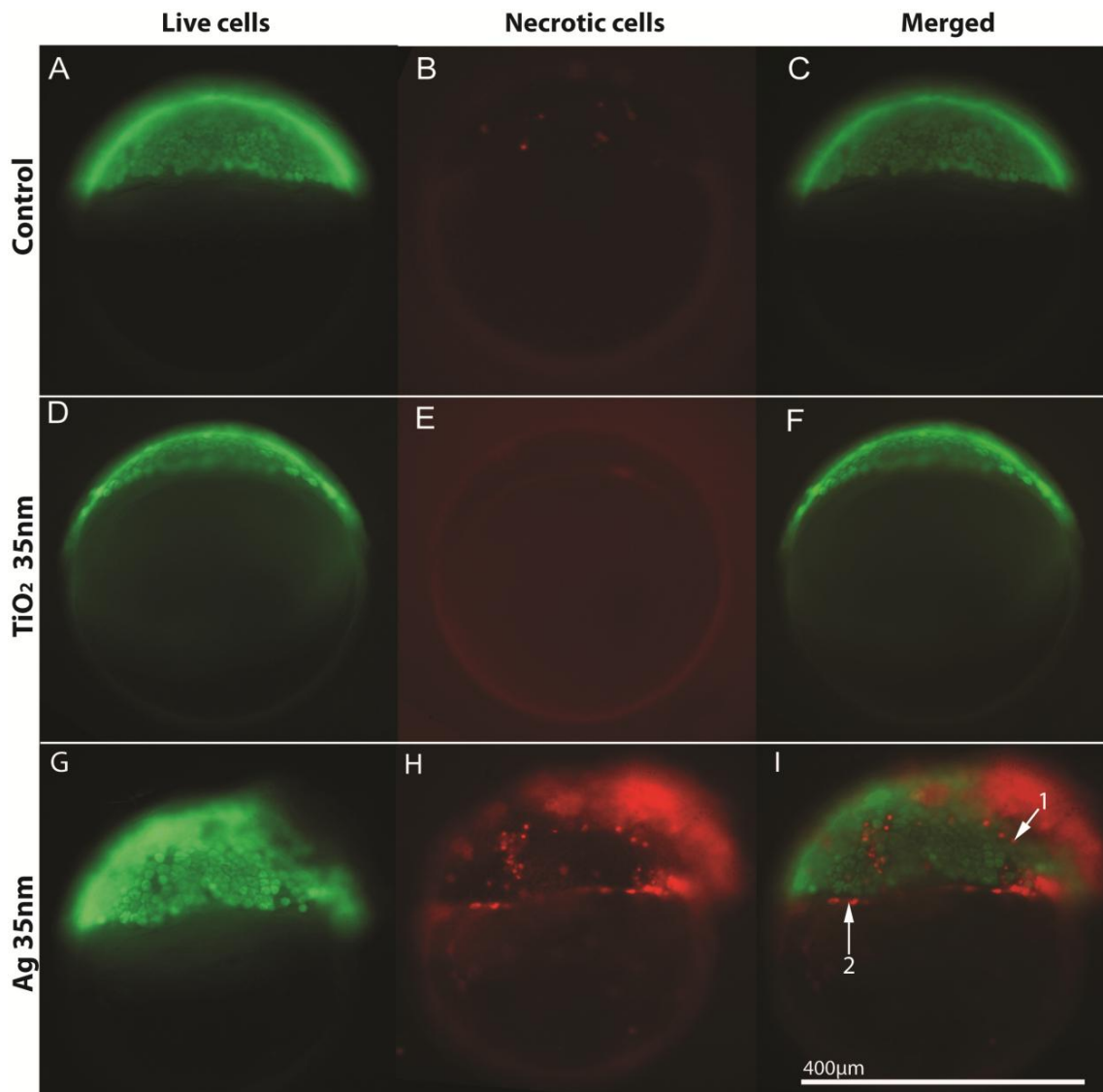


Figure 3. Images showing live (green)/necrotic (red) cells in embryos at shield stage (Lateral View). **A.** control-live cells **B.** control-necrotic cells **C.** control -merged images live/necrotic cells **D.** TiO₂ (35nm 500 µg/l) -live cells **E.** TiO₂ (35nm 500 µg/l) - necrotic cells **F.** TiO₂ (35nm 500 µg/l) – merged live/necrotic cells **G.** Ag (35nm 500 µg/l) -live cells **H.** Ag (35nm 500 µg/l) - necrotic cells **I.** Ag (35nm 500 µg/l) – merged live/necrotic cells, showing -1. necrotic nuclei in blastoderm, 2. necrotic nuclei in yolk syncytial layer (YSL)

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Particle uptake (CARS)

The studies showing cell damage for the high concentration exposures suggested that material (particles and/or free silver) entered the embryo from the culture medium. CARS microscopy however, showed no detectable particles contained within the exposed embryos (Figure 4). CARS images (Figure 4), including images that were focused at the outside edge of the chorion (panels B and C), for exposures of embryos to both AgNPs and TiO₂NPs, illustrate that the particles were associated with the outer edge of the embryo and not contained within the embryo itself.

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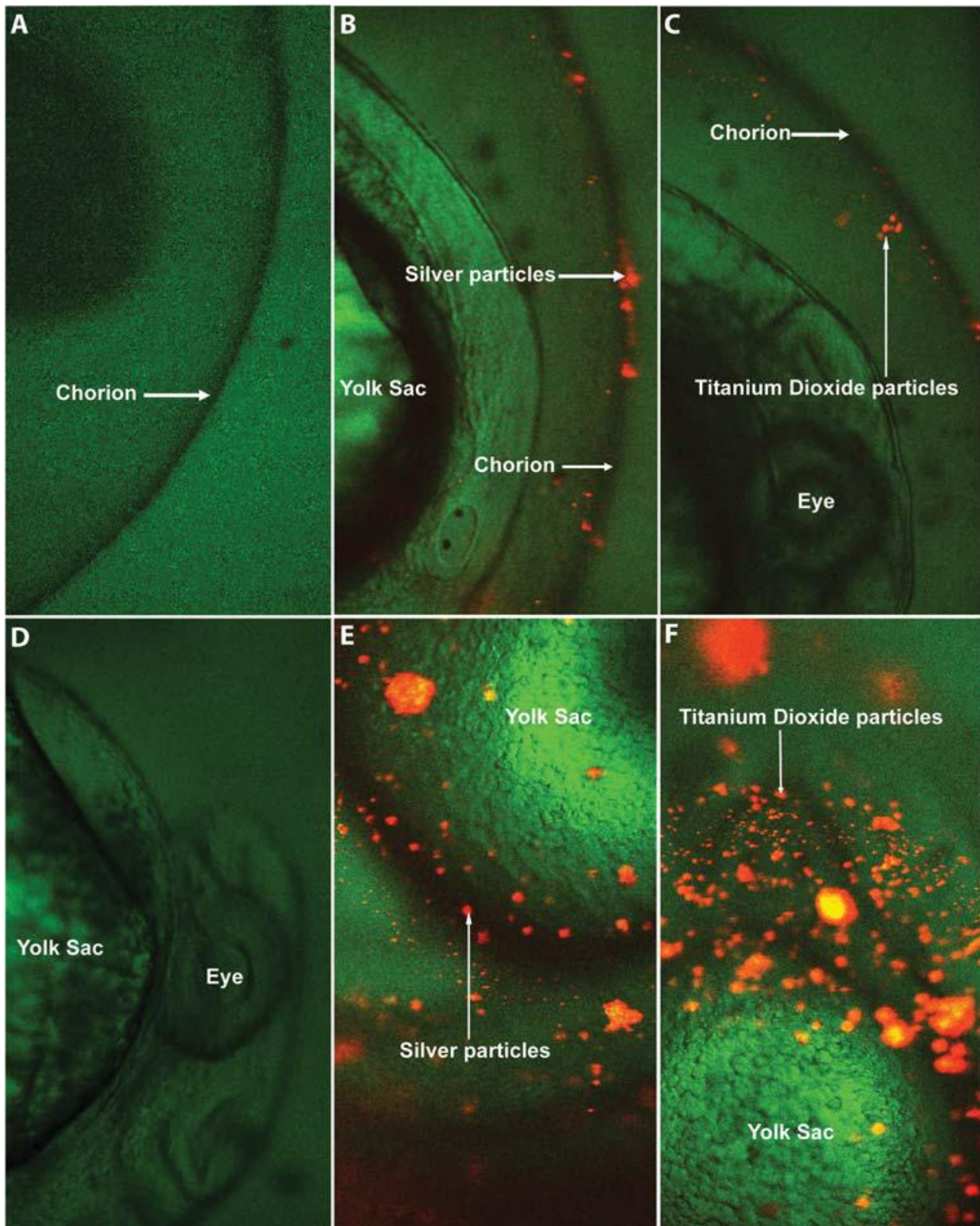


Figure 4. Coherent Antistokes Raman Scattering images of embryos exposed to silver and titanium nanoparticles after 24h. Aggregates of NPs appear as coloured (yellow/red) patches on the image. NPs were visible only on the outside of chorion membrane. **A.** Control embryo - showing chorion margin, **B.** Embryo exposed to Ag (35nm, 25 000 µg/l) -

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showing chorion margin, **C**. Embryo exposed to TiO₂ (35nm, 25 000 µg/l) -showing chorion margin, **D**. Control embryo showing chorion surface of embryo. **E**. Embryo exposed to Ag (35nm, 500 µg/l) showing silver particles on chorion surface of embryo **F**. Embryo exposed to TiO₂ (35nm, 500 µg/l) showing titanium dioxide particles on chorion surface of embryo. CARS revealed nanomaterial on the surface of the chorion, likely as aggregates of nano particles **B, C**), but none were detected internally to the chorion within the embryo itself.

Expression of metallothionein

We conducted *in situ* hybridisation with Mt2 to identify possible tissue targets for metal responses induced by exposure to Ag nano. For the exposures to all silver treatment groups at sub-lethal doses, and for which no significant morphological defects were found, significant induction of Mt2 was detected in the YSL especially at the posterior extension. Exposure to 35nm Ag (Figure 5) induced a 3.9-fold increase, Ag bulk material induced a 2.7-fold increase and Ag ion (12µg L⁻¹) induced a 2.8 fold increase. TiO₂ (500 µg L⁻¹) did not show any enhanced expression of Mt2.

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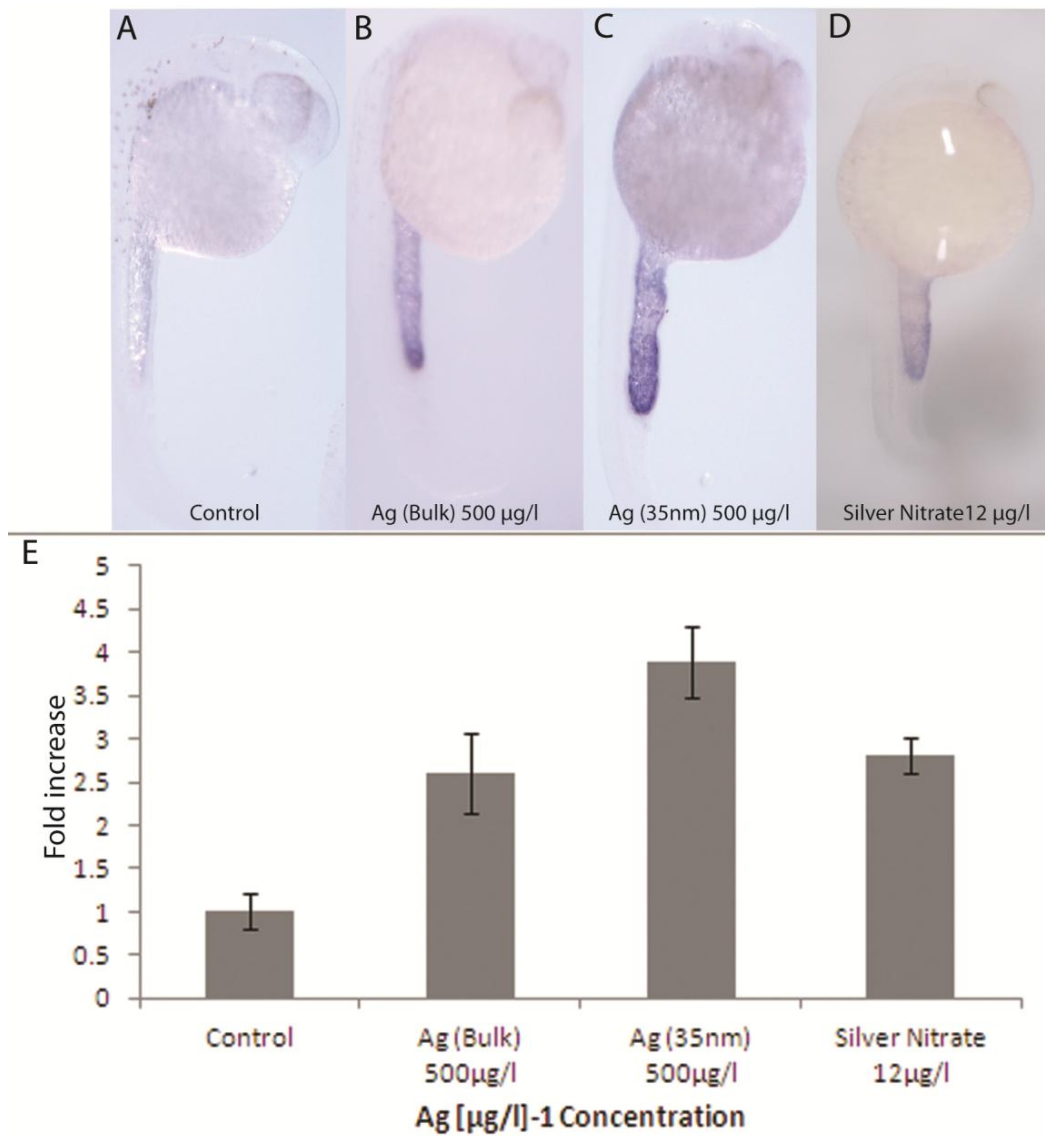


Figure 5. Embryos (24hpf) after applying the technique *in-situ* hybridisation to investigate for expression of metallothionein 2 as a measure of metal exposure/toxicity. There was a very low expression signal in the YSL especially at the posterior extension in the control embryos and in the embryos exposed to TiO_2 , but high expression in the Ag nano/bulk exposed embryos. **A.** Control embryo, **B.** Embryo-exposed to Ag (Bulk 500 µg/l), **C.** Embryo-exposed to Ag (35nm, 500 µg/l), **D.** Embryo-exposed to AgNO_3 (12 µg/l), **E.** Graphical representation of the fold-increase Mt2 expression in the different treatments.

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Discussion

We found TiO₂NPs had little or no toxicity in zebrafish embryos on the endpoints measured and at exposure levels far exceeding those predicted to occur in some of the most polluted environments (Colvin, 2003). Our data support the majority of previous studies in this regard and would suggest therefore that in natural environments exposure to the TiO₂ particles tested are unlikely to pose any obvious health threat to fish embryos, which are widely accepted as highly sensitive to the effects of a wide range of toxicants.

In contrast Ag induced a dose dependent toxicity in both nano and bulk form. One possible explanation for the enhanced toxicity of the nano Ag is that the particles themselves may interfere with biological processes because they have the potential to by-pass barriers which normally prevent larger molecules from entering (Scown et al., 2010b). It is thought that nanoparticles can enter via pathways such as tight junctions (Luhmann et al., 2008) and if this is the case, in turn block the channel pathways of epithelial membranes (Hunziker et al., 2009). But for these particles, this is unlikely as they were aggregated. Furthermore; an enhanced ability to cross cell membranes was not supported by the CARS imaging in this study where at 24hpf we found no evidence for uptake of Ag (or TiO₂) nanoparticles into the embryo (Figure 4).

Time lapse video analysis of the embryos exposed to nano and bulk silver established that it was during gastrulation, when the yolk sac folds in on itself over the cells, where the greatest mortality occurred. The necrosis assay confirmed a high incidence of damaged nuclei both in the blastoderm and the YSL in the Ag exposed embryos at this development period (7hpf). We recently reported that deformity of the YSL often results in failure of gastrulation cell movement which leads to embryonic lethality at the gastrula stage (Takesono et al., 2012). The developmental morphologies seen for exposure to Ag particles (i.e. bent tails and a small head) are common for embryos exposed to xenobiotic compounds (Yeo and Kang, 2008) and some abnormalities likely result from failed epiboly movement.

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Citrate is used widely to stabilize NPs to prevent /reduce their aggregation (Baveye and Laba, 2008) and here we found that coating 10nm Ag particles with citrate reduced significantly rates of mortality and abnormalities in exposed embryos compared with uncoated 35nm Ag; the LC50 of 35nm Ag was $500 \mu\text{g L}^{-1}$ compared with $5\,000 \mu\text{g L}^{-1}$ for 35nm Ag coated with citrate (i.e. 10-fold lower). No studies were undertaken to investigate the aggregation behaviour of the different particles in the embryo incubation water, but some other studies have shown that in high ionic strength water there can be an enhanced aggregation rate for particles coated with citrate (Christian et al., 2008). It is possible therefore, that an enhanced aggregation of the citrate coated particles resulted in a lower bioavailability of Ag particles/ions for uptake. Subtle differences in the nature of NPs have also been found to profoundly affect biological effects responses (Moore, 2006). An alternative hypothesis is that the toxicity of the silver nanoparticles derives from the dissolution of silver ions from the particles and the rate of this process is much reduced in citrate coated silver particles (Treuel et al., 2010, Studer et al., 2010, Kittler et al. 2010). Similarly, addition of fulvics to the medium also reduced the toxicity of the AgNPs to the fish embryos. Such a coating could affect the particles by reducing Ag particle dissolution rates, and/or complexing free Ag ions after dissolution.

It is well established that fish and many other aquatic animals are sensitive to the toxic effects of silver ions, with LC10 concentrations reported for rainbow trout (*Oncorhynchus mykiss*) between 0.7 to $0.8 \mu\text{g L}^{-1}$ and LC50 between $10 \mu\text{g L}^{-1}$ to $240 \mu\text{g L}^{-1}$ for freshwater fish species (Birge and Zulderveen, 1996). The degree of dissolution (up to 2%) we found for Ag 35nm equates well with previous literature (Kittler et al., 2010, Fabrega et al., 2009). Based on the amount of silver ions in solution, it appears that they do not explain all of the toxicity observed. However; the Ag particles settle on the embryo surface (as evidenced by the CARS imaging), and therefore the local concentration of dissolved Ag ions is likely to be higher at the membrane surface compared with the surrounding medium and therefore may explain all toxicity observed in our experiments. Nevertheless, it is still possible that the NP is having an effect directly on toxicity. These discrepancies further highlight the need for stringent reporting on the physio-chemical characterization of materials used. A further difficulty in relating the toxicity effects with the measured Ag^+ is the embryo medium

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contained relatively high levels of chloride ion ($626.2 \mu\text{mol L}^{-1}$) and this can complex Ag^+ and in turn reduce its toxicity.

CARS images (Figure 4) illustrated that the nanomaterials, generally appearing as aggregates, were associated with the outer edge of the embryo (panels B and C) with no evidence for penetration of the embryo itself. This was supported by CARS images of embryos that were dechorionated and showed an absence of any nanomaterial at the embryo surface and again no evidence of body penetration. Contrasting with this however, expression of Mt2, that plays a central role in metal transport, storage and detoxification (Ngu and Stillman, 2006), strongly supports an intracellular presence of silver ions in exposed embryos/larvae.

We identified Mt2 expression in the YSL of the embryo, a body region where processing of xenobiotic compounds is known to occur in zebrafish embryos (Chen et al., 2004a). The YSL was both the target for a toxicology response (cell necrosis) to AgNPs at the gastrula stage of development and location of Mt2 expression later in development (24hpf), for exposure to the lower Ag exposure concentration. We found low level and more diffuse expression of Mt2 at 7.2hpf compared with at 24hpf and this may confer a lower resistance of earlier life stage embryos to the toxic effects of Ag, but this would need further investigation to confirm this hypothesis. No such gene up-regulation was seen in TiO_2 exposed embryos. These findings provide further evidence that at least some of the Ag toxicity relates to the bioavailability of silver ions that may be more readily released from nanoform silver. This would indicate the possibility for greater health effects associated with silver for AgNP exposure. Our data further show Mt2 as an effective biomarker for exposure to silver nanoparticles in fish embryos. Where the release of silver ions occurs to induce the response in Mt2 is not known, it may potentially occur outside of the embryo from where the silver ions are then transported into the embryo or be released from AgNPs that have penetrated the embryo, or a combination of both.

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Conclusion

Our findings indicate that TiO₂NPs are not likely to have adverse biological effects in fish in the natural environment. In contrast; AgNPs at sublethal exposure concentrations have the potential to induce harmful effects, disrupting embryo development predominantly at gastrula stage, inducing embryonic deformity at 1-2dpf stage and inducing the heavy metal stress response gene Mt2 in the YSL. These reported effects occur predominantly at exposure levels exceeding those currently found (or estimated) in the most aquatic environments but with the rapid expansion in the use and discharge of AgNPs, concentrations in the aquatic environment are likely to rise in the near future (Simonet and Valcárcel, 2009a); reviewed in Fabrega et al. (2011) heightening potential health concerns. Collectively, our data would suggest that silver ions play a major role in the toxicity of AgNPs and furthermore we show that coating of the particles, here with citrate or natural organic matter (here fulvics) can reduce significantly associated toxicity with major implications for understanding toxicity of metal NPs in the natural environment.

Acknowledgments

The NERC funded Facility for Environmental Nanoscience Analysis and Characterisation (FENAC) is acknowledged for their support in generating the characterisation data.

Declaration of Interest

This work was supported by the UK Environment Agency and NERC (NE/H013172/1) on grants to CRT.

Supportive Information

The supportive information contains extra information on the materials and methods. In addition it contains data for the characterization of the titanium dioxide particles.

References

AshaRani PV, Hande MP, Valiyaveetil S. 2009. Anti-proliferative activity of silver nanoparticles. BMC Cell Biology 10:65.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Baveye P & Laba M. 2008. Aggregation and toxicology of titanium dioxide nanoparticles. Environmental health perspectives 116: A152.
- Birge W & Zulderveen J. (1996). The Comparative Toxicity of Silver to Aquatic Biota. University of Wisconsin System, Sea Grant Institute. Available at: <http://images.library.wisc.edu/EcoNatRes/EFacs/Argentum/Argentumv03/reference/econatres.argentumv03.birgetoxicity.pdf> Accessed on: October 2011
- Braydich-Stolle L, Hussain S, Schlager J, Hofmann, M. 2005. In vitro cytotoxicity of nanoparticles in mammalian germline stem cells. Toxicological Sciences 88: 412.
- Chen W, John J, Lin C, Lin H, Wu S, Chang, C. 2004. Expression of metallothionein gene during embryonic and early larval development in zebrafish. Aquatic Toxicology 69:215-227.
- Choi JE, Kim S, Ahn JH, Youn P, Kang JS, Park K, Yi J, Ryu DY. 2009. Induction of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. Aquatic Toxicology 100:151-159.
- Christian P, Von der Kammer F, Baalousha M, Hofmann T. 2008. Nanoparticles: structure, properties, preparation and behaviour in environmental media. Ecotoxicology 17:326-343.
- Colvin VL. 2003. The potential environmental impact of engineered nanomaterials. Nature Biotechnology 21:1166-1170.
- Fabrega J, Fawcett SR, Renshaw JC, Lead JR. 2009. Silver Nanoparticle Impact on Bacterial Growth: Effect of pH, Concentration, and Organic Matter. Environmental Science & Technology 43:7285-7290.
- Fabrega J, Luoma SN, Tyler CR, Galloway TS, Lead JR. 2011. Silver nanoparticles: Behaviour and effects in the aquatic environment. Environment International, 37:517-531.
- Heinlaan M, Ivask A, Blinova I, Dubourguier HC, Kahru A. 2008. Toxicity of nanosized and bulk ZnO, CuO and TiO₂ to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. Chemosphere 71:1308-1316.
- Hu Z. (2010) Impact of Silver Nanoparticles on Wastewater Treatment. Water Intelligence Online 9:58. Available at: <http://www.mendeley.com/research/impact-silver-nanoparticles-wastewater-treatment/> Accessed on: November 2011

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Hunziker W, Kiener T, Xu J. 2009. Vertebrate animal models unravel physiological roles for zonula occludens tight junction adaptor proteins. *Annals of the New York Academy of Sciences* 1165:28-33.
- Hussain S, Hess K, Gearhart J, Geiss K, Schlager J. 2005. In vitro toxicity of nanoparticles in BRL 3A rat liver cells. *Toxicology in vitro* 19:975-983.
- Jin X, Li M, Wang J, Marambio-Jones C, Peng F, Huang X, Damoiseaux R, Hoek EMV. 2010. High-throughput screening of silver nanoparticle stability and bacterial inactivation in aquatic media: influence of specific ions. *Environmental Science & Technology* 44:7321–7328.
- Ju-Nam Y, Baalousha M, Cole P, Tyler C, Stone V, Fernandes T, Jepson M, Lead J. 2012. Characterisation of cerium dioxide NPs . Part I : Size measurements. *Environmental Toxicology and Chemistry*. (*In Press*)
- Kittler S, Greulich C, Diendorf J, Köhler M, Epple M. 2010. Toxicity of Silver Nanoparticles Increases during Storage Because of Slow Dissolution under Release of Silver Ions. *Chemistry of Materials* 22:4548-4554.
- Lee KJ, Nallathamby PD, Browning LM, Osgood CJ, Xu XHN. 2007. In Vivo Imaging of Transport and Biocompatibility of Single Silver Nanoparticles in Early Development of Zebrafish Embryos. *ACS Nano* 1:133-143.
- Long T, Tajuba J, Sama P, Saleh N, Swartz C, Parker J, Hester S, Lowry G, Veronesi B. 2007. Nanosize titanium dioxide stimulates reactive oxygen species in brain microglia and damages neurons in vitro. *Environmental health perspectives* 115:1631.
- Lovern SB, Strickler JR, Klaper R. 2007. Behavioral and physiological changes in *Daphnia magna* when exposed to nanoparticle suspensions (titanium dioxide, nano-C60, and C60HxC70Hx). *Environmental Science & Technology* 41:4465-4470.
- Luhmann C, Chun M, Yi D, Lee D, Wang X. 2008. Neural dissociation of delay and uncertainty in intertemporal choice. *Journal of Neuroscience* 28:14459.
- Luoma SN, Rainbow P. (2008). *Metal contamination in aquatic environments: science and lateral management*, Cambridge University Press.
- Marambio-Jones C, Hoek EMV. 2010. A review of the antibacterial effects of silver nanomaterials and potential implications for human health and the environment. *Journal of Nanoparticle Research* 12:1531-1551.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Moore M. 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? *Environment International* 32:967-976.
- Ngu T, Stillman M. 2006. Arsenic binding to human metallothionein. *J. Am. Chem. Soc.* 128:12473-12483.
- Nowack B, Bucheli TD. 2007. Occurrence, behavior and effects of nanoparticles in the environment. *Environmental Pollution* 150:5-22.
- Pinero J, Lopez-Baena M, Ortiz T, Cortes F. 1997. Apoptotic and necrotic cell death are both induced by electroporation in HL60 human promyeloid leukaemia cells. *Apoptosis* 2:330-336.
- Purcell TW, Peters JJ. 1999. Historical impacts of environmental regulation of silver. *Environmental Toxicology and Chemistry* 18:3-8.
- Scown T, Van Aerle R, Johnston B, Cumberland S, Lead J, Owen R, Tyler C. 2009. High doses of intravenously administered titanium dioxide nanoparticles accumulate in the kidneys of rainbow trout but with no observable impairment of renal function. *Toxicological Sciences* 109:372.
- Scown T, Van Aerle R, Tyler C. 2010. Review: Do engineered nanoparticles pose a significant threat to the aquatic environment? *Critical reviews in toxicology* 40:653-670.
- Simonet B, Valcárcel M. 2009. Monitoring nanoparticles in the environment. *Analytical and Bioanalytical Chemistry* 393: 17-21.
- Skebo J, Grabinski C, Schrand A, Schlager J, Hussain S. 2007. Assessment of metal nanoparticle agglomeration, uptake, and interaction using high-illuminating system. *International journal of toxicology* 26:135.
- Studer AM, Limbach LK, Van Duc L, Krumeich F, Athanassiou EK, Gerber LC, Moch H, Stark WJ. 2010. Nanoparticle cytotoxicity depends on intracellular solubility: Comparison of stabilized copper metal and degradable copper oxide nanoparticles. *Toxicology letters* 197:169-174.
- Takesono A, Moger J, Farooq S, Cartwright E, Dawid IB, Wilson SW, Kudoh T. 2012. Solute carrier family 3 member 2 (Slc3a2) controls yolk syncytial layer (YSL) formation by regulating microtubule networks in the zebrafish embryo. *Proceedings of the National Academy of Sciences*, 109: 3371-3376.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

Treuel L, Malissek M, Gebauer J, Zellner R. 2010. The Influence of Surface Composition of Nanoparticles on their Interactions with Serum Albumin. *ChemPhysChem* 11:3093-3099.

Yeo M, Kang M. 2008. Effects of nanometer sized silver materials on biological toxicity during zebrafish embryogenesis. *Bull. Korean Chem. Soc* 29: 1179–1184.

Zhao J, Bowman L, Zhang X, Vallyathan V, Young S, Castranova V, Ding M. 2009. Titanium Dioxide (TiO₂) Nanoparticles Induce JB6 Cell Apoptosis Through Activation of the Caspase-8/Bid and Mitochondrial Pathways. *Journal of Toxicology and Environmental Health Part A* 72: 1141-1149.

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SUPPLEMENTARY MATERIAL

**Effects of particle size and coating on nanoscale Ag and TiO₂ exposure
in zebrafish (*Danio rerio*) embryos**

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Supplementary Material

S1

Fish source, culture, and husbandry

Wild-type WIK strain zebrafish embryos were obtained from the Max Planck Institute, Tubingen, Germany and maintained at University of Exeter. Fry from approximately 2 days post-hatch (dph) were fed on a microencapsulated diet (ZM Advanced Fry feed; ZM Ltd., Hampshire, U.K.). This was supplemented from approximately 7 dph with freshly hatched *Artemia nauplii* (ZM Premium Grade Artemia; ZM Ltd.). From approximately 21 dph onwards to adulthood fish were fed with freshly hatched *Artemia nauplii* to satiation twice daily. As adults, fish were fed daily on both freshly hatched *Artemia nauplii* and TetraMin tropical flake food (TetraMin, Tetra Werke, Melle, Germany). Embryos for use in the toxicity assessments were collected from naturally spawning colonies.

S2

Time lapse video analysis using compound microscopy

To gain an insight into the timing of mortality and developmental effects induced by the exposures to Ag, time lapse video analysis was used. 20 embryos were exposed at the 1 cell stage to 35nm Ag at 25 000 $\mu\text{g L}^{-1}$ and incubated at 28 \pm 1 °C for 5 hours. Four embryos were then taken at random and embedded into 1% low melting agarose in a petri dish. The petri dish was placed on a hotplate (in order to maintain the temperature at 28 \pm 1 °C) and images were taken every 150 seconds for 3 hours. A petri dish containing non exposed control embryos was run under identical conditions for comparison. Embryos were photographed using Leica DMI4000 B Compound Microscope equipped with a digital camera.

S3

Whole mount in-situ hybridization

For the hybridizations, embryos were fixed using 4% PFA in PBS at 4°C overnight, then dechorionated and placed in methanol for 2 hours. The embryos were placed in

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hybridisation buffer for 1 hour and incubated with the Mt2 probe overnight at 65°C. The embryos were then washed in 50% formamide 2XSSC, 0.1%Tween 20 wash for 30minutes at 65°C, followed by a 2XSSC 0.1%Tween 20 wash at 65°C and then 2x 30 minutes wash at 65°C with 0.2XSSC 0.1%Tween 20. Blocking solution (2% Blocking reagent (Roche) 2.5ml of calf serum in MAB: component here) was added for 1 hour. Anti-DIG antibody (Roche) (x5000, dilute 1/100 with pre-absorption with fixed embryos) was then added for 2 hours. A series of 4x 30minutes of PBS + 0.1%Tween20 washes was conducted before a 10 minute wash with AP buffer (Tris 0.1M pH 9.5, NaCl 0.1M, MgCl₂ 50mM, Tween 20 0.1%). Embryos were transferred to a 24 well plate and placed in the staining solution of BM-Purple AP Substrate (Roche REF 11442074001) to reveal the probe.

S4

CARS microscopy

CARS microscopy derives chemically specific contrast from the vibrational frequencies of molecular bonds within a sample (for reviews see Zumbusch et al., (1999)). Briefly, imaging was performed using a modified commercial inverted microscope and confocal laser scanner (IX71 and FV300, Olympus UK), as described previously (Moger et al., 2008, Majewska et al., 2000, Wang et al., 2005). 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 the CARS generation, simultaneous forwards- and epi-detection is desirable (Cheng and Xie, 2004). 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).

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Supplementary Tables:

Table S1. Characterisation measurements on 3 nm TiO₂, 10 nm TiO₂, 35 nm TiO₂ and Bulk TiO₂ in distilled water

Original S Mitov swap	3 nm TiO₂	10 nm TiO₂	35 nm TiO₂	Bulk TiO₂
CAS No	1317-70-0	1317-70-0	1317-70-0	1317-70-0
Primary particle size by TEM (nm)	*	19.1 ± 13.8	*	
Mean particle size by AFM (nm)	-	46.0 ± 2.6	-	518.1 ± 65.9
Mean particle size calculated from SSA (nm)	10.8	32.5	4.4	115.5
Crystallite size by XRD (nm)	20.7 ± 4.1**	42.4 ± 3.9**	8.1 ± 1.1**	63.8 ± 2.3**
Specific Surface Area (SSA) by BET (m² g⁻¹)	142.6	47.3	350.0	10.6
Particle number by NTA mL⁻¹	(1.8 ± 0.3) × 10 ⁸	(1.0 ± 0.2) × 10 ⁸	(0.9 ± 0.2) × 10 ⁸	(0.6 ± 0.4) × 10 ⁸
Hydrodynamic diameter by NTA (nm)	38 ± 14	65 ± 14	475 ± 128	393 ± 354
Aggregate size by TEM (nm)	1242 ± 682	1377 ± 1113	959 ± 570	

* particles formed sheets of aggregates with aligned orientation and grain boundaries could not be resolved.

** Estimates of particle size by XRD using peak 101 and 200. Strain effect is ignored. Samples are composed primarily of Anatase

Density = 3.9

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

Table S2. ICP-MS results of mean recoveries of silver for AgNO₃ control standards measured in Milli-Q water

AgNO₃ in Milli-Q water	CONCENTRATION (ppb)
Sample	Ag / 107
0 ppb	< 0.1
15 ppb	11.75
30 ppb	30.29
60 ppb	63.60
120 ppb	129.2
240 ppb	249.8

Table S3. ICP-MS results of mean recoveries of silver for AgNO₃ control standards measured in embryo culture water

AgNO₃ in culture water	CONCENTRATION (ppb)
Sample	Ag / 107
0 ppb	< 0.1
15 ppb	9.93
30 ppb	13.13
60 ppb	19.61
120 ppb	23.63
240 ppb	23.79

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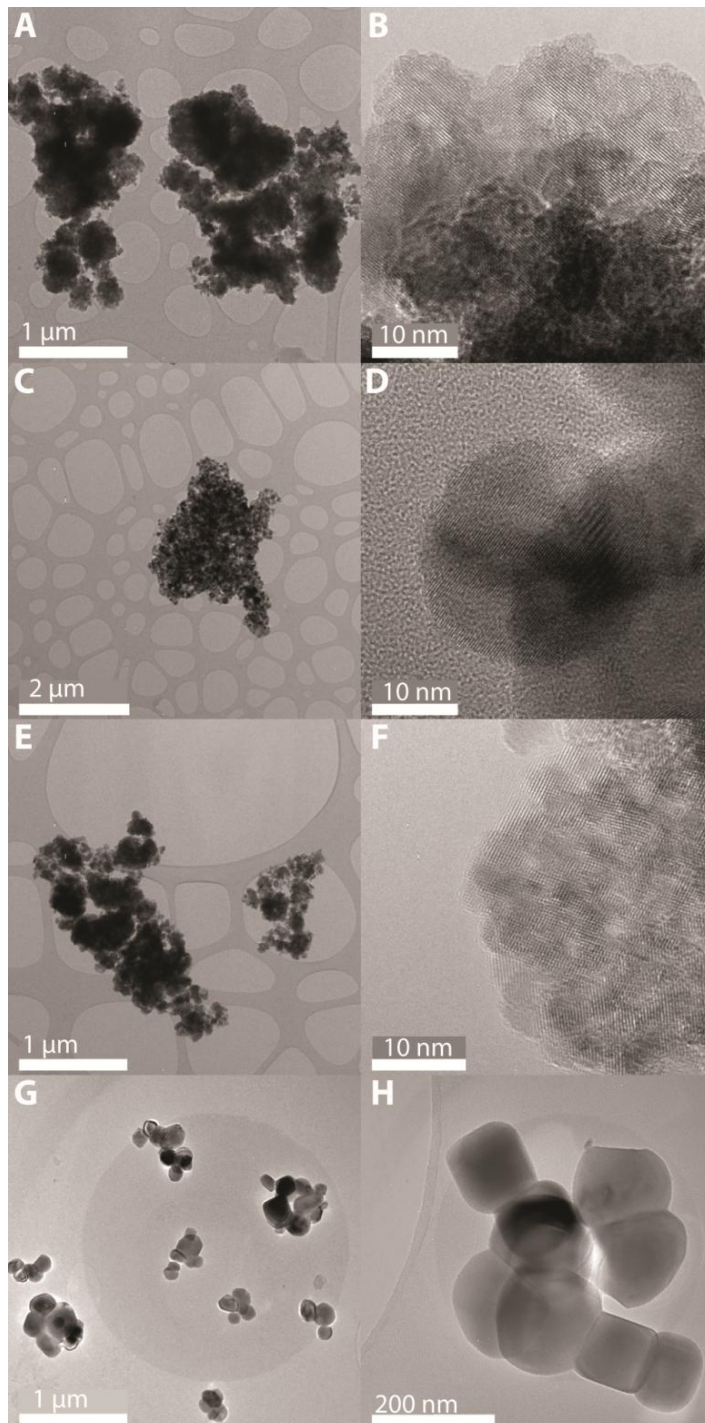
Table S4. Characterisation measurements on 10nm Ag, 35 nm Ag and Bulk Ag in distilled water

	10nm Ag	35nm Ag	Bulk Ag
Zeta Potential	-12.52 ± 2.7	-6.5 ± 1.8	-2.8 ± 0.6
pH	7.11	7.34	6.40
Hydrodynamic Diameter (nm) DLS	589 ± 101	2029 ± 524	938 ± 230
Polydispersant Index	0.54	0.93	0.69
Hydrodynamic diameter (nm) NTA technique	158 ± 76	166 ± 72	217 ± 130
Particle per ml (NTA technique)	1.07 x 10 ⁸	0.21 x 10 ⁸	0.27 x 10 ⁸
Mean particle size (nm) (TEM)	49 ± 18.5	114 ± 65.3	137 ± 62.0
Mean particle size (nm) (AFM)	46.3 ± 10.7	90.0 ± 15.6	147.5 ± 82.3
Crystallite size (nm) (XRD technique)	21.2 ± 0.5	68.0 ± 2.0	60.0 ± 4.6
Surface area (m²/g)(BET)	2.0 ± 0.2	2.9 ± 0.2	0.6 ± 0.1

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

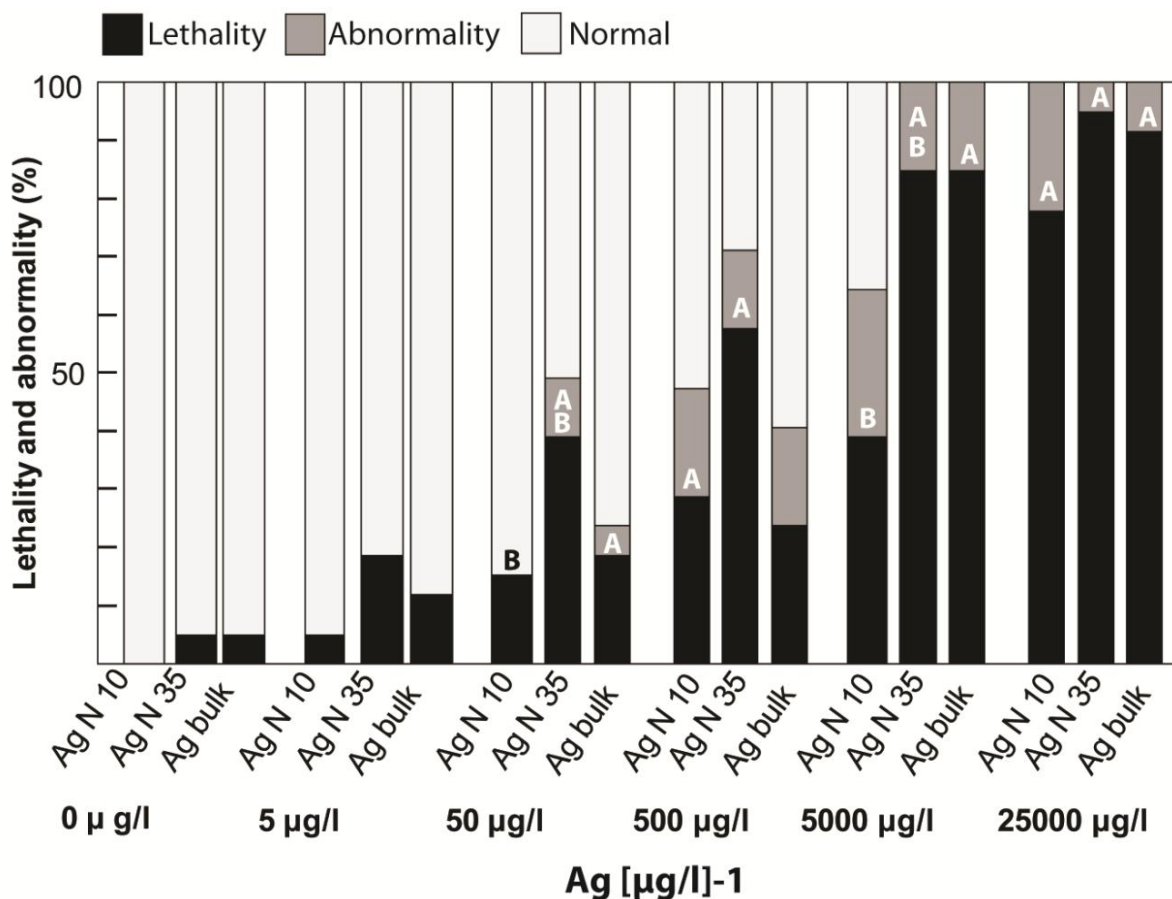
Supplementary Figures :-

Figure S1 Typical TEM micrographs of TiO₂NPs aggregates **(A)** 3 nm, **(C)** 10 nm, **(E)** 35 nm and **(G)** Bulk. The corresponding HRTEM of TiO₂ NPs **(B)** 3 nm, **(D)** 10 nm and **(F)** 35 nm and **(H)** Bulk.



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Figure S2 Combined mortality and developmental abnormality data for exposure to silver particles



References

- Cheng JX, Xie XS. 2004. Coherent Anti-Stokes Raman Scattering Microscopy: Instrumentation, Theory, And Applications. *The Journal Of Physical Chemistry B* 108:827-840.
- Majewska A, Yiu G, Yuste R. 2000. A Custom-Made Two-Photon Microscope And Deconvolution System. *Pflügers Archiv European Journal Of Physiology* 441:398-408.
- Moger J, Johnston BD, Tyler CR. 2008. Imaging Metal Oxide Nanoparticles In Biological Structures With Cars Microscopy. *Optics Express* 16:3408-3419.
- Wang H, Fu Y, Zickmund P, Shi R, Cheng JX. 2005. Coherent Anti-Stokes Raman Scattering Imaging Of Axonal Myelin In Live Spinal Tissues. *Biophysical Journal* 89:581-591.
- Zumbusch A, Holtom GR, Xie XS. 1999. Three-Dimensional Vibrational Imaging By Coherent Anti-Stokes Raman Scattering. *Physical Review Letters* 82:4142-4145.

CHAPTER 3

**SENSORY SYSTEMS AND IONOCYTES ARE AMONG THE TARGET TISSUES FOR
SILVER NANOPARTICLES IN FISH**

To be submitted to *Nanotoxicology*

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

Sensory systems and ionocytes are among the target tissues for silver nanoparticles in fish

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BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

Abstract

There is concern internationally that some nanoparticles (NPs) may induce adverse health effects in exposed organisms, but to date the evidence for this in wildlife is minimal. Silver nanoparticles (AgNPs) comprise more than 30% of marketed nano products and they can be toxic to aquatic organisms, including fish, at concentrations relevant for some environmental exposures. Major challenges in the ecotoxicology of nanomaterials include identifying where NPs interact in the body and establishing their effect mechanisms. Here, we applied whole mount *in-situ* hybridisation (*WISH*) in zebrafish embryos and larvae for a suite of genes involved with detoxifying processes and oxidative stress including, metallothionein (*mt2*), glutathione S-transferase pi (*gstp*), glutathione S-transferase mu (*gstm1*), heme oxygenase (*hmox1*) and ferritin heavy chain 1 (*fth1*) to identify potential target tissues and effect mechanisms of AgNPs compared with a bulk counterpart and ionic silver (AgNO₃). AgNPs caused upregulation in the expression of *mt2*, *gstp* and *gstm1* and down regulation of expression of both *hmox1* and *fth1* and there were both life stage and tissue specific responses. Responding tissues included olfactory bulbs, lateral line neuromasts, ionocytes in the skin, yolk sac and regions of the head. Target sites of AgNPs thus included sensory systems, with the potential for effects on olfaction, behaviour, and maintaining ion balance. Silver ions affected the same target tissues and induced the same gene responses as AgNPs, albeit there were differences in the levels of gene responses. Silver particles without coating (where a more rapid rate of silver ion dissolution occurs) invoked levels of gene responses more similar to silver treatments compared with coated silver NPs. These findings indicate the gene responses seen for the silver materials were due to silver ions. Expression of *mt2* (24 hpf) and *gstp* (3 dpf) were either non-detectable or were at lower levels, in an *Nrf2* zebrafish mutant compared with wild type zebrafish for exposures to AgNPs indicating these gene responses are controlled through the Nrf2-Keap pathway.

Keywords: silver nanoparticles, *Danio rerio*, oxidative stress, target tissues, Nrf2 pathway

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Introduction

Given the rapid expansion in global markets in nanotechnology, an increasing number of NPs will enter aquatic systems. There is concern internationally that some NPs, may induce adverse health effects in exposed organisms, but to date the evidence for this in wildlife is minimal. More than 30% of nano products in the marketplace contain silver nanoparticles (AgNPs) (Wijnhoven et al., 2009) and current global use is approximately 500 tonnes per year (Fabrega et al., 2011a). AgNPs are of particular concern to wildlife as most will be discharged via wastewater treatment works into surface waters where dissolution will release silver ions and these are toxic to aquatic species (Hogstrand and Wood, 2009).

Studies on the acute toxicity for AgNPs in zebrafish embryos and in subsequent early life stages have established adverse effect concentrations ranging between 50 µg/l and 500 µg/l, and have suggested greater effects compared with bulk counterparts (Osborne et al., 2013, Asharani et al., 2008b). Developmental effects of AgNP exposure in zebrafish include stunted growth, a reduced yolk sac and distortions in the tail, albeit for high exposure concentrations (between 5 000 µg/l and 25 000 µg/l). These effects on embryogenesis have also been reported in other fish, such as the medaka (Kashiwada et al., 2012). Reported effects of AgNPs are believed to result from silver ions dissociating from AgNPs, but there is limited evidence also for direct effects of the particles themselves (Beer et al., 2012, van Aerle et al., 2013).

Heavy metals, including silver, induce oxidative stress (Ercal et al., 2001) in a wide range of organisms spanning algae (Pinto et al., 2003) to fish (Sanchez et al., 2005) and usually they do so via generation of Reactive Oxygen Species (ROS) resulting in lipid peroxidation. AgNPs have been shown to induce oxidative stress *in vitro* through an increase in the production of ROS (Foldbjerg et al., 2009). In their detoxification in the body, metals usually bind to thiol-containing compounds such as metallothionein (MT) and glutathione (GSH).

Standard approaches for testing the effects of NPs do not inform on material partitioning within the body or the target organs affected in an integrated manner. Whole mount *in-situ* hybridisation (*WISH*) as applied to zebrafish embryos and early life stages potentially offers a highly integrative, systems-wide approach to assess the toxicity of NPs through effects on

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gene expression. The method offers the ability to target where in the body nanomaterials induce biological responses to help inform on their potential health effects (Nakajima et al., 2011b). Application of the technique of *in situ* hybridisation to assess for effects of toxicants on target genes, however, requires understanding on the ontogeny of the expression of the target genes, and this is known for very few genes of toxicological relevance.

In this study, we applied *WISH* in zebrafish embryos and early life stages exposed to AgNPs, a bulk counterpart and silver ions, for a suite of genes known to respond to metals (toxicity, transport and storage), oxidative stress and other markers of cellular stress. The genes selected were metallothionein (*mt2*), glutathione *S*-transferase pi (*gstp*), glutathione *S*-transferase mu (*gstm1*), heme oxygenase (*hmox1*) and ferritin heavy chain 1 (*fth1*). *Mt2* is involved with transport and storage of heavy metals (Andrews, 2000) and has been shown previously to be responsive to AgNPs in zebrafish embryos (Osborne et al., 2013). Glutathione *S*-transferases are a major group of detoxification enzymes that catalyze the nucleophilic addition of the tripeptide GSH to many xenobiotics and endogenous electrophiles. *Gstm1* functions in the detoxification of electrophilic compounds and *gstp* plays roles in xenobiotic metabolism and oxidative stress (Garner and Di Giulio, 2012). *Gstp* is also known to be responsive to silver (Cheng et al., 2006). The *gstm1* gene encodes for the carcinogen detoxification enzyme glutathione *S*-transferase M1. Heme oxygenase (encoded by *hmox1*) is an enzyme that catalyzes the degradation of heme which in turn produces iron and protects against oxidative stress (Ponka et al., 1998). We initially studied the ontogeny and tissue expression profiles for each of these target genes in unexposed animals up to 12 days post fertilization (dpf) to determine their expression dynamics and identify the most appropriate life stages for studies on the effects of silver materials.

Nrf2 plays a role in the protection of cells against oxidative stress (Kobayashi and Yamamoto, 2005) and regulates our marker genes such as *gstp*. Others include the heavy metal response gene *mt2*. Under normal conditions Nrf2 is nestled in the cytoplasm by cytoskeletal protein Keap1. When ROS occurs this causes the dissociation of Keap1 as a consequence Nrf2 is translocated to the nucleus which leads to an activation of antioxidants i.e. stress response genes. To investigate the potential involvement of the Nrf2 pathway in

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the stress response against AgNP we have used mutant zebrafish which has defect in the Nrf2 gene (Mukaigasa et al., 2012).

The Nrf2 mutant line was already well established in the same laboratory that had supplied us with some of the plasmids for the probes for stress response genes; therefore, it was a good opportunity to take advantage of this to further explore some mechanisms of AgNP toxicity.

Materials and Methods

Nano particle source and characterization

Citrate-covered Ag 10-nm nanoparticles (measured diameter of 9.9 ± 3.1 nm; AgNPCi) and larger sized citrate-covered Ag particles (measured diameter of 160 nm AgBCi) were acquired from the University of Birmingham. Uncoated Ag 35 nm nanoparticle (measured diameter 114 ± 65 nm; AgNP) and uncoated Ag bulk (measured diameter of 137 ± 62.0 nm; AgB) were acquired from Nanostructured and Amorphous Materials Inc. Houston, USA. Detailed information on the characteristics of the particles derived from Nanostructured and Amorphous Materials are provided in Scown et al., (2010c) and Osborne et al., (2013). A series of techniques were applied to quantify and characterize AgNPCi and AgBCi particles including, Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS) and Zeta Potential. For TEM, 0.1 mg/L suspensions of AgNPCi (0.1 mg/L and AgBCi were prepared in milli Q-water and embryo media (0.60 mg marine salts [Tropic marin] per 1 litre of deionised water with 10 μ l of methylene blue). 10 mL of each suspension were deposited on Formvar/Carbon coated TEM-grids using ultracentrifugation (Beckman L-75) at 30 000 rpm for 60 min. Thereafter, the grids were rinsed by gently immersion in milli Q-water, and then dried overnight. Micrographs were acquired at 30 k, 75 k, 300 k and 500 k magnification (5-10 micrographs at each magnification from each sample) at 80 keV accelerating voltage using a JEOL 1200EX TEM-instrument. Methods for deriving information on size (DLS), charge (Zeta Potential) are given in the supplementary material, S1.

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Dissolution of silver

(Currently awaiting data for dissolution of AgNPCi in embryo culture medium) However; a study using the same AgNPCi in seawater was carried out which gives approximate dissolution estimations. For materials and methods see supplementary materials S3.

Fish source, culture and husbandry

Wild type Indian Calcutta (WIK) strain embryos were obtained from the Max Planck Institute, Tubingen, Germany and maintained at the University of Exeter (see supplementary material S2 for details on fish maintenance). Nrf2 mutant zebrafish were supplied from the University of Tsukuba, Japan (Mukaigasa et al., 2012).

Embryo handling and Ag exposures

Embryos for gene expression ontogeny analysis and silver material exposures were collected from breeding colonies and transferred into a Petri dish and washed twice with embryo culture water. For all gene expression analyses and exposure studies, 15 to 20 embryos were studied for each gene/exposure (collected at the 1-2 cell stage, 1-1.5 hours post fertilisation (hpf)) per treatment well (5 ml) and each exposure study was replicated at least 3 times. The materials underwent sonication prior to use in the exposures using a Cole and Palmer Ultrasonicator Processor a full amplification and pulsing for two 10-second bursts. The required amount of silver material was then introduced into the embryo culture medium containing the embryos. The embryos for both gene expression ontogeny analyses and ecotoxicology studies were incubated at 28 +/-1°C and the embryos were then fixed with 4% PFA at the appropriate collection time. The expression ontogeny analyses were conducted in embryos from fertilisation up to 12 dpf.

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Ag exposures

Based on the ontogeny of expression of the individual target genes in control animals (see results section) *in situ* expression analysis for the silver materials was adopted for the different target genes at specific life stages (see results section). The silver dosing regimen adopted for assessing target gene responses using *WISH* analysis was between 500 µg/l and 1 000 µg/l based on previous findings for sublethal effect concentrations (Osborne et al., 2013). Citrate-coated nanomaterials were included as most NPs in industry are coated with a surfactant to reduce aggregation (Christian et al., 2008). Silver ions (AgNO₃) (Perkin Elmer) were used as an ionic control at 20 µg/l, calculated to provide an approximate dissolution equivalence (1-2%) for the dosing of AgNPCi, based on previous dissolution experiments with these materials, exposure medium and exposure periods (Osborne et al., 2013). *WISH* responses of the *gstp* gene were also analysed with non-coated AgNP and AgB particles for a dosing series of 0 µg/l, 5 µg/l, 50 µg/l, 500 µg/l, 5 000 µg/l and 25 000 µg/l and for *mt2* with AgNP encompassing exposure concentrations spanning 0 µg/l to 32 µg/l for sensitivity analysis. To investigate the role of the *nrf2* transcription factor in the toxicology responses to the silver materials tested, *Nrf2* mutant zebrafish embryos were exposed to AgNPCi (500 µg/l) and AgNO₃ (20 µg/l) at the 1-2 cell stage for a period of 24 h for *mt2* and 3 days for *gstp*. As for the studies on wild type zebrafish, all exposures for *Nrf2* mutants were repeated 3 times.

Synthesis of gene probes: mt2, gstm1, gstp, hmox1 and fth1

The *mt2 in situ* probe was prepared as described previously (Osborne et al., 2013). For *gstm1*, a clone (IRBOP991A0110D) was obtained from Bioscience Life Sciences. To prepare the RNA probe for this gene, *gstm1* cDNA was amplified by PCR using two primers, Sugano_F1: CTG CTC CTC AGT GGTGT TGC CTT TAC and T3_Sugano_R1: GGA TCC ATT AAC CCT CAC TAA AGG CAG GTT CAG GGG GAG GTG TGG. *Gstp*, *hmox1* and *fth1* were acquired from University of Tsukuba (Mukaigasa et al., 2012). To make antisense probes for *WISH*, plasmids encoding *gstp*, *hmox1* and *fth1* were digested and transcribed with the following sets of restriction enzymes and RNA polymerases: *gstp* with BamH1/T7, *fth1* with BamH1/T3, *hmox1* with Xho1/ T3. Using a G50 column (GE Healthcare) the RNA was purified

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and precipitated using Lithium Chloride. The probes were then diluted 1/200 with hybridisation buffer (Osborne et al., 2013).

Whole mount in-situ hybridization

For the hybridizations, embryos were fixed using 4% PFA in PBS at 4°C overnight. They were then dechorionated and placed in methanol for 2 hours. Embryos/larvae were incubated in proteinase K (5 µg/ml) for either 30 minutes (for embryos between 24 hpf to 5 dpf) or 40 minutes (for larvae between 6 dpf and 12 dpf). They were then washed two times with PBS+0.1% Tween 20 (PBSTw), placed in hybridisation buffer for 1 hour and incubated with the desired probe overnight at 65°C. Embryos were then washed in 50% formamide 2XSSC, 0.1%Tween 20 wash for 30 minutes at 65°C, followed by a 2X SSC 0.1% Tween 20 wash at 65°C and received 2x 30 minutes washes at 65°C with 0.2X SSC 0.1%Tween 20. Blocking solution (2% Blocking reagent (Roche) containing 2.5 ml of calf serum in MAB (Osborne et al., 2013) was added and the embryos were incubated for 1 hour. Anti-DIG antibody (5000x diluted with Blocking solution) (Roche) was then added and the embryos incubated for 2 hours. Embryos subsequently received a series of 4 x 30 minutes washes in PBTw before a 10-minute wash with AP buffer (Tris 0.1 M pH 9.5, NaCl 0.1 M, MgCl₂ 50 mM, Tween 20 0.1%). Embryos were transferred to a 24 well plate and placed in staining solution (BM-Purple AP Substrate, Roche) to reveal the probe, and photographed using a Nikon SMZ1500 stereo microscope equipped with a digital camera. For the studies on gene ontogeny, the levels of gene expression were assessed on a qualitative basis. For the silver materials exposure work the levels of expression of the different genes studied were quantified using Image-J 1.44 P. Here, the expression intensity for a target gene was determined for a specific tissue area for 15-20 embryos/larvae, subtracting any background. The intensity value obtained was then converted to a fold change (FC) compared with controls to give an approximate quantification of effect for the different silver material treatments for that gene and tissue. Given the variation in the background expression of the target genes, only measured expressions differing from controls by more than 20% (i.e. fold changes above 1.2 or below 0.8) were considered as significant.

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Antibody staining of skin cells: vHATPase and NaKATPase

Double immunohistochemistry was used to characterise skin ionophores, specifically vHATPase and NaKATPase. Ionophores are involved with ion transport and the exchange of other molecules in the zebrafish larvae (Esaki et al., 2007). Larvae were fixed with 4% PFA in PBS at 4°C overnight and then dehydrated in 100% methanol. After rehydration in PBT (PBS+0.2% Triton X-100) and incubation in PBT containing 10% sheep serum (Sigma) for 1 h, the larvae were incubated overnight at 4°C with rat anti-dace vHATPase and rabbit anti-eel NaKATPase (diluted 1:1000 with PBT containing 10% sheep serum). Following washing with PBT, the larvae were further incubated at room temperature for 2 hours with anti-rabbit IgG, Alexa Fluor 543-conjugated anti-rabbit IgG, Alexa Fluor 488-conjugated anti-rat IgG (diluted 1:1000, Invitrogen) and then washed with PBT, embedded in 2% methylcellulose and photographed under Zeiss Microscope Observer Z.1.

Results

Particle Characterization

A summary of the physiochemical properties of the AgNP, AgB, AgNPCi and AgBCi particles is provided in the supplementary information (supplementary information Figure S1 and Tables S1 and S2). DLS data illustrated that AgNPCi had a mean diameter of 20 nm and AgBCi a mean diameter of 160 nm (supplementary information, Table S1). In contrast, AgNPCi in the embryo culture medium had a mean diameter of 92.25 ± 1.8 nm and AgBCi had a mean diameter of 2456.2 ± 1287.5 nm. When dispersed in the embryo medium the measured sizes as determined via TEM were 52^{93}_{22} nm for AgNPCi and 145^{509}_{114} nm for AgBCi. Zeta potential for AgNPCi was -47.78 ± 4.25 and for AgBCi was -35.97 ± 6.2 mV. In the embryo culture medium, the zeta potential for AgNPCi was -18.38 ± 1.6 and -21.54 ± 3.9 mV for AgBCi. TEM micrographs illustrated that individual nanomaterials could be distinguished in liquid form, but there was evidence of aggregation when they were placed in the embryo exposure medium. Micrographs of bulk particles showed they occurred as large aggregates in the embryo culture medium. It is possible that some of the aggregation seen in the TEM

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images for both the nano and bulk materials occurred as a consequence of the collection and fixing for the TEM processing (Bozzola and Russell, 1999).

Dissolution of silver

Please refer to supplementary materials S4 and Graph S1 for full details.

Expression of target genes in zebrafish during early life (0-12 dpf):

mt2 (metallothionein 2) was expressed at 3-4 hpf in cells of the blastoderm, and then at a relatively low level at 24 hpf in the extended yolk sac region of the embryo. No expression was seen after 2 dpf up to 12 dpf (Supplementary Figure 2, Ai-Aii; see also Osborne et al. (2013)).

gstm1 (glutathione S-transferase mu) There was low-level expression at 3-4 hpf in cells of the blastoderm which was more intense at 8-10 hpf. Low detectable expression of this gene occurred at 24 hpf in the extended yolk sac region. No detectable expression occurred during the subsequent development up to 12 dpf (Supplementary Figure 2 Bi-Bii).

gstp (glutathione S-transferase pi) Expression was detected throughout the ontogeny period studied and occurred in olfactory bulbs, ventricles in the brain, neuromasts, jaw fins and gut. The patterns of expression and intensity of expression differed over the life stages studied. At 24 hpf *gstp* expression occurred in the extended yolk sac region and at 48 hpf in the olfactory bulbs and brain ventricles. At 3 dpf expression occurred in neuromasts expressed along the lateral line and at 96 hpf also in the jaw. At 3 dpf some weak expression also occurred in the region of the olfactory bulb. *Gstp* expression occurred in the pectoral fins at 120 hpf, and at 144 hpf was also associated with the region of the gut. At 168 hpf, 192 hpf and 240 hpf there was relatively weak *gstp* expression and this occurred predominantly in neuromasts (Supplementary Figure 2, Ci-Cviii).

hmox 1 (heme oxygenase 1) There was little detectable expression of *hmox1* in embryos until 24 hpf, when a relatively high level of expression was observed in the retina and at the tip of extended yolk sac. At 48 hpf expression also occurred in the retina, in the yolk sac and in some neuromasts. At 72 hpf onwards some (18%) of the embryos studied showed a low

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level, or no observable expression of *hmox1*, until 7 dpf when there was a relatively high level expression in the liver (Sadler et al., 2007) which was continued subsequently to 12 dpf (Supplementary Figure 2, Di-Dvii).

fth1 (ferritin heavy chain 1) Expression of *fth1* occurred 3-4 hpf in the entire blastoderm. At 7-9 hpf low level expression was detected in the germ ring and at 24 hpf there was a pronounced expression in the forebrain, which persisted in the brain at 48 hpf with additional expression in the extended yolk sac. No expression of *fth1* was detected at 72 hpf or at 96 hpf, but from day 6 to 11 variable and relatively low level expression occurred in the yolk sac and/or gills. *Fth1* expression occurred in the liver from 144 hpf to 216 hpf, was absent in this tissue at 240 hpf, but detected again at 264 hpf (See supplementary Figure 2 Ei-Eviii).

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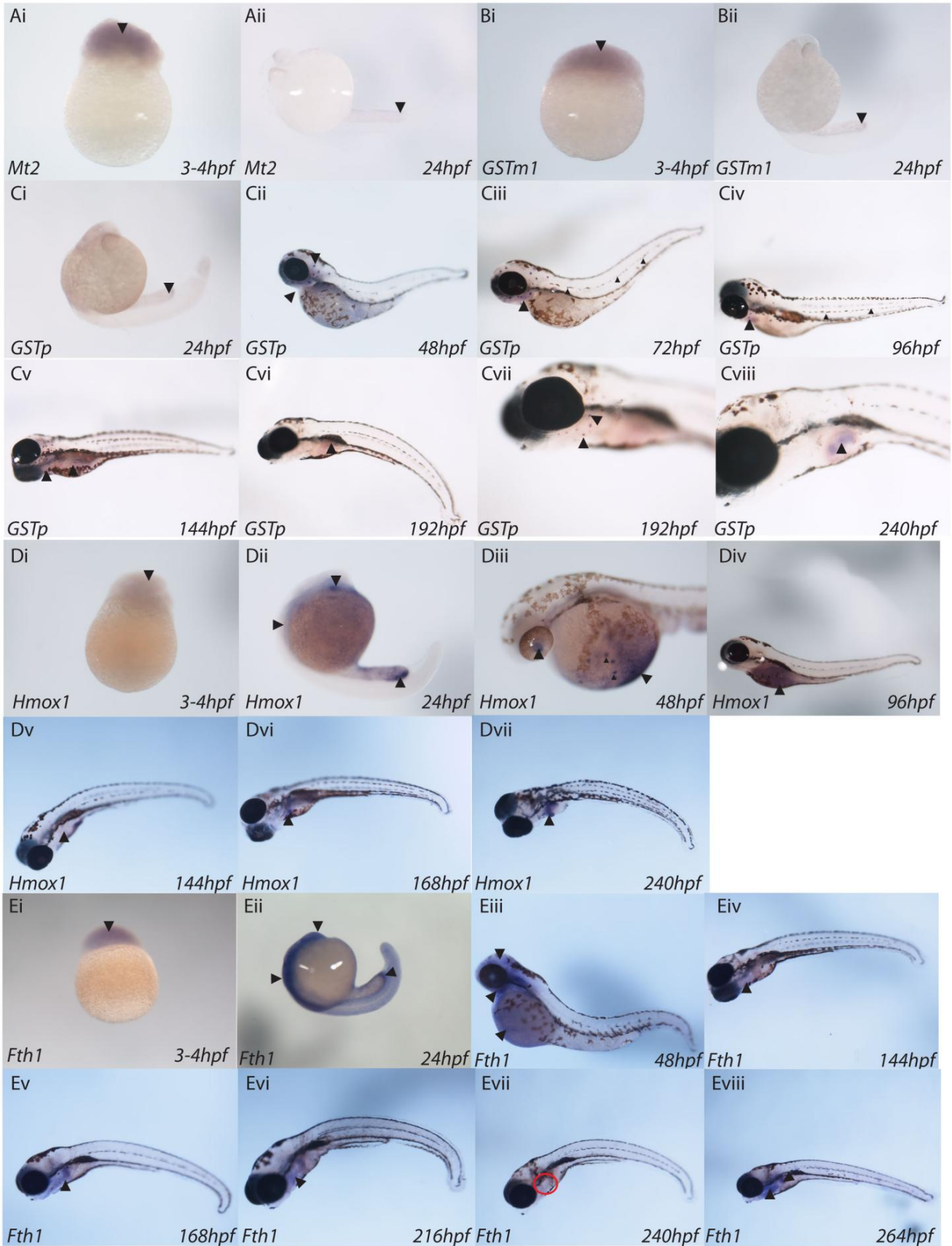


Figure S2

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Figure S2: Representative *in situ* hybridisation images showing ontogeny of expression of *mt2*, *gstp*, *gstm1*, *fth1* and *hmox1* for up to 12 dpf in zebrafish: *mt2* in 3-4hpf (**Ai**) and 24hpf (**Aii**) embryos; *gstm1* in 3-4hpf (**Bi**) and 24hpf (**Bii**) embryos; *gstp* in a 24hpf (**Ci**), 48hp, (**Cii**), 72hpf (**Ciii**) 96hpf (**Civ**) 144hpf (**Cv**), 192hpf (**Cvi**, and **CVii** with head close up, showing neuromasts , and 240hpf) (**Cviii**, showing extended yolk sac) embryos/larvae; *hmox1* in 3-4hpf (**Di**), 24hpf (**Dii**), 48hpf (**Diii**) 96hpf (**Div**), 144hpf (**Dv**) 168hpf (**Dvi**), and 240hpf (**Dvii**) embryos/larvae; *fth1* in 3-4hpf (**Ei**), 24hpf (**Eii**), 48hpf (**Eiii**), 144hpf (**Eiv**), 168hpf (**Ev**), 216hpf (**Evi**), 240hpf (**Evii**) and 264hpf (**Eviii**) embryos/larvae. ▲ indicates focal areas of expression for the target genes studied.

Developmental life stages adopted for assessing exposure responses to silver materials

Based on the ontogeny of expression of the different genes, appropriate life stages were chosen for exposure studies on silver materials. These stages allowed for analysis assessing possible stimulation and/or suppression of target gene expression. *Mt2*: 24 hpf and 4 dpf, *gstp*: 24 hpf, 48 hpf and 4 dpf, *hmox1* and *fth1*: 24 hpf and 5 dpf, *gstm1*: 24 hpf, 4 dpf and 5 dpf.

Quantifying Ag effects

An overview of threshold responses for the different target genes and responsive genes showing the fold change (FC) difference to that compared with controls is shown in Table I.

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A 24hpf

Gene	Region	AgNPCi	AgBCi	AgNO ₃
<i>mt2</i>	YS	↑ (4.6 FC)	↑ (4.7 FC)	↑ (2.7 FC)
<i>gstp</i>	YS	↑ (1.7 FC)	↑ (1.5 FC)	↑ (1.6 FC)
<i>gstm1</i>	YS	↑ (1.8 FC)	↑ (1.6 FC)	↑ (1.7 FC)
	Head	↑ (1.5 FC)	↑ (1.2 FC)	↑ (4.8 FC)
<i>hmox1</i>	YS	→ (1.0 FC)	→ (1.0 FC)	→ (1.1 FC)
	Lens	→ (1.1 FC)	→ (1.0 FC)	→ (0.9 FC)
<i>fth1</i>	Head	→ (1.0 FC)	→ (1.1 FC)	→ (0.9 FC)

B 48hpf

Gene	Region	AgNP	AgB	AgNO ₃
<i>gstp</i>	Head	↑ (2.1 FC)	↑ (1.5 FC)	↑ (1.5 FC)
	Pectoral fin	↑ (1.4 FC)	↑ (1.3 FC)	↑ (1.6 FC)

C 4 dpf

Gene	Region	AgNPCi	AgBCi	AgNO ₃
<i>mt2</i>	YS	↑ (2.3 FC)	-	-
	Head	↑ (2.5 FC)	-	-
<i>gstp</i>	YS	↑ (2.6 FC)	-	-
	Head	↑ (2.7 FC)	-	-
	Pectoral fin	↑ (2.7 FC)	-	-
<i>gstm1</i>	YS	↑ (1.2 FC)	↑ (1.7 FC)	↑ (1.6 FC)
	Head	↑ (2.0 FC)	↑ (2.1 FC)	↑ (1.9 FC)

D 5 dpf

Gene	Region	AgNPCi
<i>gstm1</i>	YS	↑ (2.3 FC)
	Head	↑ (3.1 FC)
<i>hmox1</i>	YS	↓ (0.7 FC)
	Head	↓ (0.5 FC)
<i>fth1</i>	YS	↓ (0.4 FC)
	Head	↓ (0.5 FC)

Key

↑ = Upregulated
 ↓ = Downregulated
 → = No change

FC = Fold change
 YS = Yolk Sac

Table I. Quantification of target gene responses derived from *in situ* hybridisations showing fold change in expression compared with controls. **A.** *mt2*, *gstp*, *gstm1*, *hmox1* and *fth1* at 24 hpf-dosings: 500 µg AgNPCi/l, 500 µg AgBCi/l, 20 µg AgNO₃/l; **B.** *gstp* at 48 hpf-dosings: 500 µg AgNPCi/l, 500 µg AgBCi/l, 20 µg AgNO₃/l; **C.** *mt2*, *gstp* and *gstm1* at 4 dpf-dosings: 1 000 µg AgNPCi/l, 1 000 µg AgBCi/l, 20 µg AgNO₃/l; **D.** *gstm1*, *hmox1* and *fth1* at 5 dpf dosings: 1 000 µg AgNPCi/l.

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Effects of silver materials on gene expression

mt2

At 24 hpf, in non-exposed embryos 57% showed a detectable expression of *mt2* in the extended yolk sac region and this was consistently (80% + embryos) elevated for exposures to 500 μg AgNPCi/l (4.6-fold), 500 μg AgBCi/l (4.7-fold) and 20 μg AgNO₃/l (2.7-fold) (Figure 1 Ai-Aiv) (Table I). *Mt2* showed an enhanced level of expression in the extended yolk sac region for exposures to AgNPs as low as 4 μg /l (the lowest concentration tested; Figure 2 Bii). At 4 dpf (Figure 2 Ci-Cii) a 24-hour exposure to AgNPCi resulted minimally in a 2-fold higher level of *mt2* expression (Table I) across various regions of the larval body including head, jaw and yolk sac (and consistently so, in 92% of the embryos).

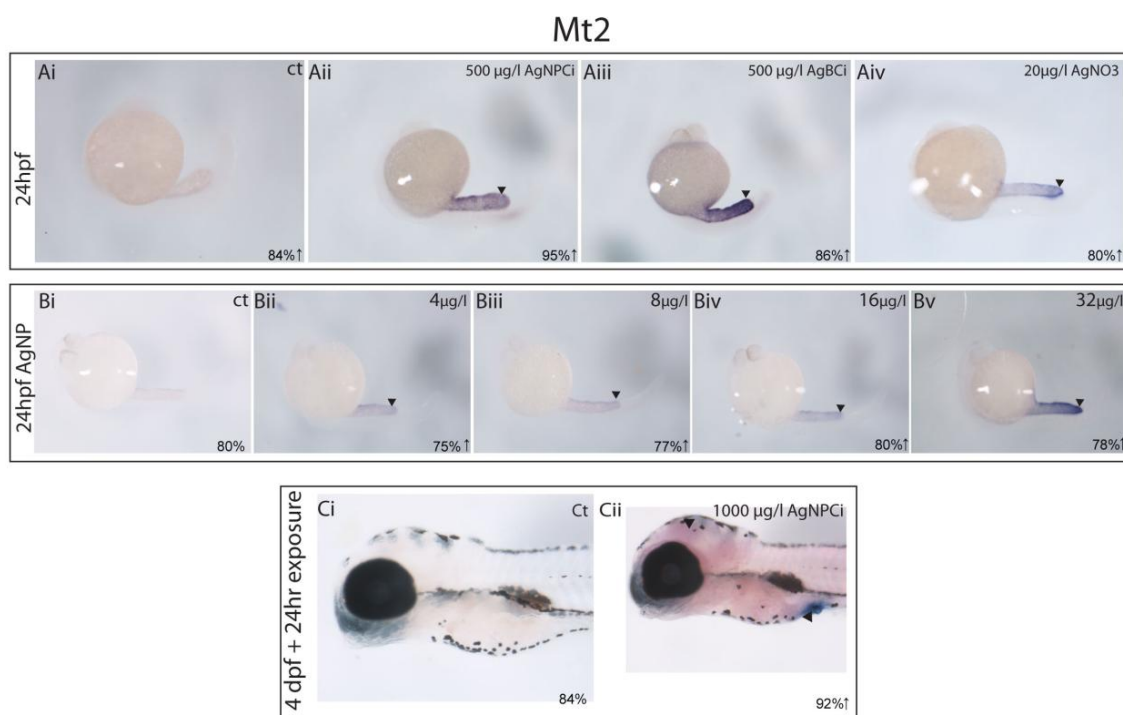


Figure 1. Expression of *mt2* in zebrafish embryos/larvae at 24 hpf-after exposure to AgNPCi, AgBCi and AgNO₃ as determined by whole mount *in situ* hybridisation (n=60 for each treatment group). ▲Indicates target tissue/focal areas where *mt2* expression was affected by the treatments. **Ai.** Control embryo, **Aii.** 500 μg AgNPCi/l, **Aiii.** 500 μg AgBCi/l, **Aiv.** 20 μg AgNO₃/l (all 24 hours post fertilisation). **Bi.** Control embryo, **Bii-Bv** AgNP, at (**Bii**) 4 μg /l, (**Biii**) 8 μg /l, (**Biv**) 16 μg /l and (**Bv**) 32 μg /l (all 24 hours post fertilisation). **Ci-ii** AgNPCi, **Ci.** Control

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larvae, Cii 1 000 µg/l (all exposed at 4 days post fertilisation for 24h). Numbers represent percentage of embryos affected in the treatment (n = 15-20). Upwards and downwards pointing arrows indicate enhanced or reduced gene expression respectively compared with controls (see also Table I).

gstp

At 24 hpf *gstp* expression was enhanced in the extended yolk sac by between 1.5 and 1.7-fold above controls (Table I) for exposures to 500 µg AgNPCi/l, 500 µg AgBCi/l and 20 µg AgNO₃/l, and in a consistent manner (more than 90% of the embryos responding in the same manner) (Figure 2 Ai-Aiv). For exposures to non-coated silver materials, at 48 hpf (Fig.2 Bi-Cv) AgNP activated *gstp* expression in regions of the head and pectoral fin in a concentration related manner (75% of embryos showing this response). Levels of *gstp* expression for embryos exposed to AgNP were 2.1-fold greater in the head, and 1.4-fold greater in the pectoral fin compared with controls. Exposure to AgB induced a 1.5-fold higher expression in the head and 1.3-fold higher expression in the pectoral fin compared with controls (Table I). For ionic silver, there were 1.5-fold and 1.6-fold higher levels of expression in the head and pectoral fin, respectively (Figure 2 Ci) (Table I). Antibody staining of ionocytes with vHATPase (shown in green) NakATPase (shown in red, Figure 2 Di-Dv) showed that focal areas of *gstp* expression on the yolk sac, extended yolk sac and some other body regions of zebrafish larvae were co-localised with vHATPase and NakATPase skin cells. In 4 dpf larvae, (Figure 2 Ei-Eii) 24-h exposure to AgNPCi (1 000 µg/l) resulted in a 2-fold higher expression of *gstp* (Table I) across various body of the larvae (and in 100% of the embryos examined; Figure 2 Ei-Eii).

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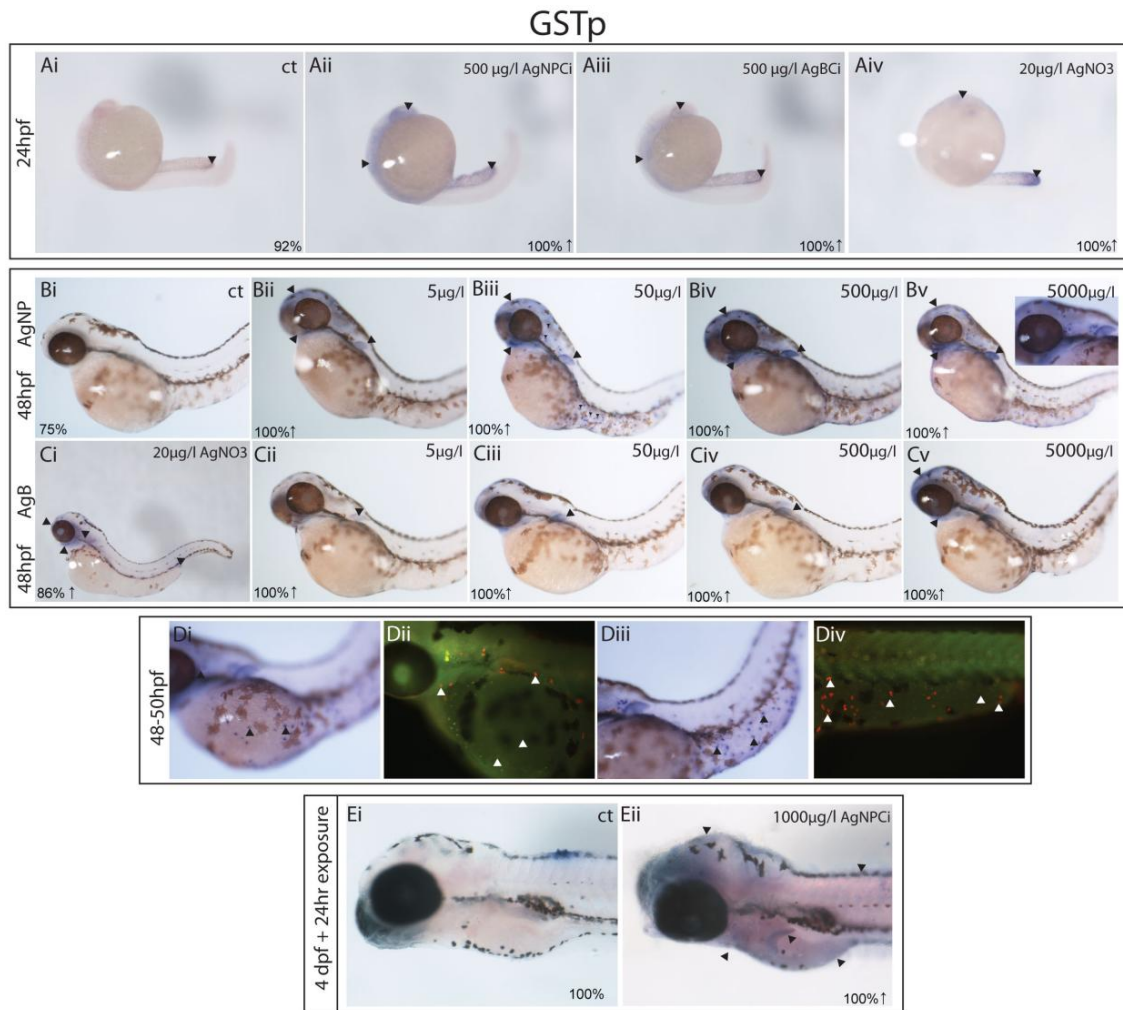


Figure 2. Expression of *gstp* in zebrafish embryos/larvae at 24 hpf-after exposure to AgNPCi, AgBCi and AgNO₃ as determined by whole mount *in situ* hybridisation (n=60 for each treatment group). ▲Indicates target tissue/focal areas where *gstp* expression was affected by the treatments. **Ai.** Control embryo, **Aii.** 500 µg AgNPCi/l, **Aiii.** 500 µg AgBCi/l, **Aiv.** 20 µg AgNO₃/l (all 24 hours post fertilisation). **Bi.** Control embryo, **Bii-Bv** AgNP, at (**Bii**) 5 µg /l, 50 µg/l (**Biii**), 500 µg/l (**Biv**) and 5 000 µg/l (**Bv**); **Cii-Cv** AgB, (**Cii**) at 5 µg /l, (**Ciii**) 50 µg/l, (**Civ**) 500 µg/l and (**Cv**) 5 000µg/l and (**Cvi**) 20 µg AgNO₃/l (all 48 hours post fertilisation). **Di-Div** Embryos exposed to AgNPs and subjected to *in situ* hybridisation for *gstp* and antibody staining with vHATPase (green) and NaKATPase (red) to detect ionocytes. **Di.** Exposure to 500 µg AgNP/l showing *in-situ* staining at 50 hpf, **Dii.** Close up of embryo at 50 hpf after application of antibody staining (green -vHATPase, and red -NaKATPase) to reveal ionocytes on yolk sac, **Diii.** 500 µg AgNP/l dosed embryo showing skin cell staining on extended yolk

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sac, **Div.** embryo after application of antibody staining (green -vHATPase, red -NaKATPase) indicating ionocytes on yolk sac. **Ei-ii** AgNPCi, **Ei.** Control larvae, **Eii.** 1 000 µg/l (all exposed at 4 days post fertilisation for 24 h). Numbers represent percentage of embryos affected in the treatment. Upwards and downwards pointing arrows indicate enhanced or reduced gene expression respectively compared with controls (see also Table I).

gstm1

At 24 hpf *gstm1* was expressed in controls (57% of the embryos) at detectable (albeit low) levels in the extended yolk sac region. Exposure to 500 µg AgNPCi/l, 500 µg AgBCi/l and 20 µg AgNO₃/l induced enhanced expression between 1.6 and 1.8-fold (Table I) higher in the extended yolk sac region. AgNPCi and AgBCi induced between 1.2 and 1.8-fold higher expression in the head region (occurring in over 60% of embryos). The same pattern occurred for exposures to AgNO₃ but with higher fold inductions (4.8-fold in the head for 40% of the embryos; Figure 3 Ai-Aiv, Table I). At 4 dpf larvae showed a consistently (70% of the embryos) enhanced expression of *gstm1* for exposures to 1 000 µg AgNPCi/l, 1 000 µg AgBCi/l and 20 µg AgNO₃/l with focal activity in regions of the head (between a 1.9 and 2.1 fold increase) and yolk sac (between 1.2-1.7-fold increase) (Table I). There was no detectable expression of *gstm1* in control embryos (Figure 3 Bi-Biv). Exposure of 5 dpf larvae to AgNPCi (1 000 µg/l) for 6 h induced expression of *gstm1* in both the head region (by a 3.1-fold) and in the yolk sac (2.3-fold, Figure 3 Ci-Cii, Table I) in all of the larvae.

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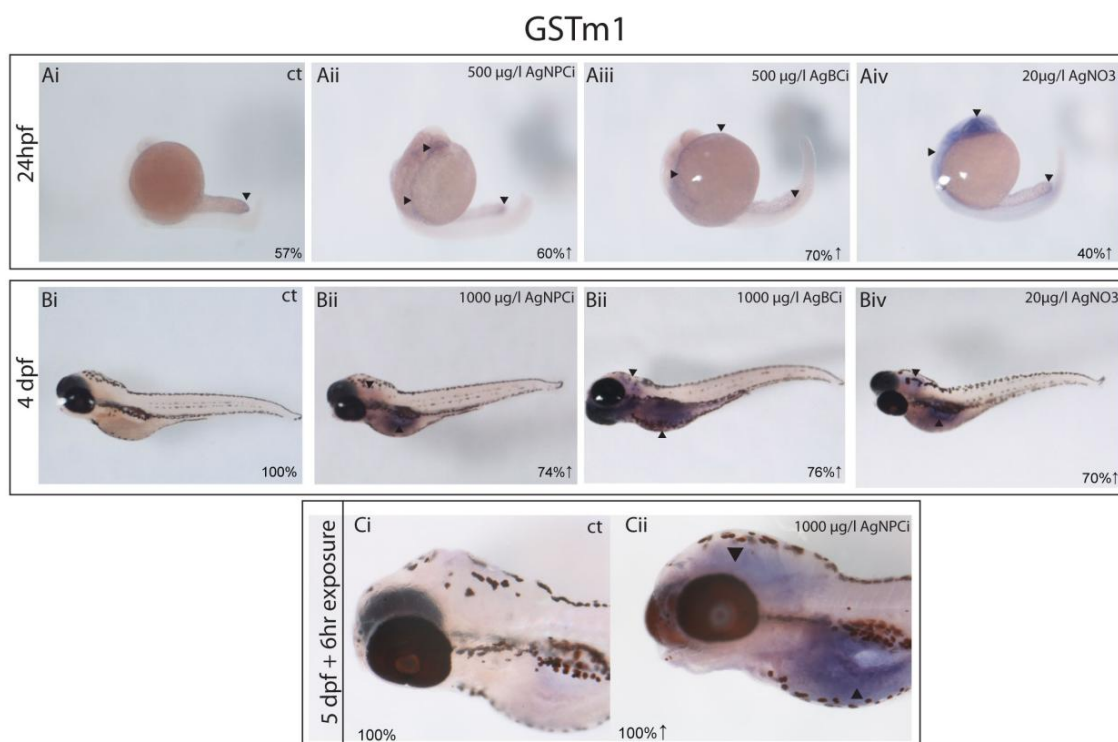


Figure 3. Expression of *gstm1* in zebrafish embryos/larvae at 24 hpf-after exposure to citrate-coated AgNPCi, AgBCi and AgNO₃ as determined by whole mount *in situ* hybridisation (n=60 for each treatment group). Indicates target tissue/focal areas where *gstm1* expression was affected by the treatments. **Ai.** Control embryo, **Aii.** 500 µg AgNPCi/l, **Aiii.** 500 µg AgBCi/l, **Aiv.** 20 µg AgNO₃/l (all 24 hours post fertilisation). **Bi.** Control embryo, **Bii.** 1 000 µg AgNPCi/l, **Biii.** 1 000 µg AgBCi/l, **Biv.** 20 µg AgNO₃/l (all 4 days post fertilisation). **Ci-ii** AgNPCi, **Ci.** Control larvae, **Cii.** 1 000 µg/l (all exposed at 5 days post fertilisation for 6 h). Numbers represent percentage of embryos affected in the treatment (n = 15-20). Upwards and downwards pointing arrows indicate enhanced or reduced gene expression respectively compared with controls (see also Table I).

hmox1

At 24 hpf there was no effect of any of the silver treatments (AgNPCi, AgBCi nor AgNO₃) on the expression of *hmox1* in the yolk sac region or the lens region of the eye (Figure 4 Ai-Aiv). At 5 dpf, *hmox1* expression was downregulated in all of the larvae in the head region (0.5 of controls and yolk sac (0.7 of controls) (Figure 4 Bi-Bii, Table I).

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fth1

At 24 hpf, *fth1* expression occurred mainly in the head and in almost all (97%) of control embryos (Figure 4 Ci-Civ). There was also some more minor expression seen on the skin. There were no obvious effects of exposure to AgNPCi, AgBCi nor AgNO₃ on the expression pattern or intensity for *fth1* compared to controls. However, at 5 dpf a 6 h exposure to AgNPCi (1 000 µg/l) resulted in a suppressive effect (in all larvae) on the expression of *fth1* in both the head and yolk sac region (expression was between 0.4 and 0.5 of controls; Figure 4 Di-Dii, Table I).

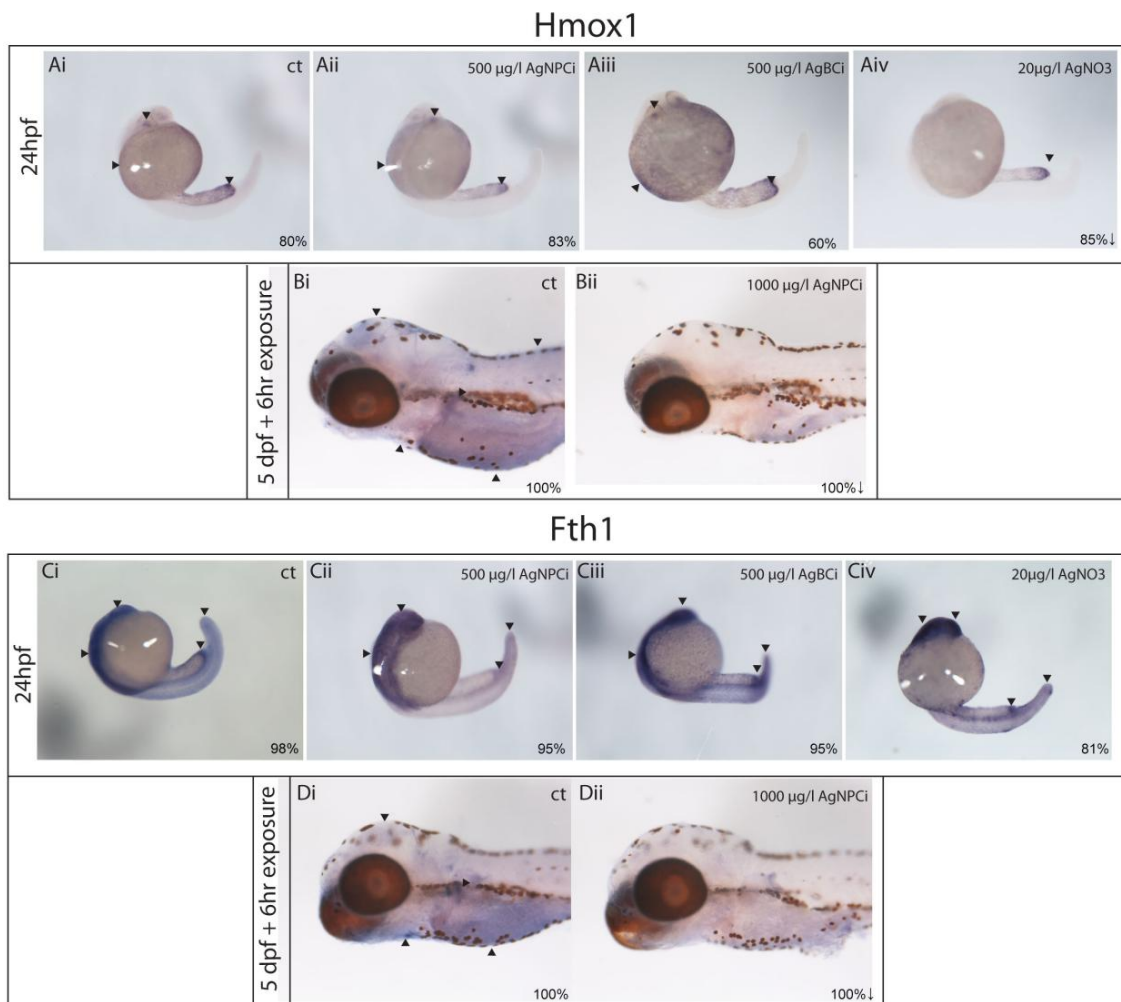


Figure 4. Expression of *hmox1* and *fth1* in zebrafish embryos/larvae at 24 hpf-after exposure to coated AgNPCi, AgBCi and AgNO₃ as determined by whole mount *in situ* hybridisation (n=60 for each treatment group). ▲Indicates target tissue/focal areas of *hmox1* and *fth1*

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expression. *Hmox1*: **Ai**. Control embryo, **Aii**. 500 µg AgNPCi/l, **Aiii**. 500 µg AgBCi/l, **Aiv**. 20 µg AgNO₃/l (all 24 hours post fertilisation). **Bi-ii** AgNPCi, **Bi**. Control larvae, **Bii**. 1 000 µg/l (all exposed at 5 days post fertilisation for 6 h). *Fth1*: **Ci**. Control embryo, **Cii**. 500 µg AgNPCi/l, **Ciii**. 500 µg AgBCi/l, **Civ**. 20 µg AgNO₃/l (all 24 hours post fertilisation). **Di-ii** AgNPCi, **Di**. Control larvae, **Dii**. 1 000 µg/l (all exposed at 5 days post fertilisation for 6 h). Numbers represent percentage of embryos affected in the treatment (n = 15-20). Upwards and downwards pointing arrows indicate enhanced or reduced gene expression respectively compared with controls (see also Table I).

Effects of AgNP on expression of mt2 and gstp in the nrf2 mutant

We examined expression of two of our study genes, *mt2* and *gstp*, in an Nrf2 mutant zebrafish to investigate their regulatory mechanism. In wild type (WIK) zebrafish at 24 hpf *mt2* was expressed in the extended yolk sac region in response to AgNPCi and AgNO₃ (Figure 5 Ai and Aiii) but in an Nrf2 mutant the same exposures induced no detectable expression in the extended yolk sac region in the majority of embryos (over 70%; Figure 5 Aii and Aiv). There was variable, but comparatively minor expression by up to 0.7 of *mt2* in the remaining Nrf2 mutant embryos compared with wild type fish. In wild type zebrafish (at 3 dpf), exposure to AgNPCi and AgNO₃ induced *gstp* expression in the olfactory region, pectoral fin, cloaca, and neuromasts of the head region (Figure 5 Bi and Biii). In contrast with this, in the Nrf2 mutant, *gstp* expression was not observed in either the olfactory region or the cloaca in the AgNPCi treatment group in any embryo (Figure 5 Bii). For exposure to 20 µg AgNO₃/l (Figure 5 Biv), there was expression of *gstp* in the neuromasts and olfactory region in the Nrf2 mutant, albeit at a lower level compared with the wild type zebrafish, and no detectable *gstp* expression in the cloaca.

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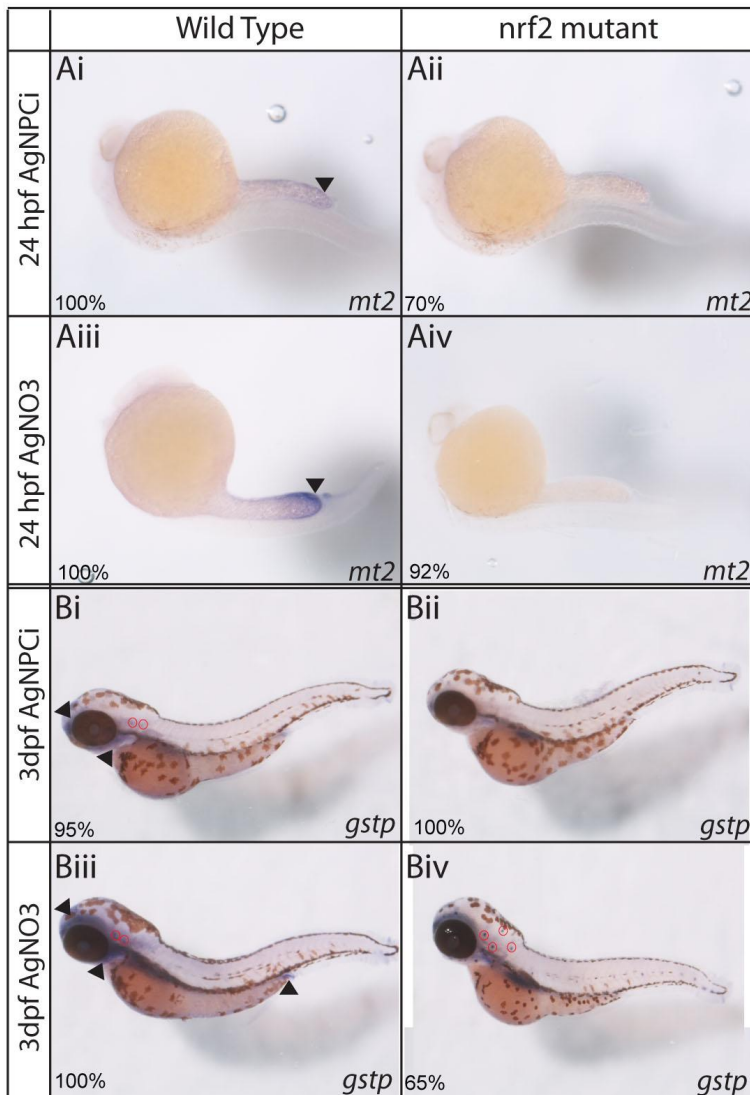


Figure 5. Expression of *mt2* and *gstp* in zebrafish embryos (WIK and Nrf2 mutant) -after exposure to AgNPCi and AgNO₃ as determined by whole mount *in situ* hybridisation (n=60 for each treatment group). ▲Indicates target tissue affected. -*Mt2*: **Ai.** AgNPCi (WIK) **Aii.** AgNPCi (Nrf2 mutant), **Aiii.** AgNO₃ (WIK), **Aiv.** AgNO₃ (Nrf2 mutant) –all at 24 hpf. *Gstp*: **Bi.** AgNPCi (WIK) **Bii.** AgNPCi (Nrf2 mutant) **Biii.** AgNO₃ (WIK) **Biv.** AgNO₃ (Nrf2 mutant)- all at 3 dpf. Numbers represent percentage of embryos affected in the treatment (n = 15-20).

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Discussion

Adopting *WISH* as a technique we identified target tissues for exposure to AgNPs, a bulk counterpart and silver ions via responses in genes associated with metal handling, detoxification and oxidative stress (Valko et al., 2006). We provide evidence that key sensory systems and ionocytes are targets for these materials in fish exposed via the water and that the toxicological responses to the silver materials tested are due principally to silver ions. Furthermore, we show that the Nrf2 pathway is involved in the toxicological response to AgNPs.

Whole organism gene expression ontogeny

Detailed expression ontogeny analysis on more than 2 700 zebrafish embryos and early life stages (15 to 20 individuals x 12 stages x 5 markers x triplicate analyses) identified the potential receptive tissues and life stages for toxicological effects of silver materials (Supplementary information, Figure S2). In unexposed embryos, we found that *mt2* was weakly expressed in the extended yolk sac (at 24 hpf), as we have shown previously (Osborne et al., 2013). Low level expression of *gstp* also occurred in the yolk sac and in head, pectoral fin, jaw, olfactory and gill and this probably serves to provide constitutive protection against oxidative damage in the developing embryo/larvae. Similarly, the expression of *gstm1* (at 24 hpf) in the yolk sac seen in control embryos may serve to help protect against electrophilic compounds during early life development. Overall, the oxidative stress genes *gstp* and *gstm1* shared similar tissue expression patterns.

Hmox1 and *fth1* that both play roles in maintaining cellular iron and in the control of the porphyrin metabolic pathway, showed similar patterns of expression in non-exposed embryos and larvae, but the patterns differed from the genes associated with oxidative stress. Expression of *hmox1* and *fth1* in the head, yolk sac and liver is consistent with the roles of iron in processes including growth and immunity. The finding of the liver as the major tissue expressing *fth1* in 6 dpf fish is consistent with this tissue containing the highest levels of ferritin (Neves et al., 2009). The variable expression of *hmox1* and *fth1* between different tissues and within tissues over time is reflective of the variable requirement for metals such as iron in development and growth.

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For all genes studied, the yolk sac was a major site of activity which may relate to its role in processing of metabolites generally (Chen et al., 2004a). Based on the patterns of expression in control embryos and larvae we selected zebrafish early life stages between 24 hpf and 5 dpf that allowed for effective assessment of silver material effects on their expression. The expression of the target genes detected at 3-4 hpf in the blastoderm probably represented maternally derived mRNA.

Genes and tissues responsive to Ag materials

There were concentration dependent responses to the silver materials for *mt2* (Figure 1) and all silver treatments induced *mt2*, principally in the region of the extended yolk sac (Figure 1 Ai-Aiv). *Mt2* was relatively sensitive with responses detected down to exposures of 4 µg/L for AgNP. These levels that are environmentally relevant Ag concentrations from predicted modelling studies (Gottschalk et al., 2009, Mueller and Nowack, 2008). In oyster embryos, Ag has been shown to induce MT down to only 0.16 µg/l, as detected via real time quantitative PCR analyses, which is a more sensitive detection method (Ringwood et al., 2010). In adult fish, responsive tissues to Ag reported in previous studies include the liver and gills (Hogstrand et al., 1996).

Gstp expression was induced by Ag materials in a variety of tissues, consistent with findings from previous studies showing oxidative stress in fish exposed to AgNPs (Choi et al., 2009, Foldbjerg et al., 2009). Induction of this gene was also seen in olfactory bulbs, an established tissue target for heavy metals and potentially affecting olfaction (Gobba, 2006).

An interesting finding was that *gstp* activation co-localised with other parts of the sensory system, including neuromasts (Figure 2 Biii). Neuromasts are receptors comprised of groups of hair cells usually found in the lateral line and head region of the zebrafish. These sensory receptors are essential for various behaviours, social interactions, prey detection and predator avoidance (Froehlicher et al., 2009). It is known that heavy metals can alter vision, taste and olfaction, orientation and auditory functions (Kasumyan and DÖving, 2003). Our data indicate that exposure to silver particles could impact on the neuromasts and other associated sensory functions in the zebrafish.

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We also observed enhanced *gstp* expression co-localised with ionocytes (skin cells) specifically ion transferring NaKATPase channels (Lin et al., 2008) in zebrafish larvae (Figure 2 Di-Dv). The distribution of ionocytes in zebrafish larvae detected using antibody staining concurred with that reported previously (Esaki et al., 2009), with wide distribution over the skin and yolk sac (Hiroi et al., 1998). In adult fish, ionocytes are principally located in the gill area and are involved in the molecular transfer and exchange of ions (Dymowska et al., 2012). In trout gills, exposure to silver can inhibit basolateral membrane Na⁺/K⁺ ATPase activity in ionophores (Wood et al., 1999). Responses in *gstp* expression in zebrafish larvae indicate that silver might affect ionic regulation in the skin. In gill ionophores, Ag ion affects on the Na⁺/K⁺ pumps reduces active N⁺ and Cl⁻ uptake, which can consequently lead to an imbalance of Na⁺/Cl⁻ ions in the blood plasma and in extreme cases, even death (Hogstrand and Wood, 2009).

Gstp induction also occurred in response to Ag in regions of the head, yolk sac, skin and the pectoral fin (48 hpf). Recently, *gstp* upregulation was shown to occur during the regeneration of caudal fin, indicating a role in repair in response to tissue damage (Timme-Laragy et al., 2012). *Gstm1* is upregulated in the presence of large number of xenobiotics (Higgins and Hayes, 2011) indicating a general role in the prevention of xenobiotic induced oxidative stress. Our studies on the zebrafish embryo support a wider body of literature showing AgNPs cause oxidative stress in various cell systems (Foldbjerg et al., 2009). In human liver cells, it has been reported that PVP coated AgNPs can generate ROS in only 30 minutes and this effect is maintained for 12 hours (Piao et al., 2011). In this study, we also showed a clear inductive response for *gstm1* after a 6-hour exposure to AgNPCi.

We found a reduced expression of both *hmox1* (Figure 4 Bi-Bii) and *fth1* (Figure 4 Di-Dii) in the head region and yolk sac (Table I) after a 6-hour exposure to AgNPCi s in 5 dpf zebrafish. Oxidative stress (occurring through ROS) and inflammatory responses have been shown also to have suppressive effects on *hmox1* in mice, for example in chronic inflammatory illness (Poss and Tonegawa, 1997). Intracellular excess iron causes oxidative stress by generating Fe²⁺ and in turn a hydroxyl radical in the Fenton reaction (Harrison and Arosio, 1996). It is still uncertain whether ferritin causes more oxidative stress by releasing Fe²⁺ as part of its

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cytoprotective role of oxidative stress (Arosio et al., 2009). It has been suggested that this is why *fth1* down-regulation or over expression can be seen during oxidative stress (Orino and Watanabe, 2008). In our study we observed suppressed effect of *fth1* (Figure 4 Di-Dii).

The toxicity of AgNP is via silver ion

Increasing evidence suggests that the toxicity of AgNPs is a direct effect of dissociating silver ions (Kittler et al., 2010, Osborne et al., 2013) and our data for the expression of the various genes studied would support this.

In the first instance at 24 hpf, *gstp* and *mt2* induction in the yolk sac region was common across all silver treatments (Figure 2 Ai-Aiv) for equivalent estimated availability of silver ions. Secondly, AgNP induced stronger *gstp* responses compared with the bulk counterpart for the same affected tissues (Figure 2 Bi-Cv) and this too is consistent with the response being due to silver ions, as dissolution tends to be faster for NPs compared with their bulk counterparts (Choi et al., 2008). Furthermore, we found coating of particles in citrate reduced toxicity, and coating of particles reduces the level (rate) of dissolution and thus bioavailability of silver ions (Marambio-Jones and Hoek, 2010). Also, there were no differences between the tissues affected for *gstm1* induction for the nano and bulk silver material exposures (4 dpf) i.e. the response patterns were the same.

Nrf2 plays a key role in the toxicological response to Ag

We saw expression of *nrf2* in the head, nose, yolk sac and gills of fish. Another recent study on *nrf2* reported expression in the nose, liver and gill of fish (Nakajima et al., 2011a). In mammals, it is established that Nrf2 plays a major role in mediating the oxidative stress response in cells (Theodore et al., 2008) and activation of this transcriptional response is triggered in the presence of reactive oxygen species (ROS) (Motohashi and Yamamoto, 2004) (Knörr-Wittmann et al., 2005). Studies with mice have indicated that the metallothionein gene contains an antioxidant response element (ARE) (Ohtsuji et al., 2008) to which Nrf2 binds. Here, we established that *mt2* and *gstp* were both induced by Ag in wild type (WIK strain) zebrafish, but in an Nrf2 mutant zebrafish, their expression was diminished considerably at 24 hpf (*mt2*) and 3 dpf (*gstp*) supporting Nrf2 mediating the role

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of Nrf2 in the oxidative stress cascade for *mt2* and *gstp* in fish. Our findings concur with studies in *nrf1* mutant mice, where expression of both metallothionein (Ohtsuji et al., 2008) and GSH were decreased (Itoh et al., 1997) and indicating similarities in the response mechanisms between mice and fish. In the Nrf2 mutant zebrafish exposed to AgNO₃ (at 3 dpf) *gstp* expression was still clearly evident in the neuromasts, suggesting that the response in this tissue could be mediated by a transcriptional oxidative stress pathway different than that of Nrf1 (Biswas and Chan, 2010). Collectively data for the Nrf2 mutant however, shows that the Nrf2 pathway plays an important role in mediating the toxicological response to Ag materials in fish.

Conclusion

Applying *WISH*, we identify target tissues for silver nanomaterials, and they include tissues involved in environmental sensing (olfactory bulbs and neuromasts) and ionocytes involved with ion transport. Furthermore, the gene response associated with detoxification and oxidative stress appears to occur as a consequence of silver ions rather than a physical effect of the materials. We further show that, mutant lines used in combination with whole mount *in situ* hybridisation can be an effective way to better delineate pathways of effect for nanomaterials. Here, using an Nrf2 mutant zebrafish we show that *Nrf2* is an important transcription factor in the toxicological response of *mt2* and *gstp* to AgNPs.

Acknowledgements

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Supportive Information

The supportive information contains extra information on the materials and methods, the ontogeny of the 5 stress response genes up to 12 dpf. In addition it contains data for the characterization of the AgNPCi and AgBCi particles.

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References

- Andrews, G. K. 2000. Regulation of metallothionein gene expression by oxidative stress and metal ions. *Biochemical pharmacology*, 59, 95-104.
- Arosio, P., Ingrassia, R. & Cavadini, P. 2009. Ferritins: a family of molecules for iron storage, antioxidation and more. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1790, 589-599.
- Asharani, P., Wu, Y. L., Gong, Z. & Valiyaveetil, S. 2008. Toxicity of silver nanoparticles in zebrafish models. *Nanotechnology*, 19, 255102.
- Beer, C., Foldbjerg, R., Hayashi, Y., Sutherland, D. S. & Autrup, H. 2012. Toxicity of silver nanoparticles—nanoparticle or silver ion? *Toxicology letters*, 208, 286-292.
- Biswas, M. & Chan, J. Y. 2010. Role of Nrf1 in antioxidant response element-mediated gene expression and beyond. *Toxicology and applied pharmacology*, 244, 16-20.
- Bozzola, J. J. & Russell, L. D. 1999. *Electron microscopy: principles and techniques for biologists*, Jones & Bartlett Learning.
- Chen, W., John, J., Lin, C., Lin, H., Wu, S. & Chang, C. 2004. Expression of metallothionein gene during embryonic and early larval development in zebrafish. *Aquatic Toxicology*, 69, 215-227.
- Cheng, W., Guo, L., Zhang, Z., Soo, H. M., Wen, C., Wu, W. & Peng, J. 2006. HNF factors form a network to regulate liver-enriched genes in zebrafish. *Developmental biology*, 294, 482-496.
- Choi, J. E., Kim, S., Ahn, J. H., Youn, P., Kang, J. S., Park, K., Yi, J. & Ryu, D. Y. 2009. Induction of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. *Aquatic Toxicology*, 100, 151-159.
- Choi, O., Deng, K. K., Kim, N.-J., Ross Jr, L., Surampalli, R. Y. & Hu, Z. 2008. The inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth. *Water research*, 42, 3066-3074.
- Christian, P., Von der Kammer, F., Baalousha, M. & Hofmann, T. 2008. Nanoparticles: structure, properties, preparation and behaviour in environmental media. *Ecotoxicology*, 17, 326-343.
- Dymowska, A., Hwang, P.-P. & Goss, G. G. 2012. Structure and function of ionocytes in the freshwater fish gill. *Respiratory physiology & neurobiology*.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Ercal, N., Gurer-Orhan, H. & Aykin-Burns, N. 2001. Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. *Current topics in medicinal chemistry*, 1, 529-539.
- Esaki, M., Hoshijima, K., Kobayashi, S., Fukuda, H., Kawakami, K. & Hirose, S. 2007. Visualization in zebrafish larvae of Na⁺ uptake in mitochondria-rich cells whose differentiation is dependent on foxi3a. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 292, R470-R480.
- Esaki, M., Hoshijima, K., Nakamura, N., Munakata, K., Tanaka, M., Ookata, K., Asakawa, K., Kawakami, K., Wang, W. & Weinberg, E. S. 2009. Mechanism of development of ionocytes rich in vacuolar-type H⁺ATPase in the skin of zebrafish larvae. *Developmental biology*, 329, 116-129.
- Fabrega, J., Luoma, S. N., Tyler, C. R., Galloway, T. S. & Lead, J. R. 2011. Silver nanoparticles: Behaviour and effects in the aquatic environment. *Environment International*, 37, 517-531.
- Foldbjerg, R., Olesen, P., Hougaard, M., Dang, D. A., Hoffmann, H. J. & Autrup, H. 2009. PVP-coated silver nanoparticles and silver ions induce reactive oxygen species, apoptosis and necrosis in THP-1 monocytes. *Toxicology letters*, 190, 156-162.
- Froehlicher, M., Liedtke, A., Groh, K., Neuhaus, S., Segner, H. & Eggen, R. 2009. Zebrafish (*Danio rerio*) neuromast: promising biological endpoint linking developmental and toxicological studies. *Aquatic Toxicology*, 95, 307-319.
- Garner, L. V. & Di Giulio, R. T. 2012. Glutathione transferase pi class 2 (GSTp2) protects against the cardiac deformities caused by exposure to PAHs but not PCB-126 in zebrafish embryos. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 155, 573-579.
- Gobba, F. 2006. Olfactory toxicity: long-term effects of occupational exposures. *International archives of occupational and environmental health*, 79, 322-331.
- Gottschalk, F., Sonderer, T., Scholz, R. W. & Nowack, B. 2009. Modeled environmental concentrations of engineered nanomaterials (TiO₂, ZnO, Ag, CNT, fullerenes) for different regions. *Environmental Science & Technology*, 43, 9216-9222.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Harrison, P. M. & Arosio, P. 1996. The ferritins: molecular properties, iron storage function and cellular regulation. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 1275, 161-203.
- Higgins, L. G. & Hayes, J. D. 2011. Mechanisms of induction of cytosolic and microsomal glutathione transferase (GST) genes by xenobiotics and pro-inflammatory agents. *Drug metabolism reviews*, 43, 92-137.
- Hiroi, J., Kaneko, T., Uchida, K., Hasegawa, S. & Tanaka, M. 1998. Immunolocalization of vacuolar-type H⁺-ATPase in the yolk-sac membrane of tilapia (*Oreochromis mossambicus*) larvae. *Zoological science*, 15, 447-453.
- Hogstrand, C., Galvez, F. & Wood, C. M. 1996. Toxicity, silver accumulation and metallothionein induction in freshwater rainbow trout during exposure to different silver salts. *Environmental Toxicology and Chemistry*, 15, 1102-1108.
- Hogstrand, C. & Wood, C. M. 2009. Toward a better understanding of the bioavailability, physiology, and toxicity of silver in fish: Implications for water quality criteria. *Environmental Toxicology and Chemistry*, 17, 547-561.
- Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K. & Hatayama, I. 1997. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochemical and biophysical research communications*, 236, 313-322.
- Kashiwada, S., Ariza, M. E., Kawaguchi, T., Nakagame, Y., Jayasinghe, B. S., Gärtner, K., Nakamura, H., Kagami, Y., Sabo-Attwood, T. & Ferguson, P. L. 2012. Silver nanocolloids disrupt medaka embryogenesis through vital gene expressions. *Environmental Science & Technology*, 46, 6278-6287.
- Kasumyan, A. O. & Döving, K. B. 2003. Taste preferences in fishes. *Fish and Fisheries*, 4, 289-347.
- Kittler, S., Greulich, C., Diendorf, J., Köllner, M. & Epple, M. 2010. Toxicity of Silver Nanoparticles Increases during Storage Because of Slow Dissolution under Release of Silver Ions. *Chemistry of Materials*, 22, 4548-4554.
- Knörr-Wittmann, C., Hengstermann, A., Gebel, S., Alam, J. & Müller, T. 2005. Characterization of Nrf2 activation and heme oxygenase-1 expression in NIH3T3 cells

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- exposed to aqueous extracts of cigarette smoke. *Free Radical Biology and Medicine*, 39, 1438-1448.
- Kobayashi, M. & Yamamoto, M. 2005. Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxidants & redox signaling*, 7, 385-394.
- Lin, T.-Y., Liao, B.-K., Horng, J.-L., Yan, J.-J., Hsiao, C.-D. & Hwang, P.-P. 2008. Carbonic anhydrase 2-like a and 15a are involved in acid-base regulation and Na⁺ uptake in zebrafish H⁺-ATPase-rich cells. *American Journal of Physiology-Cell Physiology*, 294, C1250-C1260.
- Marambio-Jones, C. & Hoek, E. M. V. 2010. A review of the antibacterial effects of silver nanomaterials and potential implications for human health and the environment. *Journal of Nanoparticle Research*, 12, 1531-1551.
- Motohashi, H. & Yamamoto, M. 2004. Nrf2–Keap1 defines a physiologically important stress response mechanism. *Trends in molecular medicine*, 10, 549-557.
- Mueller, N. C. & Nowack, B. 2008. Exposure Modeling of Engineered Nanoparticles in the Environment. *Environmental Science & Technology*, 42, 4447-4453.
- Mukaigasa, K., Nguyen, L. T., Li, L., Nakajima, H., Yamamoto, M. & Kobayashi, M. 2012. Genetic evidence of an evolutionarily conserved role for Nrf2 in the protection against oxidative stress. *Molecular and cellular biology*, 32, 4455-4461.
- Nakajima, H., Nakajima-Takagi, Y., Tsujita, T., Akiyama, S.-I., Wakasa, T., Mukaigasa, K., Kaneko, H., Tamaru, Y., Yamamoto, M. & Kobayashi, M. 2011a. Tissue-restricted expression of Nrf2 and its target genes in zebrafish with gene-specific variations in the induction profiles. *PloS one*, 6, e26884.
- Nakajima, H., Nakajima-Takagi, Y., Tsujita, T., Akiyama, S. I., Wakasa, T., Mukaigasa, K., Kaneko, H., Tamaru, Y., Yamamoto, M. & Kobayashi, M. 2011b. Tissue-Restricted Expression of Nrf2 and Its Target Genes in Zebrafish with Gene-Specific Variations in the Induction Profiles. *PloS one*, 6, e26884.
- Neves, J. V., Wilson, J. M. & Rodrigues, P. N. 2009. Transferrin and ferritin response to bacterial infection: the role of the liver and brain in fish. *Developmental & Comparative Immunology*, 33, 848-857.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Ohtsuji, M., Katsuoka, F., Kobayashi, A., Aburatani, H., Hayes, J. D. & Yamamoto, M. 2008. Nrf1 and Nrf2 play distinct roles in activation of antioxidant response element-dependent genes. *Journal of Biological Chemistry*, 283, 33554-33562.
- Orino, K. & Watanabe, K. 2008. Molecular, physiological and clinical aspects of the iron storage protein ferritin. *The Veterinary Journal*, 178, 191-201.
- Osborne, O. J., Johnston, B., Moger, J., Baalousha, M., Lead, J., Kudoh, T. & Tyler, C. 2013. Effects of particle size and coating on nanoscale Ag and TiO₂ exposure in zebrafish (*Danio rerio*) embryos. *Nanotoxicology*, 7, (8) 1315-1324.
- Piao, M. J., Kang, K. A., Lee, I. K., Kim, H. S., Kim, S., Choi, J. Y., Choi, J. & Hyun, J. W. 2011. Silver nanoparticles induce oxidative cell damage in human liver cells through inhibition of reduced glutathione and induction of mitochondria-involved apoptosis. *Toxicology letters*, 201, 92-100.
- Pinto, E., Sigaud-kutner, T., Leitao, M. A., Okamoto, O. K., Morse, D. & Colepicolo, P. 2003. Heavy metal-induced oxidative stress in algae¹. *Journal of Phycology*, 39, 1008-1018.
- Ponka, P., Beaumont, C. & Richardson, D. R. Year. Function and regulation of transferrin and ferritin. *In: Seminars in hematology*, 1998. 35.
- Poss, K. D. & Tonegawa, S. 1997. Heme oxygenase 1 is required for mammalian iron reutilization. *Proceedings of the National Academy of Sciences*, 94, 10919-10924.
- Ringwood, A. H., McCarthy, M., Bates, T. C. & Carroll, D. L. 2010. The effects of silver nanoparticles on oyster embryos. *Marine environmental research*, 69, S49-S51.
- Sadler, K. C., Krahn, K. N., Gaur, N. A. & Ukomadu, C. 2007. Liver growth in the embryo and during liver regeneration in zebrafish requires the cell cycle regulator, uhrf1. *Proceedings of the National Academy of Sciences*, 104, 1570-1575.
- Sanchez, W., Palluel, O., Meunier, L., Coquery, M., Porcher, J.-M. & Aït-Aïssa, S. 2005. Copper-induced oxidative stress in three-spined stickleback: relationship with hepatic metal levels. *Environmental Toxicology and Pharmacology*, 19, 177-183.
- Scown, T. M., Santos, E. M., Johnston, B. D., Gaiser, B., Baalousha, M., Mitov, S., Lead, J. R., Stone, V., Fernandes, T. F. & Jepson, M. 2010. Effects of aqueous exposure to silver nanoparticles of different sizes in rainbow trout. *Toxicological Sciences*, 115, 521-534.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Theodore, M., Kawai, Y., Yang, J., Kleshchenko, Y., Reddy, S. P., Villalta, F. & Arinze, I. J. 2008. Multiple nuclear localization signals function in the nuclear import of the transcription factor Nrf2. *Journal of Biological Chemistry*, 283, 8984-8994.
- Timme-Laragy, A. R., Karchner, S. I. & Hahn, M. E. 2012. Gene Knockdown by Morpholino-Modified Oligonucleotides in the Zebrafish (*Danio rerio*) Model: Applications for Developmental Toxicology. *Developmental Toxicology*. Springer.
- Valko, M., Rhodes, C., Moncol, J., Izakovic, M. & Mazur, M. 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-biological interactions*, 160, 1-40.
- van Aerle, R., Lange, A., Moorhouse, A., Paszkiewicz, K. H., Ball, K., Johnston, B. D., de-Bastos, E., Booth, T., Tyler, C. R. & Santos, E. M. 2013. Molecular Mechanisms of Toxicity of Silver Nanoparticles in Zebrafish Embryos. *Environmental Science & Technology*.
- Wijnhoven, S. W. P., Peijnenburg, W. J. G. M., Herberts, C. A., Hagens, W. I., Oomen, A. G., Heugens, E. H. W., Roszek, B., Bisschops, J., Gosens, I. & Van De Meent, D. 2009. Nano-silver-a review of available data and knowledge gaps in human and environmental risk assessment. *Nanotoxicology*, 3, 109-138.
- Wood, C. M., Playle, R. C. & Hogstrand, C. 1999. Physiology and modeling of mechanisms of silver uptake and toxicity in fish. *Environmental Toxicology and Chemistry*, 18, 71-83.

SUPPLEMENTARY INFORMATION

**Sensory systems and ionocytes are among the target tissues for
silver nanoparticles in fish**

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Supplementary information

S1

Particle Characterization

0.1 mg L⁻¹ suspensions of AgNPCi and 1mg L⁻¹ AgBCi were prepared in embryo media. The nanoparticles in 10 mL of each suspension were deposited on Formvar/Carbon coated TEM-grids using 30,000 rpm ultracentrifugation (Beckman L-75) during 60 min. Thereafter the grids were rinsed by gently immersing in milli Q-water, and dried overnight. Micrographs were acquired at 30k, 75k, 300k and 500k magnification (5-10 micrographs at each magnification from each sample) at 80 keV accelerating voltage using a JEOL 1200EX TEM-instrument.

S2

Fish source, culture and husbandry

Wild-type WIK strain zebrafish embryos were obtained from the Max Planck Institute, Tubingen, Germany and maintained at University of Exeter. Fry from approximately 2dpf were fed on a microencapsulated diet (ZM Advanced Fry feed; ZM Ltd., Hampshire, U.K.). This was supplemented from approximately 7dpf with freshly hatched *Artemia nauplii* (ZM Premium Grade Artemia; ZM Ltd.). From 21dpf onwards to adulthood fish were fed with freshly hatched *Artemia nauplii* to satiation twice daily. As adults, fish were fed daily on both freshly hatched *Artemia nauplii* and TetraMin tropical flake food (TetraMin, Tetra Werke, Melle, Germany). Embryos for use in the toxicity assessments were collected from naturally spawning colonies.

S3

Silver dissolution of AgNPCi

Two plastic containers were filled with 500 mL of 16 ppt salinity synthetic seawater. In each container, 2 dialysis bags (Spectra/Por, 1 kDa MWCO), labelled A and B respectively, each filled with 25 mL of 16 ppt synthetic seawater, were placed. The solutions in the containers (outside the dialysis bags) was stirred. 0.5 mL blank samples were taken, inside dialysis bag

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A and B, and from the solution outside the bags, in each container, in order to check background Ag concentrations. In one of the containers, 50 mL of the synthetic seawater was removed, and was replaced by 50 mL of a suspension of 9.0 ppm 10nm Ag-labelled citrate capped Ag-nanoparticles (AgNPCi), resulting in 0.9 ppm AgNPCi in suspension in the containers. Synthetic seawater salt was added to the container to adjust for the dilution resulting from the AgNPCi-addition. 0.5 mL samples were taken inside the dialysis bags, and from the suspension/solution outside the dialysis bags, in both containers, immediately after the silver-additions. The containers were thereafter kept dark (inside a dark plastic bag) and under stirring for 27 days. The sampling was repeated 14 times during this period, to monitor the Ag-concentration inside and outside the dialysis bags.

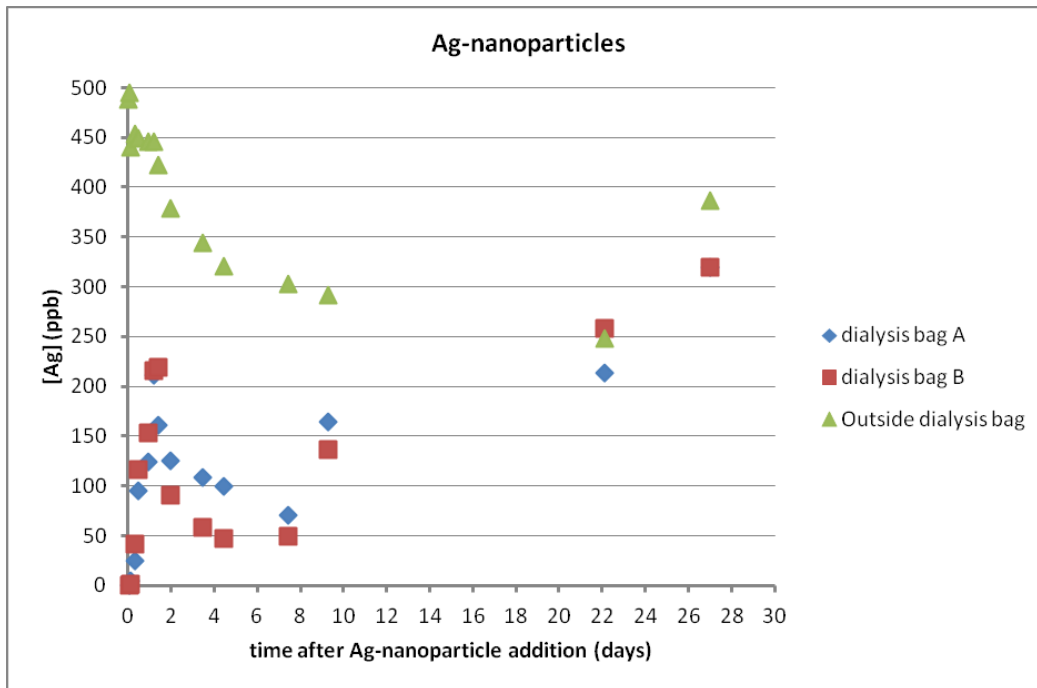
S4

Results of silver dissolution of AgNPCi in container

Mean recovery of Ag was 54%. It was concluded that this low recovery could be due to the AgNPs aggregating and adsorbing onto the container. Dissolution of the silver ions from AgNPCis in the first 23-34 hours was around 0.12-0.22 ppm in the dialysis membrane bags concluding a rapid dissolution of the AgNPs. At 4.5-7.5 days the Ag concentrations decreased and were around 0.047-0.071 ppm. Soon after Ag concentration started to increase and reached 0.32 ppm after 27 days which was similar to the outside solution at 0.39ppm. This suggests that Ag only fully “dissolved” at day 27 (Graph S1).

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Graph S1



Graph S1. Graph showing dissolution over time for citrate coated Ag nanoparticles showing the variations of Ag concentrations inside and outside dialysis membrane in the container.

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Table S1: Summary of AgNPCi and AgBCi particle characteristics

	AgNPCi	AgNPCi in embryo medium	AgBCi	AgBCi in embryo medium
Zeta Potential (mV)	-47.78±4.3	-18.38 ± 1.6	-35.97±6.2	-21.54 ± 3.9
Z average diameter (DLS)	20	92.25 ± 1.8	160	2456.2 ± 1287.5
TEM (nm)	9.9 ± 3.1	52 ⁹³ ₂₂ Median value Upper quartile Lower quartile	-	145 ⁵⁰⁹ ₁₁₄ Median value Upper quartile Lower quartile

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Table S2: Summary of characterisation measurements on AgNP (35nm) and AgB in distilled water

	AgNP 35nm	AgB (Ag Bulk)
Zeta Potential	-6.5 ± 1.8	-2.8 ± 0.6
pH	7.34	6.40
Hydrodynamic Diameter (nm) DLS	2029 ± 524	938 ± 230
Polydispersant Index	0.93	0.69
Hydrodynamic diameter (nm) NTA technique	166 ± 72	217 ± 130
TEM (nm)	114 ± 65.3	137 ± 62.0

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Figure S1

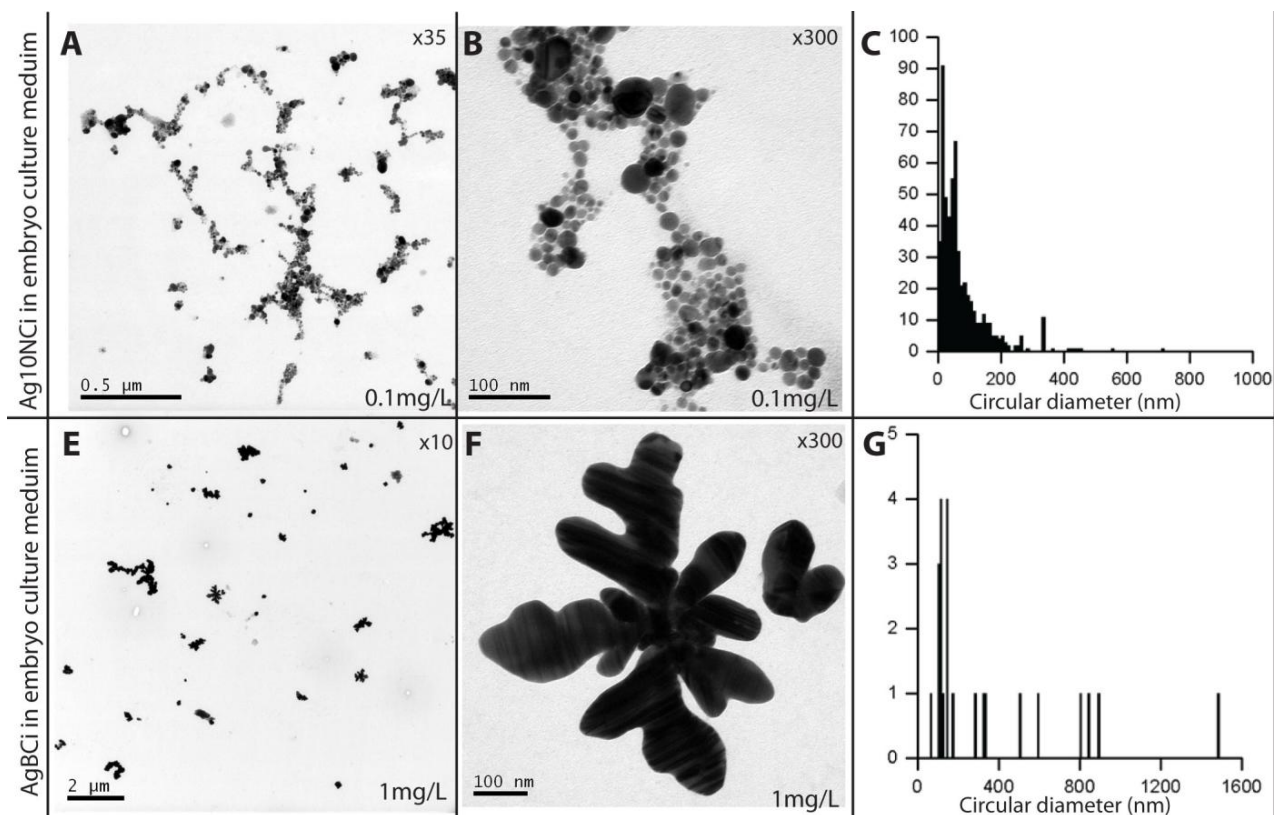


Figure S1: Characterization of the AgNPCi and AgBCi. TEM of AgNPCi at 0.1 mg/l embryo culture medium x35 magnification **(A)**, and x300 magnification **(B)**; DLS separation of AgNPCi in embryo exposure medium at 0.1 mg/l showing circular particle size distribution **(C)**; Circular particle size distribution determined via TEM analysis **(D)**; TEM of AgBCi at 1 mg/l embryo culture medium, x35 magnification **(E)**, and x300 magnification **(F)**; DLS separation of AgBCi in embryo exposure medium at 1 mg/l showing circular particle size distribution **(G)**.

CHAPTER 4

**ALTERED TOXICOLOGICAL RESPONSIVENESS IN LARVAL ZEBRAFISH AFTER PARENTAL
DIETARY EXPOSURE TO SILVER NANOPARTICLES**

To be submitted to *Nanotoxicology* or *ACS Nano*

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Altered toxicological responsiveness in larval zebrafish after parental dietary exposure to silver nanoparticles

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Abstract

Increasing amounts of products containing silver nanoparticles (AgNPs) in the market means that inevitably discharges will increase into surface waters. Predicated amounts ending up in surface waters pose a potential health risk to our aquatic systems. This study investigated the effects of a dietary exposure to citrate coated AgNPs (10nm, 62.6±3.20 mg/kg) and a bulk counterpart (600-1600nm, 43.9±0.33 mg/kg) on breeding in zebrafish (*Danio rerio*), and assessed for maternal transfer and subsequent biological consequences in their offspring. ICP-MS analysis detected silver in the liver (up to 2.1 µg/g in males) and gonad (up to 0.5 µg/g in males) of adult fish dosed with ciAgN only (detection limit > 0.1 µg /g) indicating an enhance bioavailability for the silver (Ag) nanomaterial compared with the bulk counterpart. Dosing of adult zebrafish for three weeks with the Ag materials had no effect on fecundity (numbers of eggs spawned) or on fertility (numbers of eggs fertilised). Ag was detected in the embryos for the ciAgN treated adults only (up to 0.43 ng/per embryo) confirming maternal transfer. Gene responses of metallothionein 2 (*mt2*) and glutathione S Transferase Pi (*gstp*) were measured in the subsequent offspring via whole mount *in-situ* hybridisation (*WISH*) at 24 hours post fertilisation (hpf). This showed significant *mt2* upregulation in the offspring during the period of adult dosing with AgNPs. Challenging the subsequent embryos to the same Ag materials indicated de-sensitisation of *mt2* to Ag in offspring for adults treated previously (over 26 days) with nano and bulk forms of Ag. Overall, we show that dosing breeding fish with the different Ag materials had no effect on obvious measures of reproductive fitness but there was an enhanced Ag accumulation in selected tissues of adult fish exposed to AgNP compared with a bulk counterpart and an

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altered response sensitivity of the *mt2* gene to Ag in their offspring which may have implications for their ability to process metal based materials.

Keywords: Silver nanoparticles, zebrafish, fecundity, stress response, transgenerational effects

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Introduction

Nanoparticles (NPs) are being used in a very wide range of applications and the nanotechnology industry is growing at a rapid rate. One of the consequences of this will be that NPs will increasingly end up in aquatic environments due to their direct discharge and via leaching from products containing nanomaterials as they age and deteriorate. There is much uncertainty as to how NPs will behave or impact in our environment. The fate and transport of these NPs will be determined, at least in part, by environmental factors and the manner in which they are disposed (Nowack et al., 2012).

Most research has focused on short-term biological effects of NPs and generally adverse effects for acute exposures are observed only for concentrations that far exceed those with environmental relevance (Scown et al., 2010b). There is less certainty however, regarding the ability of NPs to accumulate in exposed organisms or on longer-term exposures and even less information on the trophic transfer of NPs.

There is some evidence that NPs can accumulate in fish. An example of this, is a study on carp exposed to titanium dioxide nanoparticles (TiO₂NPs) (50nm) at 10 mg/L for 20 days via the water column showed titanium concentrated mainly in their gills by a bioconcentration factor (BCF) of 0.74 and the viscera 1065 which is extremely high (Zhang et al. 2006). A further study on zebrafish exposed via the diet to gold (Au) NPs (12nm), dosed at 5 µg Au/g given at 2% of their body weight for 36 days showed they accumulated in the brain and liver with resulting tissues concentrations of 4.6±2.3 µg Au/g and 3.0±2.4 µg Au/g, respectively. Studies in medaka too exposed via the water to fluorescent NPs (solid latex solution) at 10 mg/L over a period of 7 days, provided evidence via fluorescence intensity for accumulation mainly in the gills, gallbladder, liver and testis (Kashiwada, 2006). Further studies on fish have clearly established the liver as a target tissue for NPs, including Ag materials (Wood et al., 1999). In studies on Ag, the liver has consistently been identified as a target site. Early studies showed in juvenile rainbow trout exposed to 9.3 µg AgNO₃/L for 7 days Ag was found subsequently in the liver at 20 µg/g with an approximate BCF of 2.1 (Hogstrand et al., 1996). Another example is a study by (Scown et al., 2010a); where a 10 day exposure to AgNPs (10nm, 35nm) via the water column (100 µg/L) to rainbow trout showed Ag uptake in the liver at 1.50 ± 0.30 µg/g and 0.92 ± 0.16 µg/g respectively.

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A few studies have now also demonstrated that some NPs can undergo trophic transfer. Examples of this include TiO₂NPs that have been shown to pass into body tissues of zebrafish fed daphnia pre-dosed with TiO₂NPs (Zhu et al., 2010b). Furthermore; trophic transfer of AuNPs (15nm) initially uptaken from the water column into different organisms (clams, snails, shrimp and fish) via sea grass, biofilms and microbes has been observed through a series of mesocosms studies (Ferry et al., 2009).

One potentially key route of entry for nanomaterials into organisms is into developing eggs and or offspring via maternal transfer. Studies using rodents have shown NPs can cross the placenta to the foetus, depending on the NPs size and coating. One study showed TiO₂NPs (25-75nm) administered into pregnant mice via subcutaneous injection at 1 mg/L were detected via Field Electron Scanning Electron Microscopy (FE-SEM) in the testis and brain of the offspring (Takeda et al., 2009). A further study on TiO₂NPs (2570nm) administered to pregnant mice at 1 µg/µL (injected subcutaneously for the gestational period), found upregulation and down regulation in the foetuses/pups brain in a number of genes, specifically related with development and function of nervous system which included apoptosis, oxidative stress and motor activity (Shimizu et al., 2009). It is being increasingly recognised that various NPs can translocate membrane barriers such as the blood brain barrier and placental-foetal barrier and this is now being exploited for the development of drug delivery and nanomedicines (Silva, 2007).

Ag is used in 34% of the 1 000 plus nano products (Wijnhoven et al., 2009) on the market currently, making it a priority material for toxicology research. Ag can be persistent in aquatic ecosystems (Kothe and Varma, 2012, Luoma et al., 1995) and resulting concentrations in the aquatic environment can be very significant indeed. In a recent study, up to 175 µg/L of Ag was measured in leachates from painted facades (Kaegi et al., 2010), and it has been reported that AgNP socks release up to 1 300 µg Ag/L (Benn and Westerhoff, 2008). Estimates of Ag in natural waters range from 0.03 and 500 ng/L (Luoma and Nanotechnologies, 2008). Ag is a well known toxicant inducing biological effects including apoptosis, oxidative stress in the liver (Choi et al., 2009), DNA damage and morphological abnormalities in zebrafish (Asharani et al., 2008b) at concentrations of 5-500 µg/L. AgNPs at concentrations 50 µg/L cause lethality in zebrafish embryos (Osborne et al.,

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2013) and cause Metallothionein 2 (*mt2*) upregulation via *WISH* analysis at sublethal doses (Osborne et al., 2013).

Here we carried out a dietary exposure of breeding zebrafish to Ag particles to investigate their uptake into selected body tissues and effects on breeding. We further assessed for evidence for both maternal transfer into the developing eggs and effects in their subsequent offspring. Fish were dosed with citrate coated 10nm AgNPs and a bulk counterpart and effects determined on cumulative egg number and fertilisation rates. Responses of genes involved in detoxification (*mt2*) and oxidative stress (*gstp*), both of which are targets for Ag materials, were measured in resulting embryos for these exposures and for *mt2* gene also in embryos further exposed to Ag materials. The rationale for the latter is being to establish whether parental exposure to Ag materials impacts on the response of the *mt2* gene. Dietary exposure to AgNPs is an expected natural exposure route.

Materials and Methods

Nano particle source and characterization

Citrate covered Ag 10nm NPs (measured size of 9.9 ± 3.1 nm; AgNPCi) were acquired from the University of Birmingham and larger sized Ag particles (measured size of 600-1600nm; AgB) acquired from Nanostructured and Amorphous Materials Inc. Houston, USA. These materials were characterised in the following manner: Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS), as further detailed in the results section and supplementary information (Figure S1 and Table S1).

Flake preparation and flake dosing

Three treatments of artificial flake were made up: Citrate control, Ag Nano and Ag Bulk. A citrate control was used because the Ag materials were coated in citrate to facilitate stabilisation. Many AgNPs used for industrial applications are coated with materials such as citrate, PVP, Poly (Ethylene Glycol) (PEG), silica and thiol to name a few. The diet comprised of TetraMin Tropical Flakes-ground to a fine powder mixed with flour and the required amount of Ag and citrate (0.15mM-the known concentration for the AgNPCi treatment).

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Each treatment was made up as follows: In the citrate control, 40g of ground flake was added to 4g of flour combined with 80ml of citrate; in the Ag nano treatment, 250ml of AgNPCi at 11ppb (colloidal form) was added to 40g of ground flake and 4g of flour; for the bulk treatment, 2.32mg of AgB was added to 40g of ground flake, 4g of flour and 80ml of citrate. The amalgam for each treatment was mixed together and then spread evenly onto baking paper and dried in an oven at 50°C. Once dried, the flakes were crumbled and placed in a falcon tube for storage at 4°C. Three 3g samples of each synthesised flake treatment analysed by ICP-MS to determine Ag levels. Fish were fed at 3% of their bodyweight per day. For the pre dosing period, the adult breeding fish were fed TetraMin Tropical Flakes and subsequently during the 21 day exposure period they were fed the artificially synthesised diet containing TetraMin Tropical Flake, flour and the dosing materials.

Fish source and husbandry

Wild type WIK strain zebrafish embryos were obtained from the Max Planck Institute, Tubingen, Germany and maintained at the University of Exeter. Fry from approximately 2 days post fertilisation (dpf) were fed on a microencapsulated diet (ZM advanced fry feed; ZM Ltd. Hampshire, U.K). This was supplemented from approximately 7 dpf with freshly hatched *Artemia nauplii*. From 21 dpf fish were fed twice daily to satiation with freshly hatched *Artemia nauplii* and then throughout with freshly hatched *Artemia nauplii* in the morning and with TetraMin Tropical Flake Food in the afternoon. Once the exposure to the Ag materials had been initiated the breeding fish were fed/dosed TetraMin Tropical Flake in the morning and afternoon. In addition, every 3 days the fish were fed *Artemia nauplii* to try to ensure optimal nutrition and to maintain maximum breeding output.

Fish were maintained in reconstituted water. Mains tap water was filtered by reverse osmosis (Environmental Water Systems (UK) Ltd) and reconstituted with Analar-grade mineral salts to standardized synthetic freshwater (final concentrations to give a conductivity of 300mS: 58 mg/L CaCl₂.2H₂O, 24.65 mg/L MgSO₄.7H₂O, 12.95 mg/L NaHCO₃, 1.15 mg/L KCl, 12.5 mg/L Tropic Marin Sea Salt). This water was aerated, and heated to 28°C in a reservoir before it was supplied to each aquarium using a flow-through system. Aquarium water was routinely monitored for pH, conductivity, ammonia, nitrate, and nitrite, all of which were well within acceptable limits of U.S. EPA guidelines (12). The

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photoperiod was set to 14:10h light:dark, with an artificial dawn to dusk transition of 30min. Prior to the experiment, fish of mixed sex were maintained in large holding aquaria, 900x500x300mm in dimension, with a working volume of 112L (approximately 100 fish per aquarium). Flow rates were checked every other day to ensure they did not vary 3 ml/min.

Sexing and weighing/measuring fish

Fish were sexed using differences in their colourations and differences in the behaviours that exist between males and females. Individual sexually mature fish were placed carefully in a beaker of water to obtain an accurate body weight measurement. They were then placed on a laminated graph paper to measure fork length.

Tank setup

Sixteen tanks of '4 males X 4 females' breeding colonies were established (tank dimensions: 30cm X 30 cm with a volume of 12L, with 5 full water tank replacements every 24h). Fish were allowed to acclimate for 2 weeks to ensure that all fish were spawning and behaving normally. The 12 tanks of fish showing the most consistent breeding over this time period were then used for the study. A random number generator approach was used to assign 4 replicate tanks to the three different treatments: Citrate control (cc), citrate coated Ag Nano (ciAgN) and citrate coated Ag Bulk (ciAgB) (Figure1).

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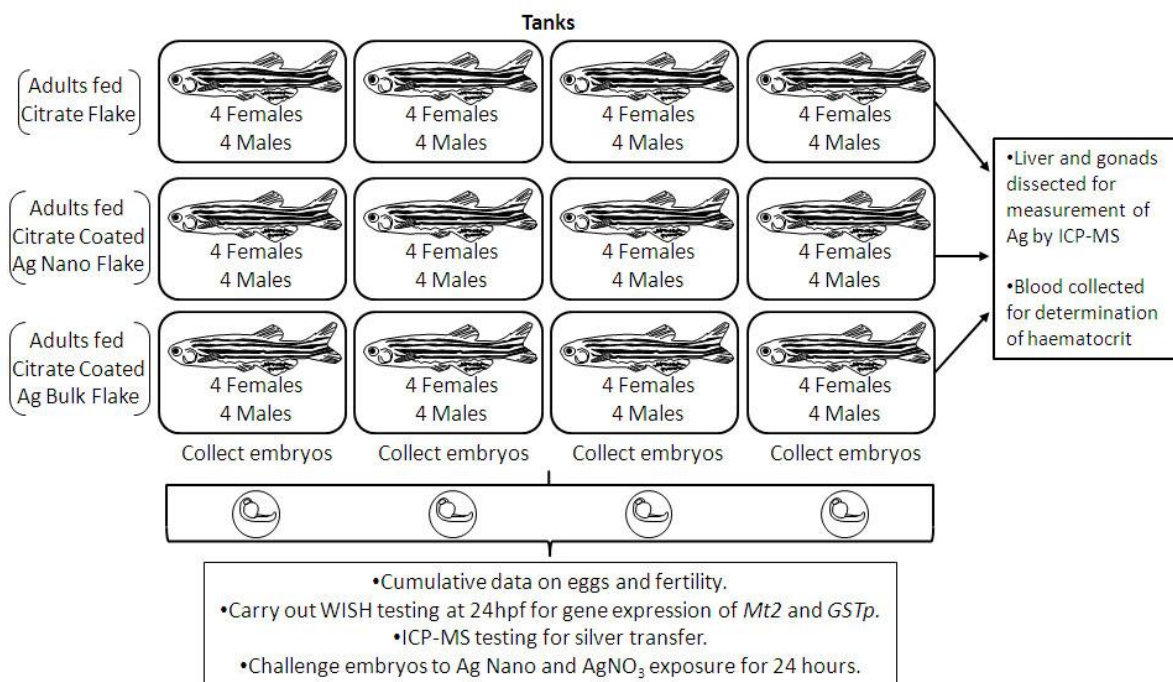


Figure 1: Schematic showing overview of experimental set up and procedures carried out during the study.

Biological Sampling

At the end of the experiment fish were sacrificed by terminal anaesthesia (Benzocaine, Sigma Poole, UK). Measurements of fork length and weight were recorded, and blood was collected from the caudal vein into haematocrit micro-capillary tubes containing heparin that were placed on ice, prior to centrifugation (Hawskley England Microhaematocrit centrifuge) at 11 800 rpm for 10 minutes, to obtain an haematocrit reading. Blood samples collected were then stored at -20 °C. Fish were then dissected and the gonads and liver, weighed and stored at -80°C until used for Ag content analysis by ICP-MS. The Gonad Somatic Index (GSI) for males and females was calculated as follows: (weight of gonad mg) / (weight of the total body)*100, the Hepato Somatic Index (HSI) for males and females was determined using the formula (weight of liver mg) / (weight of the total body)*100 and the Haematocrit Index measured for males and females as follows: (red blood cells) / (red blood cells and plasma)*100.

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Ag concentrations in adult fish tissues and embryos determined by ICP-MS

Tissue samples (liver and gonads) were defrosted at room temperature. Samples of livers and gonads were pooled in the following way: for each sex, gonads and/livers from 2 fish were collected from each tank for each treatment x (4 replicates) giving a total of 4 livers / 4 gonads per treatment for each sex. Samples were then digested by adding 1ml of 20% nitric acid (HNO₃) (AR grade, Fischer Scientific), and 200µl of hydrogen peroxide (Laboratory Reagent Grade, Fischer Scientific) to each liver/gonad sample and left at 70°C in a heat block for 3 days. Once digested the individual samples for the tissue samples were combined for each tissue and dissolved in 10ml 2% solution of HNO₃ using Milli Q water. Pooling of tissues samples for the gonad and for the liver was necessary to provide sufficient tissue for detecting the Ag materials. For the embryos, 10 embryos for each treatment group were digested with 1ml of 20% nitric acid at 70°C. Once digested the embryo samples were dissolved in 10ml 2% solution of HNO₃ using Milli Q water. Samples were analysed for Ag using Inductively Coupled Plasma Spectrometry (ICP-MS) at the University of Plymouth and the University of Birmingham. The detection limit of the ICP-MS for Ag in the tissues was >0.1 µg/g and the detection limit for embryos was >0.1 µg/g.

Measuring egg output and fertilisation

The glass aquarium was designed with sloping sides to form a 'funnel' to channel any eggs spawned in to an egg-collecting chamber. This facilitated daily embryo collection whilst minimizing disturbance to the fish. Glass marbles of 10mm diameter were placed in the 'funnel' section of the base of the aquaria at a depth of 3–4 marbles to act as a spawning substrate and to minimize oophagy. An artificial weed was placed in the tanks rooted in the marbles to act as a refuge and spawning stimulus. Eggs/embryos were collected 1h after dawn. This was done by opening the valve at the base of the egg collecting chamber, underneath which a fine mesh sieve (tea strainer) had been placed, forcing the water through the marbles where the eggs were trapped (marbles were simultaneously agitated). The eggs were captured on the fine mesh sieve and washed with embryo culture water (60 mg/L of Tropic marin salt, 10 µl of methylene blue to reduce the chance of fungal infections) to remove any waste food and/or faeces. The eggs/embryos collected were then transferred into a Petri dish with embryo culture water. Thirty embryos were separated from each tank

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collection and used to calculate fertilisation success. Following collection from all tanks, and at approximately 2 hours post fertilization (hpf), any infertile eggs /dead embryos were easily distinguished and separated from the live embryos. For each of the test aquaria, the numbers of live and dead eggs were counted giving a record of total egg output and overall egg viability for each colony of fish. The same process was conducted 30 minutes after the final feed each day in order to ensure that no further eggs had been spawned and to flush away any waste products (food/faeces) that may have built up during the day, particularly in the marbles.

Whole Mount in-situ hybridisation and synthesis of gene probes: mt2 and gstp

Twice a week during both the pre and post exposure period embryos from different tanks and different treatment groups (n=30) were collected and stored in 4% PFA for *WISH* analysis and ICP-MS analysis.

The expression of two genes (*mt2* and *gstp*) was measured to evaluate the expression over the pre and during exposure periods. The *mt2 in situ* probe was prepared as described previously (Osborne et al., 2013). *Gstp* plasmid was acquired from University of Tsukuba (Mukaigasa et al., 2012). The antisense *gstp* probe for *WISH* plasmid encoding *gstp* was digested and transcribed with restriction enzyme BamH1 and RNA polymerases T7. Using a G50 column (GE Healthcare) the RNA was purified and precipitated using Lithium Chloride. The probes were then diluted 1/200 with hybridisation buffer (Osborne et al., 2013).

Whole Mount in-situ hybridisation

For the hybridizations, embryos were fixed using 4% PFA in PBS at 4°C overnight. They were then dechorionated and placed in methanol for 2 hours. They were then washed two times with PBS+0.1% Tween20 (PBSTw), placed in hybridisation buffer for 1 hour and incubated with the desired probe overnight at 65°C. Embryos were then washed in 50% formamide 2XSSC, 0.1%Tween 20 wash for 30minutes at 65°C, followed by a 2XSSC 0.1%Tween 20 wash at 65°C and received 2x 30 minutes washes at 65°C with 0.2XSSC 0.1%Tween 20. Blocking solution (2% Blocking reagent (Roche) containing 2.5ml of calf serum in MAB (Osborne et al., 2013) was added and the embryos were incubated for 1 hour. Anti-DIG antibody (5000x diluted with Blocking solution) (Roche) was then added and the embryos incubated for 2h.

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Embryos subsequently received a series of 4 x 30minutes washes in PBTw before a 10 minute wash with AP buffer (Tris 0.1M pH 9.5, NaCl 0.1M, MgCl₂ 50mM, Tween 20 0.1%). Embryos were transferred to a 24 well plate and placed in staining solution (BM-Purple AP Substrate, Roche) to reveal the probe, and photographed using a Nikon SMZ1500 stereo microscope equipped with a digital camera. Here, the expression intensity for a target gene was determined for the specific tissue area (extended yolk sac region) of these two gene responses for 15-20 embryos/larvae, subtracting any background using Image-J 1.44 P. This value obtained was then converted to a fold change (FC) compared with controls for each week to give an approximate quantification for effect for the different Ag material treatments for that gene in the target tissue. These values were then analysed to assess for statistical differences.

Ag exposures of embryos from treated adults

In addition to assessing target gene responses in the subsequent offspring from Ag dosed parents; evaluation of the embryos responsiveness to these Ag materials was measured. To determine this, 20 embryos taken from different treatment tanks collected during week 6 of study were studied for each gene/exposure (at the 1-2 cell stage, 1-1.5 hpf) per treatment well (5ml) and the embryo exposure analysis was replicated at least 3 times. For the material treatments, sonication was conducted prior to exposure using a Cole and Palmer Ultrasonicator Processor, amplification at 100% with pulsing for two 10 second bursts. The embryos from treated adults were challenged to ciAgN and AgNO₃ and then fixed at 24 hpf for *WISH* analysis using the *mt2* probe. The dosing regime was 500 µg AgNPCi/l and 30 µg AgNO₃/l (Perkin Elmer) based on previous work showing this as an effective dose for inducing expression of the *mt2* gene.

Statistics

Unless otherwise stated all data represented, all data presented as means±S.E.M. The coefficient variant (CV) statistic was calculated for comparisons of variation, as CV= standard deviation/mean) *100. All statistical analyses were performed using Sigma Stat Version 12.0 (Jandel Scientific Software, USA). Differences among groups were analysed by one-way ANOVA, followed by Tukey's multiple comparison post-hoc test, or a non parametric

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alternative (Kruskal-Wallis one-way ANOVA on ranks) where data were not normally distributed.

Results

Particle Characterization

Characterisation of raw materials:

A summary of the physiochemical properties of the ciAgN and AgB particles is provided in the supplementary material (supplementary information Figure S1 and Table S1). It was established that AgNPCi in raw form had a zeta potential of -47.78 ± 4.3 mV, DLS data illustrated that AgNPCi had a mean diameter of 20nm, whereas TEM determined 9.9 ± 3.1 nm. AgNPCi in milli Q water had a zeta potential of -40.21 ± 9.3 mV, a mean diameter of 32.4 ± 2.6 nm measured by DLS and 36 ± 50 nm measured via TEM. AgB in milli Q water had a 137 ± 62.0 nm measured via TEM and a zeta potential of -2.8 ± 0.6 mV. For full details see supplementary information, Table S1.

Materials in the embryo culture medium (for final embryos exposures):

DLS established AgNPCi in the embryo culture medium had a mean diameter of 92.25 ± 1.8 nm. When dispersed in the embryo medium the measured sizes as determined via TEM were 52^{93}_{22} nm for AgNPCi, the zeta potential for AgNPCi was -18.38 ± 1.6 mV. TEM micrographs illustrated that individual nanomaterials could be distinguished in liquid form, but there was evidence of aggregation when they were placed in the embryo exposure medium (Table S1).

Artificial Flake -Ag concentration determined by ICP-MS

Concentrations of Ag in the dietary flake treatments were determined by ICP-MS. Final average concentrations for the artificial flake were cc (0.3 mg/kg ± 0.00), ciAgN (62.6 mg/kg ± 3.20) and ciAgB (43.9 mg/kg ± 0.33).

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Determination of Ag concentrations in fish tissues and embryos determined by ICP-MS

Trace metal (Ag) analyses carried out on digested fish tissues (liver/gonads) and embryos from treated adults using ICP-MS is shown in Tables 1 and 2.

Gonads: In the citrate control treatment group both females and males contained <0.1 µg Ag/g. In the ciAgN treatment gonads contained 0.5 µg Ag/g in males and 0.3 µg Ag/g in females. In the ciAgB treated fish gonads in both males and females contained <0.1 µg Ag/g.

Liver: In the citrate control group liver Ag content was <0.1 µg Ag/g in both males and females. In the ciAgN treatment the liver content of Ag was 2.1 µg Ag/g in males and 0.5 µg Ag/g in females. In the ciAgB treatment both males and females contained <0.1 µg Ag/g tissue.

	Males		Females	
	Livers	Gonads	Livers	Gonads
cc	<0.1	<0.1	<0.1	<0.1
ciAgN	2.1	0.5	0.5	0.3
ciAgB	<0.1	<0.1	<0.1	<0.1

Ag levels detected in liver and gonads (µg/g)
n=8 for each treatment and tissue

Table 1: Ag levels (µg/g) determined via ICP-MS in adult livers and gonads in adult fish in the different treatment groups after a dietary exposure to Ag materials. n=8 per treatment and tissue.

Embryos: In the first two weeks of the study during the pre exposure period embryo content of Ag was <0.1 ng /per embryo. After exposure to the Ag materials embryos content for the cc and ciAgB treatments was <0.1 ng/per embryo but for the ciAgN treatments, Ag content was as follows: on day 26, 0.24 ± 1.74 ng/per embryo; on day 30, 0.15 ± 4.31 ng/per embryo; on day 34, 0.14 ± 0.87 ng/per embryo; on day 41, 0.43 ± 1.92 ng/per embryo.

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	Treatment	Ag (ng/per embryo)
Week 1		
Day 2	Pre exposure	<0.1
Day 5	Pre exposure	<0.1
Week 2		
Day 9	Pre exposure	<0.1
Day 14	Pre exposure	<0.1
* Week 3	Exposure started on day 21	
Day 22	Control (Citrate)	<0.1
Day 22	ciAgN	<0.1
Day 22	ciAgB	<0.1
Day 26	Control (Citrate)	<0.1
Day 26	ciAgN	0.24 ± 1.74
Day 26	ciAgB	<0.1
Week 4		
Day 30	Control (Citrate)	<0.1
Day 30	ciAgN	0.15 ± 4.31
Day 30	ciAgB	<0.1
Day 34	Control (Citrate)	<0.1
Day 34	ciAgN	0.14 ± 0.87
Day 34	ciAgB	<0.1
Week 5		
Day 41	Control (Citrate)	<0.1
Day 41	ciAgN	0.43 ± 1.92
Day 41	ciAgB	<0.1

Table 2: Ag levels (ng/per embryo) determined via ICP-MS in embryos from adults exposed via the diet to Ag materials. Data are means ±SEM, n=30 per treatment.

Biological Effects

There was only one mortality over the 46 day study - a single male in the ciAgN treatment in Week 4 and this animal was not included in the subsequent data analysis. In controls, the condition factor in males ranged between 1.0 and 1.1 and in females between 1.1 and 1.2. No difference was found in the condition factor index ($p>0.05$) between treatment groups or within a treatment group for the different sexes when compared for the pre and post

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exposure periods (data not shown). The adult breeding fish were in good condition throughout the study.

Tissue somatic indices and Haematocrit Index

HSI and GSI values are provided in Figure 2. No significant difference (One Way ANOVA DF=2 F= 0.570 p=0.814) was found for the HSI in females (Fig.2 A), however in males treated with ciAgB treatment had significant difference compared with the control treatment (One Way ANOVA H=9.110 DF=2 p=0.011) (Fig.2 A). No significant difference was seen in the GSI across treatments for both males (One Way ANOVA H= 2.366 DF=2 p=0.386) and females (One Way ANOVA H=4.806 DF=2 p=0.097) (Fig. 2 B). The HSI and GSI in fish exposed to the ciAgN treatment showed greater variation for both sexes compared with the ciAgB treatment and cc treatment. No significant difference was seen in the haematocrit index across treatments for either males (One Way ANOVA F=0.567 DF=2 p=0.571) or females (One Way ANOVA H=4.806 DF=2 p=0.341) In the controls, the haematocrit value in both males and females was 0.39, in the ciAgN treatment slightly higher than control at 0.40 and in males and slightly lower than control at 0.37 in females. In the ciAgB treatment slightly lower at 0.38 in males and 0.38 in females.

Fecundity and fertilisation success

Approximate egg output per day for the different treatment groups pre exposure period was as follows: control - 471 ± 163 , ciAgN - 498 ± 100 , ciAgB - 443 ± 162 . There were no differences in fecundity (egg numbers) during the pre- exposure period between the different treatment groups (One Way ANOVA DF=2 F=0.639 p=0.863) and there was no difference either between treatment during the exposure period (One Way ANOVA DF=2 F=0.857 p=0.457). Overall egg production was greater in controls during the pre-exposure period versus during exposure period and this was also the case for the Ag treatment groups. During the exposure period egg output was reduced in both control (One way ANOVA DF=1 F=18.712 p=0.005) and both treatments (ciAgN -One way ANOVA DF=1 F=30.779 p=0.001, and ciAgB - One way ANOVA DF=1 H=5.333 p=0.029, Fig. 2 C-E). No differences were seen between control and treatments during the pre or during exposure period, so there was no effect of the Ag treatment. There was no significance difference

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(One Way ANOVA DF=2 F=0.778 p=0.572) in fertilisation success between treatment groups or tanks either during the pre exposure period or exposure period (Fig. 2 F-H). There were no obvious differences in the breeding behaviour between males and females. No developmental effects were observed in subsequent embryos.

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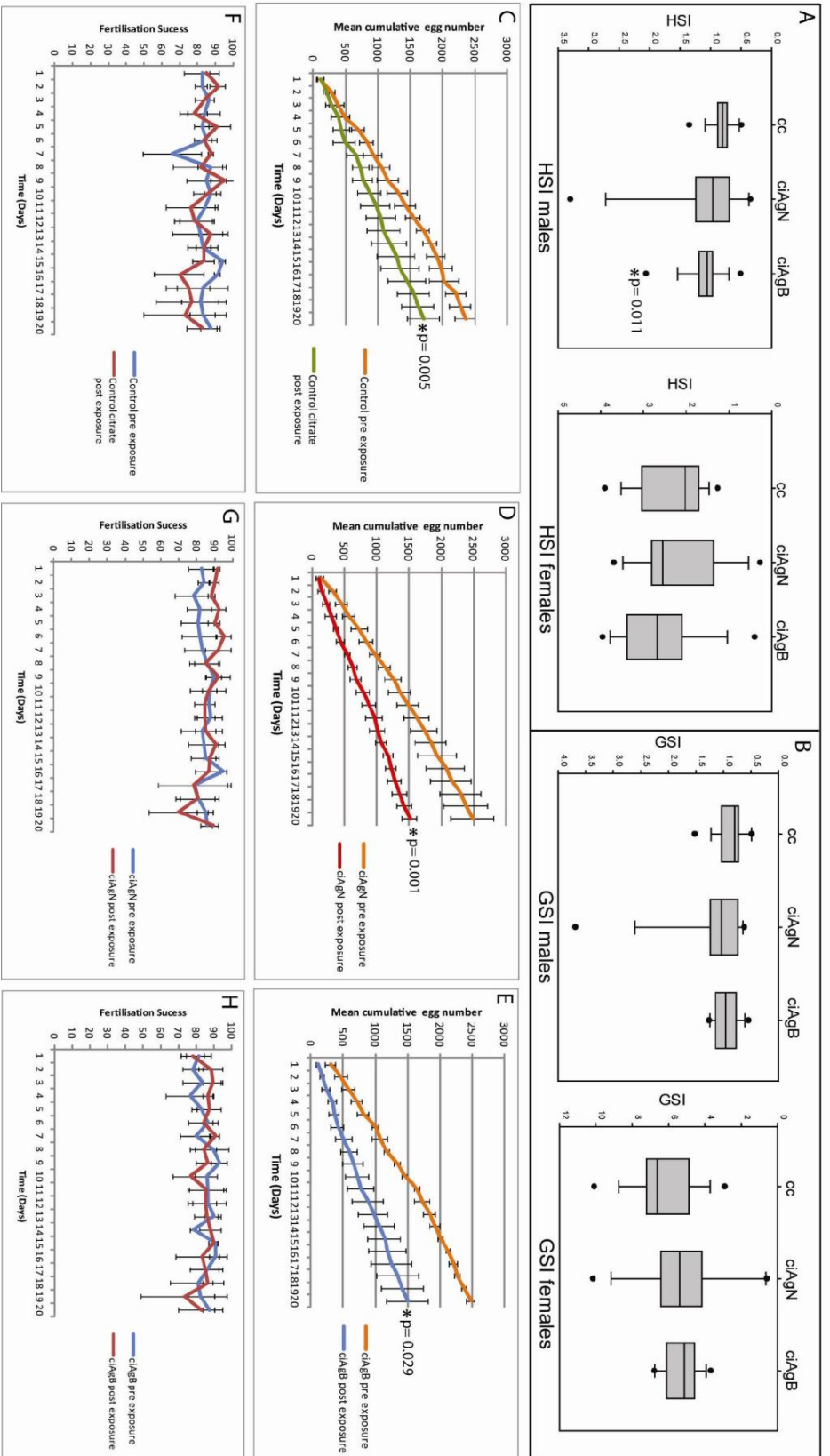


Figure 2: Box plots and line graphs of measurements on fish condition and breeding output. Panel (A) -box plot diagrams of Hepatosomatic Index (HSI) for males and females showing a significant difference between males in the ciAgB treatment and controls ($p=0.011$, Kruskal-Wallis One Way Analysis of Variance on Ranks). Panel (B) - box plots of Gonad Somatic Index (GSI) for males and females. Panel (C-E) - Line graph showing cumulative egg number for pre/post exposure periods for control and Ag treatment tanks. Panel C- controls, Panel D- ciAgN, Panel E- ciAgB. One way ANOVA analysis Variance (All Pair Wise multiple comparison procedures (Holm-Sidak method)) showed there was a statistical difference between groups pre and during exposure periods. Controls ($p<0.005$), ciAgN ($p<0.001$), ciAgB ($p<0.029$). Panel F-H Line graph showing fertilisation success pre/post exposure for controls and Ag treatment colonies; Panel F- controls, G- ciAgN, H- ciAgB treatment tanks. Statistical differences are marked with an asterisk (*).

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Responses of mt2 and gstp genes in embryos assessed via WISH

WISH data carried out on 24 hpf embryos assessing the gene responses for *mt2* and *gstp* are shown in Figure 3.

Mt2 (Fig.3 A): The variations in the controls were deemed negligible throughout the study based on previous work (Chapter 3). During the pre exposure period no significant ($p \geq 0.05$) differences were observed in *mt2* expression for any of the treatment groups. For both Ag treatment groups there was a statistically significant decrease in *mt2* expression at the onset of dosing period; *mt2* expression for ciAgN and ciAgB was 0.7 and 0.8 of that in controls. In week 4, one week after the dosing had been initiated, there was an increased level of expression of *mt2* in both Ag treatment groups (1.3 fold-difference) and in week 5 these responses were significantly (One Way ANOVA DF=2 F=1.34 $p < 0.05$) further elevated (ciAgN, *mt2* expression was 1.7 fold higher than controls and in ciAgB 1.6 fold higher than controls). This level of *mt2* induction in the Ag treatment groups persisted in the third week of the exposure period (week 6), where for both ciAgN and ciAgB expression was significantly 1.5 higher than for controls.

Gstp (Fig.3 B): During the pre exposure period, there were no significant differences in *gstp* expression between the treatments and over time. During the exposure period, there was an apparent increase in the level of *gstp* in the ciAgN treatment but this was not statistically significant.

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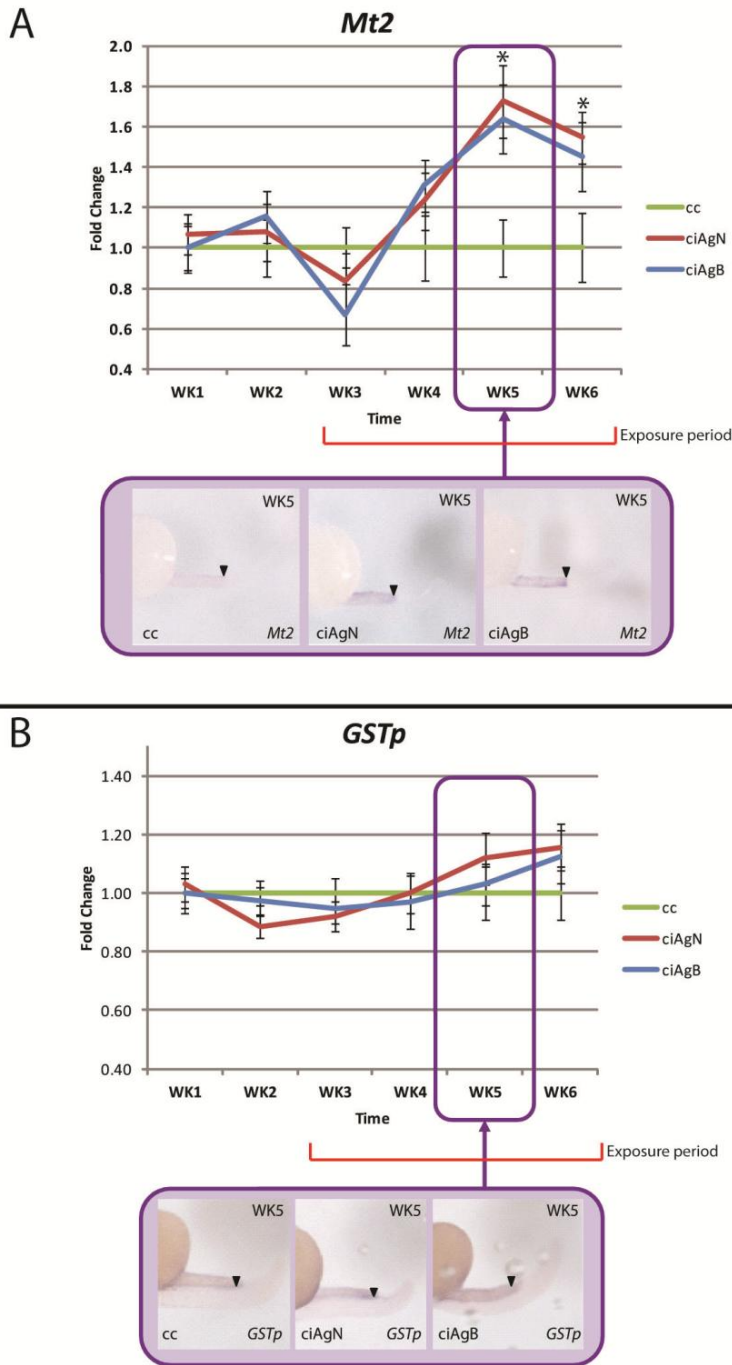


Figure 3: Linear graphs showing quantification of target gene responses (*Mt2* [A] and *GSTp* [B]) derived from *WISH* analysis and showing fold change in expression compared with controls (cc) within that week throughout study (Week1-Week6) on 24hpf embryos derived from adults treated with Ag materials. Panels below graphs show photographic examples of *WISH* analysis of the target genes analysed. (One way ANOVA, *denote $p < 0.05$). (▲) Indicates target tissue area quantified for the gene expression.

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Mt2 expression in embryos exposed directly to Ag materials as assessed via WISH

Exposure of embryos (24hpf) derived from adults treated with Ag materials to AgNPCi and AgNO₃ via the culture medium was conducted to assess effects of parental exposure on the responses of *mt2* (Figure 4). Exposure of embryos derived from control fish to both AgNPCi and AgNO₃ induced a significant induction of *mt2* (by 16-fold and 21- fold, respectively). Exposure of adults to ciAgN and ciAgB resulted in a significant reduction in the relative response of *mt2* to AgNPCi and AgNO₃ treatments. Embryos derived from parental exposure to ciAgN induced a 1.3 fold response to AgNPCi and a significant induction (3.3 fold response) to AgNO₃. Embryos derived from parental exposure to ciAgB induced a 1.3 fold response to AgNPCi and a significant 6 fold response to AgNO₃.

One way ANOVA showed there was statistical difference between groups (One Way ANOVA DF=2 F=0.640 p<0.0475). A pair means comparison test showed significant differences between the groups p<0.05 marked by an asterisk (*).

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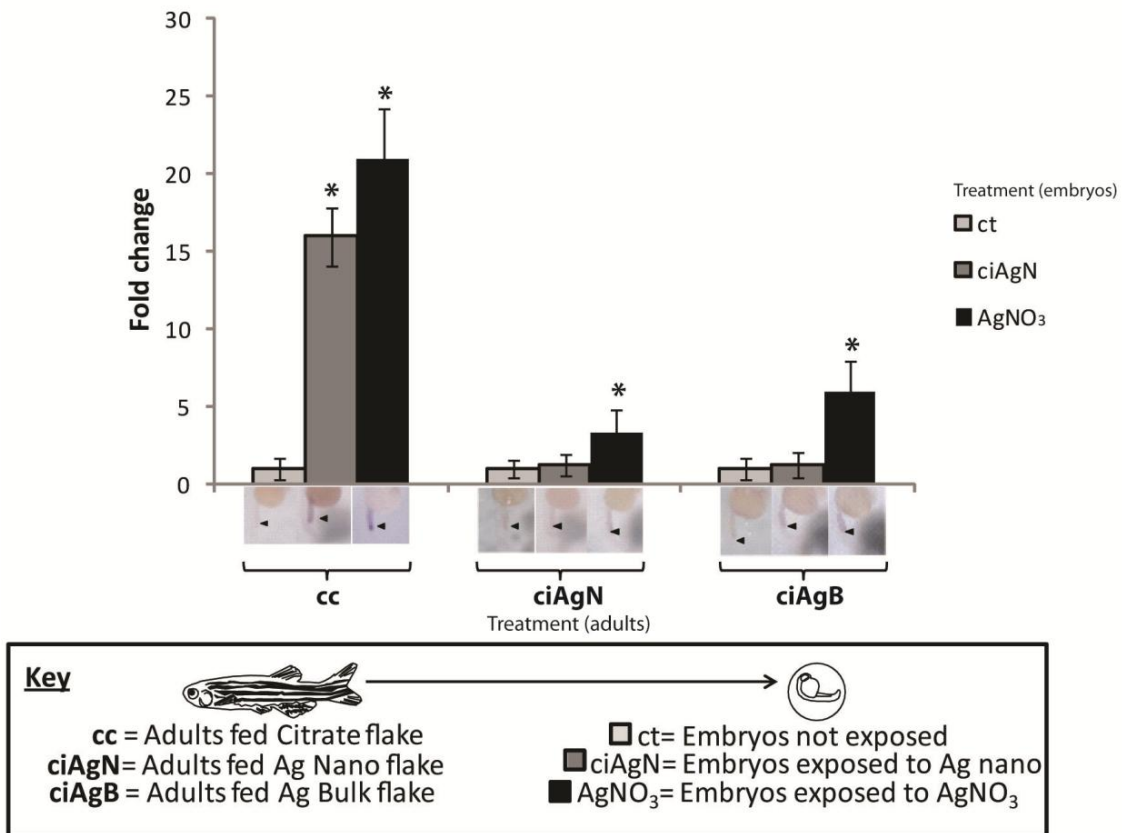


Figure 4. WISH analysis showing responses of *mt2* to exposure to AgNPCi and AgNO₃ for embryos derived from adults exposed to different Ag materials (ciAgN or ciAgB). Data presented represent fold change in expression compared with controls within treatment group. (One way ANOVA, *p<0.05). Images show WISH examples from each treatment group and (▲) indicating target tissue area quantified for the expression.

Discussion

This study set out to determine whether AgNPs and their bulk counterpart dosing the fish each day at approximately 5µg Ag/g at 3% of their body weight over a period of 3 weeks affected breeding in zebrafish. This dose is in parallel with other similar zebrafish dietary studies that explore the possibility of metal NPs toxicity (Geffroy et al., 2012) and represents environmentally relevant concentrations found in surface waters as reported in Gottschalk et al., (2009). We show that for fish dosed with ciAgN, but not ciAgB, Ag was detected in the liver and gonad indicating an enhanced deposition in these tissues compared with ciAgB and this was further reflected in the subsequent embryos where there was detectable Ag

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measured for the ciAgN exposure. We show that although there were no effects on breeding output due to the dietary Ag treatments, the adult exposures to both AgNP and AgB affected expression of *mt2* during early life and reduced the responsiveness of this gene to Ag materials on challenging these embryos to Ag materials.

Ag accumulation in adult body tissues and evidence for maternal transfer

Ag accumulated in the liver and gonads of ciAgN treated males and females, but levels in ciAgB treated fish were below the detection limit in these tissues (Table 1). The liver is well established as a the primary organ for the accumulation of Ag (Hogstrand et al., 1996), as well as other metals (Lanno et al., 1987). In murine models, studies have shown NPs are generally retained in the liver after uptake through the gastrointestinal tract (Wang et al., 2007). In a study on zebrafish dosing AgNPs (5-20nm) for 24h at 120 mg/L for 24h via the water resulted in 2.4ng Ag/mg in the liver (Choi et al., 2009). Other mammalian studies have shown how rats introduced intravenously to a range of AuNPs (10nm, 50nm, 100nm and 250nm) also accumulated in the liver (De Jong et al., 2008); showing particle size dependent differences for organ distribution specifically for 10nm size, the same size particles used in this study. Another study by Gaiser et al., (2009), showed AgNPs (35nm) had higher uptake than their bulk counterpart (600-1600nm). The reasoning for these size dependent differences seen in our study and others, could relate to the physical uptake of NPs themselves compared with their bulk counterpart (Chithrani et al., 2010) as they are smaller, and research has shown how they can readily cross physiological membrane barriers (Chen et al., 2004b). Another possibility for these differences, could be the bioavailability of NPs (Johnston et al. 2010). It is known that NPs have a greater capacity to release Ag ions in comparison to bulk counterparts (Klaine et al., 2008) and therefore a greater dissolution of the NPs could have done so in the gut of the zebrafish in this study; subsequently, Ag ions could have then easily crossed the epithelium into the blood and into liver/gonads. Past research has discussed that in the gut epithelium of fish they are able to uptake particles via endocytosis (Handy et al., 2008) which could mean the Ag is being absorbed through the gut epithelium and translocation through gut wall into the blood systems and into target organs. In this study, there is no data on dissolution of Ag from the particles in the treatment groups. Handy et al., (2008) proposed organ specific toxic effects

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for NPs including liver, also suggesting NPs to be a high free metal ion concentration delivery system. Handy et al., (2008) also proposed that certain NPs (depending if they are hydrophobic) would enhance accumulation in certain tissues such as the liver. The pattern of uptake of the Ag materials into the gonad mimicked that for the liver, with detectable quantities of Ag in the gonads in both males and females for the ciAgNP exposure, but not for the ciAgB exposure. In other studies the gonad in rats has been shown to take up a variety of NPs for dietary exposures (McAuliffe and Perry, 2007).

An interesting observation from the exposures of adult fish to the Ag materials was an apparently higher content of Ag in the liver and gonads of males compared with in females. Other studies in zebrafish too have shown gender related differences in the accumulation of toxicants, for example for Perfluorooctanoic Acid (PFOA) in Hagenars et al., (2013) study showed males to have a higher body burden than females (accumulating 9 times more). It is not known why these differences occur, but it may relate, in part, to differences in the endocrinology and metabolism that occur between the sexes. In our study, a very real possibility for the lower content of Ag in the ovary of females (per weight tissue) is likely to be related to the fact that the Ag deposited is expelled in ovulated eggs.

Consistent with the uptake of Ag into adult fish dosed, Ag in embryos was detected only in fish dosed with ciAgN. An approximate estimation of how much Ag theoretically would be embryos in relation to the amount dosed to the fish each day (taking into account: amount of Ag dosed per day X number of exposure days = (total amount of Ag) / (number of eggs produced in ciAgN treatment / the number of fish in treatment) / by the total amount of Ag) gives ~0.65 ng Ag/embryo. Our study found up to 0.43 ± 1.92 ng Ag/embryo detected (Table 2) by the last week of the exposure period. There is evidence for placental transfer of NPs (Au and C₆₀) in mammals (Ema et al., 2010). Investigations have shown how larger particles i.e. bulk particles, do not cross blood barriers or accumulate in target organs in comparison with smaller particles (Hillyer and Albrecht, 2001) which could be a possible theory as to why no Ag in the ciAgB treatment was detected and as a result did not reach/no levels were detected in target organs or embryos in this study. NPs are known to be transported across epithelium at rates up to 250 times higher (Desai et al., 1996) than their larger counterparts, and this in turn, would account for the differences in liver/gonad and embryos content for

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the different Ag materials in our study. Evidence for NPs crossing blood-brain barrier has been reported (Schroeder et al., 1998) stating the mechanism of doing so could be through passive diffusion or receptor mediated endocytosis. The reason for greater amounts of Ag in the ciAgN treated embryos could be due to the ability for smaller particles to translocate into the embryos from the Ag found in the gonads. It is known that AgNPs can pass through chorion membrane barrier of zebrafish by Brownian motion (Lee et al., 2012). The pore size of the chorion membrane is around 0.5-0.7 μm , hence it is possible ciAgN (10nm) did cross into chorionic space of the embryo. Size has been shown to play an important part in the uptake of AgNPs into zebrafish embryos in other recent studies (George et al., 2012). As discussed before; there is also the possibility that it could be due to the fact that there is a greater dissolution from the ciAgN particles in the gut and more free Ag is available for uptake into the liver and gonads and in turn the embryos. The limitation of our study is that ICP-MS doesn't measure the amount of nano but the measurement of the amount of Ag. However, we established that a relative amount of Ag reached target organs and embryos confirming maternal transfer.

Ag exposures did not affect fecundity

We found no effects of exposure to the Ag materials on measures of health or reproductive fitness. Indices of liver function (HSI), gonad development/mass (GSI) and red blood cell volume (haematocrit), showed no toxic effects for the Ag treatments. From past research, it has been shown how Ag exposures to adult marine fish have shown high tolerance to Ag (Calabrese et al., 1977). Examples include; *Tautoglabrus adspersus* (cunner) which has been known to survive up to 1 000 ppb in a 96 hr exposure and the *Pseudopleuronecles americanus* (winter flounder) 10 ppb in a 60 day exposure. Calabrese et al., (1977) concluded that adults had acquired Ag tolerance through a sequestering mechanism (metabolism can function even with a high body burden of Ag) that juveniles lack which could be a possibility why Ag did not have effect on the zebrafish health status. As seen in other species, adult stage of zebrafish are far less sensitive to Ag than in larval stages (embryo form) (Osborne et al., 2013). Our dosing was applied on an environmentally relevant basis, so it was known that Ag would not cause lethality to the zebrafish at adult stage. If we compare with previous studies, they have shown that the LC_{50} for AgNP in adult

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zebrafish is 7 mg/L for exposure via the water column (Griffitt et al., 2008). Other studies have shown how 25 µg AgNP(50nm)/L induced significant upregulation of *mt2* and heat shock protein 70 (*hsp70*) in the liver of *Oryzias latipes* (Japanese medaka). Scown et al., (2010a) reported significant upregulation of *cyp1 a2* in gills of exposed trout to 100µg AgNP(10nm)/L .

The measured fecundity of zebrafish prior to the particle treatments (4 X 4 breeding colony per day produced 87 ±7 eggs) which is comparable to that shown for previous studies with similar breeding matrices (e.g. Paull et al., (2008)).The reduced egg during the exposure period, that occurred across all treatments, was not due to treatment. This reduction in the control treatment is not usually observed in other zebrafish breeding studies (Lin and Janz, 2006), so we concluded that this reduction could have been as a result of a number of reasons including diet. Brine shrimp were only fed every 3 days in comparison to everyday pre-study period; the artificial synthesised flake contained additional chemicals to that of the commercial fish flake which was given to fish prior the exposure period; seasonal breeding ovarian cycle (Hisaoka and Firlit, 1962); and/or because fatigue over the extensive breeding period could also have been another reason. The dietary exposures to the Ag materials however, resulted in no adverse effects on the adult fish or their ability to breed and no differences in fertility rates (Fig.2 C-H). The lack of any effect on fecundity for exposure to AgNPs contrasts with some previous studies on other organisms, such as *C. elegans* (Roh et al., 2009), where decreases in fecundity have been reported. In that study, they exposed the organism via the water column at 0.5 mg AgNP/L therefore one of the contrasts could be because the dosing route was different. Another possibility could be the organism is different and it is known that lethality/toxic effects in *C. elegans* are more sensitive than those of the zebrafish (LC₅₀ 4.4mg AgNP/L) (Ellegaard-Jensen et al., 2012). It has also been discussed that exposure via the water column the toxicity is generally greater than via the diet where it is uptaken in the gut. Other studies using different NPs (TiO₂) have also reported that long term exposure (0.1 mg/L) had a negative impact on the reproduction (egg output) in zebrafish (Wang et al., 2011b). Some of the reasons for these differences could be accounted for as follows; this study was short term in comparison with Wang et al., (2011a) which ran over the course over 60 days; and different particles were used in the exposure.

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Expression of mt2 and gstp in derived embryos from adult fish dosed with Ag materials

During the pre dosing period, *mt2* and *gstp* expression was consistent and did not vary to any significant degree across the treatment groups. This basal level expression of *mt2* and *gstp* found in the extended yolk sac region is consistent with previous work (Chapter 3) and the upregulation of *mt2* seen in the extended yolk sac region (target tissue) due to Ag has been reported previously (Osborne et al., 2013). There was significant elevated expression of *mt2* in the embryos for parental exposures to Ag materials (Figure 3). The elevated *mt2* expression for the ciAgNP is consistent with the uptake of measurable levels of Ag in these embryos. The elevated expression of *mt2* in embryos derived from adults dosed with ciAgB is less easily explained. It is known however, that *mt2* is a highly sensitive marker to exposures to even low levels of Ag and the likelihood is that Ag is present in the embryos below the detection limit of ICP-MS analysis conducted ($>0.1 \mu\text{g/g}$). In other studies using real time PCR, *mt2* has been shown to be induced in zebrafish embryos at $0.16 \mu\text{g Ag/L}$, further illustrating the sensitivity of this gene to Ag (Ringwood et al., 2010). There was not a statistically significant induction of *gstp* in embryos for the parent exposures to Ag materials, but there was a trend for an effect indicating a possible low level oxidative stress response. It is known from previous work (Chapter 3) that *gstp* responds to Ag materials but not necessarily as sensitive as *mt2* at 24 hpf. Other NPs such as ZnONPs have also been shown to generate ROS causing oxidative stress in zebrafish embryos (Zhu et al., 2009a).

Embryos from Ag treated adults show desensitised response to Ag

Embryos derived from the parental exposures to the Ag materials illustrated marked differences in the responses of *mt2* when challenged with AgNPCi and AgNO₃. In embryos derived from untreated adult fish, *mt2* was highly responsive to AgNPCi and AgNO₃ which is consistent with previous studies on zebrafish embryos (Osborne et al., 2013). In contrast, for embryos derived from parental exposure to the different Ag there was a marked reduction in the responses of the *mt2* genes to the Ag challenge (Figure 4).

It is known that continuous or repeated stimulation from a chemical can constitute to desensitization (Hoffman et al., 1986), through counter-regulatory mechanisms amongst others. This means the mechanism that usually keeps the internal environment stable reacts

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in the opposite way to create a balance between thresholds. These effects are often idiosyncratic for a particular pathway and have been studied for some drugs and hormones (Sibley and Lefkowitz, 1985). It has been reported that Metallothionein can become desensitised in HeLa cells with prolonged exposure to the metal Zinc (Karin et al., 1981); they proposed inhibition of protein synthesis and as a result reduction of gene transcription. We propose that prolonged exposure to Ag at early life stages of the embryos in our study may have similarly caused Ag desensitization, through Ag inhibiting *mt2* protein synthesis and as of consequence reduction in *mt2* induction. Alternative effects have been reported in the behaviour of other fish such as *Oncorhynchus mykiss* (rainbow trout); where avoidance preference to heavy metals was seen in adult fish which had been previously exposed to heavy metals at their early life stages (Svecevicus, 2003). The desensitisation seen in this study might have occurred through epigenetics, it has been known that environmental exposure to metals has been known to modulate (Vandegheuchte and Janssen, 2011). Chemicals, in this case Ag may interact with proteins such as histone, which in turn can interfere with transcription or translation.

The implications of such would warrant further investigations into the potential risk associated with AgNPs exposures. Hagens et al., (2007) stated that it is important to assess internal exposure and the possibility of the NPs reaching target organs. Understanding the biodistribution of the NPs will be also fundamental to predicting accumulation and possible transfer through food chains. It is also key to ensure the history of the fish is well documented as responses and biological effects observed could be different, and therefore alternative interpretations could be made.

Conclusions

We show that dietary exposure to the Ag materials did not impact zebrafish condition, breeding or embryo viability. Ag was only found in the tissues and embryos of ciAgN (and not ciAgB) treated adults suggesting that size plays an important part in determining the translocation of the particles and/or Ag ions into target organs. There also appeared to be gender related differences in the accumulation of Ag, with greater body burdens in males. *WISH* analysis revealed that adult dietary exposure to Ag materials resulted in elevated *mt2*

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expression in the subsequent offspring and also a de-sensitisation to their responsiveness to Ag, the functional consequences of which warrant further investigation.

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Supportive Information

The supportive information contains extra information on the materials and methods. In addition it contains data for the characterization for the particles used in this study.

References

- Asharani, P., Wu, Y. L., Gong, Z. & Valiyaveetil, S. 2008. Toxicity of silver nanoparticles in zebrafish models. *Nanotechnology*, 19, 255102.
- Benn, T. M. & Westerhoff, P. 2008. Nanoparticle silver released into water from commercially available sock fabrics. *Environmental Science & Technology*, 42, 4133-4139.
- Calabrese, A., MacInnes, J., Nelson, D. & Miller, J. 1977. Survival and growth of bivalve larvae under heavy-metal stress. *Marine Biology*, 41, 179-184.
- Chen, Y., Dalwadi, G. & Benson, H. 2004. Drug delivery across the blood-brain barrier. *Current Drug Delivery*, 1, 361-376.
- Chithrani, D. B., Dunne, M., Stewart, J., Allen, C. & Jaffray, D. A. 2010. Cellular uptake and transport of gold nanoparticles incorporated in a liposomal carrier. *Nanomedicine: Nanotechnology, Biology and Medicine*, 6, 161-169.
- Choi, J. E., Kim, S., Ahn, J. H., Youn, P., Kang, J. S., Park, K., Yi, J. & Ryu, D. Y. 2009. Induction of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. *Aquatic Toxicology*, 100, 151-159.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- De Jong, W. H., Hagens, W. I., Krystek, P., Burger, M. C., Sips, A. J. & Geertsma, R. E. 2008. Particle size-dependent organ distribution of gold nanoparticles after intravenous administration. *Biomaterials*, 29, 1912-1919.
- Desai, M. P., Labhasetwar, V., Amidon, G. L. & Levy, R. J. 1996. Gastrointestinal uptake of biodegradable microparticles: effect of particle size. *Pharmaceutical research*, 13, 1838-1845.
- Ellegaard-Jensen, L., Jensen, K. A. & Johansen, A. 2012. Nano-silver induces dose-response effects on the nematode *Caenorhabditis elegans*. *Ecotoxicology and environmental safety*, 80, 216-223.
- Ema, M., Kobayashi, N., Naya, M., Hanai, S. & Nakanishi, J. 2010. Reproductive and developmental toxicity studies of manufactured nanomaterials. *Reproductive Toxicology*, 30, 343-352.
- Ferry, J. L., Craig, P., Hexel, C., Sisco, P., Frey, R., Pennington, P. L., Fulton, M. H., Scott, I. G., Decho, A. W. & Kashiwada, S. 2009. Transfer of gold nanoparticles from the water column to the estuarine food web. *Nature nanotechnology*, 4, 441-444.
- Gaiser, B. K., Fernandes, T. F., Jepson, M., Lead, J. R., Tyler, C. R. & Stone, V. 2009. Assessing exposure, uptake and toxicity of silver and cerium dioxide nanoparticles from contaminated environments. *Environ Health*, 8, S2.
- Geffroy, B., Ladhar, C., Cambier, S., Treguer-Delapierre, M., Brèthes, D. & Bourdineaud, J.-P. 2012. Impact of dietary gold nanoparticles in zebrafish at very low contamination pressure: The role of size, concentration and exposure time. *Nanotoxicology*, 6, 144-160.
- George, S., Lin, S., Ji, Z., Thomas, C. R., Li, L., Mecklenburg, M., Meng, H., Wang, X., Zhang, H. & Xia, T. 2012. Surface defects on plate-shaped silver nanoparticles contribute to its hazard potential in a fish gill cell line and zebrafish embryos. *ACS Nano*, 6, 3745-3759.
- Gottschalk, F., Sonderer, T., Scholz, R. W. & Nowack, B. 2009. Modeled environmental concentrations of engineered nanomaterials (TiO₂, ZnO, Ag, CNT, fullerenes) for different regions. *Environmental Science & Technology*, 43, 9216-9222.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Griffitt, R. J., Luo, J., Gao, J., Bonzongo, J. C. & Barber, D. S. 2008. Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. *Environmental Toxicology and Chemistry*, 27, 1972-1978.
- Hagenaars, A., Vergauwen, L., Benoot, D., Laukens, K. & Knapen, D. 2013. Mechanistic toxicity study of perfluorooctanoic acid in zebrafish suggests mitochondrial dysfunction to play a key role in PFOA toxicity. *Chemosphere*.
- Hagens, W. I., Oomen, A. G., de Jong, W. H., Cassee, F. R. & Sips, A. J. 2007. What do we (need to) know about the kinetic properties of nanoparticles in the body? *Regulatory Toxicology and Pharmacology*, 49, 217-229.
- Handy, R. D., Henry, T. B., Scown, T. M., Johnston, B. D. & Tyler, C. R. 2008. Manufactured nanoparticles: their uptake and effects on fish—a mechanistic analysis. *Ecotoxicology*, 17, 396-409.
- Hillyer, J. F. & Albrecht, R. M. 2001. Gastrointestinal persorption and tissue distribution of differently sized colloidal gold nanoparticles. *Journal of pharmaceutical sciences*, 90, 1927-1936.
- Hisaoka, K. & Firlit, C. 1962. Ovarian cycle and egg production in the zebrafish, *Brachydanio rerio*. *Copeia*, 788-792.
- Hoffman, B. B., Chang, H., Dall'Aglio, E. & Reaven, G. M. 1986. Desensitization of adenosine receptor-mediated inhibition of lipolysis. The mechanism involves the development of enhanced cyclic adenosine monophosphate accumulation in tolerant adipocytes. *Journal of Clinical Investigation*, 78, 185.
- Hogstrand, C., Galvez, F. & Wood, C. M. 1996. Toxicity, silver accumulation and metallothionein induction in freshwater rainbow trout during exposure to different silver salts. *Environmental Toxicology and Chemistry*, 15, 1102-1108.
- Johnston, B., Scown, T., Moger, J., Cumberland, S., Baalousha, M., Linge, K., van Aerle, R., Jarvis, K., Lead, J. & Tyler, C. 2010. Bioavailability of Nanoscale Metal Oxides TiO₂, CeO₂, and ZnO to Fish. *Environmental Science & Technology*, 44, 1144-1151.
- Kaegi, R., Sinnet, B., Zuleeg, S., Hagendorfer, H., Mueller, E., Vonbank, R., Boller, M. & Burkhardt, M. 2010. Release of silver nanoparticles from outdoor facades. *Environmental Pollution*, 158, 2900-2905.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Karin, M., Slater, E. P. & Herschman, H. R. 1981. Regulation of metallothionein synthesis in HeLa cells by heavy metals and glucocorticoids. *Journal of cellular physiology*, 106, 63-74.
- Kashiwada, S. 2006. Distribution of nanoparticles in the see-through medaka (*Oryzias latipes*). *Environmental health perspectives*, 114, 1697.
- Kim, S. W., Kwak, J. I. & An, Y.-J. 2013. Multigenerational study of gold nanoparticles in *Caenorhabditis elegans*: Trans-generational effect of maternal exposure. *Environmental Science & Technology*.
- Klaine, S. J., Alvarez, P. J., Batley, G. E., Fernandes, T. F., Handy, R. D., Lyon, D. Y., Mahendra, S., McLaughlin, M. J. & Lead, J. R. 2008. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. *Environmental Toxicology and Chemistry*, 27, 1825-1851.
- Kothe, E. & Varma, A. 2012. *Bio-geo interactions in metal-contaminated soils*, Springer.
- Lanno, R. P., Hicks, B. & Hilton, J. W. 1987. Histological observations on intrahepatocytic copper-containing granules in rainbow trout reared on diets containing elevated levels of copper. *Aquatic Toxicology*, 10, 251-263.
- Lee, K. J., Browning, L. M., Nallathamby, P. D., Desai, T., Cherukuri, P. K. & Xu, X.-H. N. 2012. In vivo quantitative study of sized-dependent transport and toxicity of single silver nanoparticles using zebrafish embryos. *Chemical research in toxicology*, 25, 1029-1046.
- Lin, L. L. & Janz, D. M. 2006. Effects of binary mixtures of xenoestrogens on gonadal development and reproduction in zebrafish. *Aquatic Toxicology*, 80, 382-395.
- Luoma, S. N., Ho, Y. & Bryan, G. 1995. Fate, bioavailability and toxicity of silver in estuarine environments. *Marine pollution bulletin*, 31, 44-54.
- Luoma, S. N. & Nanotechnologies, P. o. E. 2008. *Silver nanotechnologies and the environment: Old problems or new challenges*, Project on Emerging Nanotechnologies of the Woodrow Wilson International Center for Scholars.
- McAuliffe, M. E. & Perry, M. J. 2007. Are nanoparticles potential male reproductive toxicants? A literature review. *Nanotoxicology*, 1, 204-210.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Mukaigasa, K., Nguyen, L. T., Li, L., Nakajima, H., Yamamoto, M. & Kobayashi, M. 2012. Genetic evidence of an evolutionarily conserved role for Nrf2 in the protection against oxidative stress. *Molecular and cellular biology*, 32, 4455-4461.
- Nowack, B., Ranville, J. F., Diamond, S., Gallego-Urrea, J. A., Metcalfe, C., Rose, J., Horne, N., Koelmans, A. A. & Klaine, S. J. 2012. Potential scenarios for nanomaterial release and subsequent alteration in the environment. *Environmental Toxicology and Chemistry*, 31, 50-59.
- Osborne, O. J., Johnston, B., Moger, J., Baalousha, M., Lead, J., Kudoh, T. & Tyler, C. 2012. Effects of particle size and coating on nanoscale Ag and TiO₂ exposure in zebrafish (*Danio rerio*) embryos. *Nanotoxicology*, 7, (8) 1315-1324.
- Paull, G. C., Van Look, K. J. W., Santos, E. M., Filby, A. L., Gray, D. M., Nash, J. P. & Tyler, C. R. 2008. Variability in measures of reproductive success in laboratory-kept colonies of zebrafish and implications for studies addressing population-level effects of environmental chemicals. *Aquatic Toxicology*, 87, 115-126.
- Ringwood, A. H., McCarthy, M., Bates, T. C. & Carroll, D. L. 2010. The effects of silver nanoparticles on oyster embryos. *Marine environmental research*, 69, S49-S51.
- Roh, J.-y., Sim, S. J., Yi, J., Park, K., Chung, K. H., Ryu, D.-y. & Choi, J. 2009. Ecotoxicity of silver nanoparticles on the soil nematode *Caenorhabditis elegans* using functional ecotoxicogenomics. *Environmental Science & Technology*, 43, 3933-3940.
- Schroeder, U., Sommerfeld, P., Ulrich, S. & Sabel, B. A. 1998. Nanoparticle technology for delivery of drugs across the blood-brain barrier. *Journal of pharmaceutical sciences*, 87, 1305-1307.
- Scown, T., Santos, E., Johnston, B., Gaiser, B., Baalousha, M., Mitov, S., Lead, J., Stone, V., Fernandes, T. & Jepson, M. 2010a. Effects of aqueous exposure to silver nanoparticles of different sizes in rainbow trout. *Toxicological Sciences*, 115, 521.
- Scown, T., van Aerle, R. & Tyler, C. 2010b. Review: Do engineered nanoparticles pose a significant threat to the aquatic environment? *Critical reviews in toxicology*, 40, 653-670.
- Shimizu, M., Tainaka, H., Oba, T., Mizuo, K., Umezawa, M. & Takeda, K. 2009. Maternal exposure to nanoparticulate titanium dioxide during the prenatal period alters gene expression related to brain development in the mouse. *Part Fibre Toxicol*, 6, 20.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Sibley, D. R. & Lefkowitz, R. J. 1985. Molecular mechanisms of receptor desensitization using the β -adrenergic receptor-coupled adenylate cyclase system as a model.
- Silva, G. A. 2007. Nanotechnology approaches for drug and small molecule delivery across the blood brain barrier. *Surgical Neurology*, 67, 113-116.
- Svecevicus, G. 2003. Avoidance response of rainbow trout *Oncorhynchus mykiss* to heavy metal model mixture after long-term exposure in early development. *Bulletin of environmental contamination and toxicology*, 71, 0226-0233.
- Takeda, K., Suzuki, K.-i., Ishihara, A., Kubo-Irie, M., Fujimoto, R., Tabata, M., Oshio, S., Nihei, Y., Ihara, T. & Sugamata, M. 2009. Nanoparticles transferred from pregnant mice to their offspring can damage the genital and cranial nerve systems. *Journal of Health Science*, 55, 95-102.
- Vandegheuchte, M. B. & Janssen, C. R. 2011. Epigenetics and its implications for ecotoxicology. *Ecotoxicology*, 20, 607-624.
- Wang, J., Zhou, G., Chen, C., Yu, H., Wang, T., Ma, Y., Jia, G., Gao, Y., Li, B. & Sun, J. 2007. Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. *Toxicology letters*, 168, 176-185.
- Wang, J., Zhu, X., Zhang, X., Zhao, Z., Liu, H., George, R., Wilson-Rawls, J., Chang, Y. & Chen, Y. 2011a. Disruption of zebrafish (*Danio rerio*) reproduction upon chronic exposure to TiO₂ nanoparticles. *Chemosphere*, 83, 461-467.
- Wang, J., Zhu, X., Zhang, X., Zhao, Z., Liu, H., George, R., Wilson-Rawls, J., Chang, Y. & Chen, Y. 2011b. Disruption of zebrafish (*Danio rerio*) reproduction upon chronic exposure to TiO₂ nanoparticles. *Chemosphere*, 83, 461-467.
- Wijnhoven, S. W. P., Peijnenburg, W. J. G. M., Herberts, C. A., Hagens, W. I., Oomen, A. G., Heugens, E. H. W., Roszek, B., Bisschops, J., Gosens, I. & Van De Meent, D. 2009. Nano-silver-a review of available data and knowledge gaps in human and environmental risk assessment. *Nanotoxicology*, 3, 109-138.
- Wood, C. M., Playle, R. C. & Hogstrand, C. 1999. Physiology and modeling of mechanisms of silver uptake and toxicity in fish. *Environmental Toxicology and Chemistry*, 18, 71-83.
- Zhu, X., Wang, J., Zhang, X., Chang, Y. & Chen, Y. 2009. The impact of ZnO nanoparticle aggregates on the embryonic development of zebrafish (*Danio rerio*). *Nanotechnology*, 20, 195103.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

Zhu, X., Wang, J., Zhang, X., Chang, Y. & Chen, Y. 2010. Trophic transfer of TiO₂ nanoparticles from daphnia to zebrafish in a simplified freshwater food chain. *Chemosphere*, 79, 928-933.

SUPPLEMENTARY INFORMATION

**Altered toxicological responsiveness in larval zebrafish after parental dietary exposure
to silver nanoparticles**

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Supplementary material

S1

Particle Characterization of AgNPCi and AgB in milli Q water

0.1 mg L⁻¹ and 1 mg L⁻¹ suspensions of AgNPCi and AgB respectively in milli q water. The nanoparticles in 10 mL of each suspension were deposited on Formvar/Carbon coated TEM-grids using 30,000 rpm ultracentrifugation (Beckman L-75) during 60 min. Thereafter the grids were rinsed by gently immersing in milli Q-water, and dried overnight. Micrographs were acquired at 30k, 75k, 300k and 500k magnification (5-10 micrographs at each magnification from each sample) at 80 keV accelerating voltage using a JEOL 1200EX TEM-instrument.

Particle Characterization of AgNPCi in embryo culture medium

0.1 mg L⁻¹ suspensions of AgNPCi in embryo media. The nanoparticles in 10 mL of each suspension were deposited on Formvar/Carbon coated TEM-grids using 30,000 rpm ultracentrifugation (Beckman L-75) during 60 min. Thereafter the grids were rinsed by gently immersing in milli Q-water, and dried overnight. Micrographs were acquired at 30k, 75k, 300k and 500k magnification (5-10 micrographs at each magnification from each sample) at 80 keV accelerating voltage using a JEOL 1200EX TEM-instrument.

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Table S1: Summary of AgNPCi and AgB particle characteristics

	AgNPCi (raw)	AgNPCi in milliQ water	AgNPCi in embryo medium	AgB (Ag Bulk) in milli Q water
Zeta Potential (mV)	-47.78±4.3	-40.21±9.3	-18.38 ± 1.6	-2.8 ± 0.6
Z average diameter (DLS)	20	32.4±2.6	92.25 ± 1.8	-
TEM (nm)	9.9 ± 3.1	36 ⁶⁷ ₁₇ Median value Upper quartile Lower quartile	52 ⁹³ ₂₂ Median value Upper quartile Lower quartile	137 ± 62.0

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Figure S1. Characterization of the AgNPCi in embryo culture water (EC) and AgNPCi in Milli-Q water (MQ). TEM of AgNPCi at 0.1 mg/l embryo culture medium x35 magnification (A) , and x300 magnification (B); x500 magnification (C). TEM of AgNPCi at 1 mg/l Milli-Q water x35 magnification (D), and x300 magnification (E); x500 magnification (F).

Figure S1

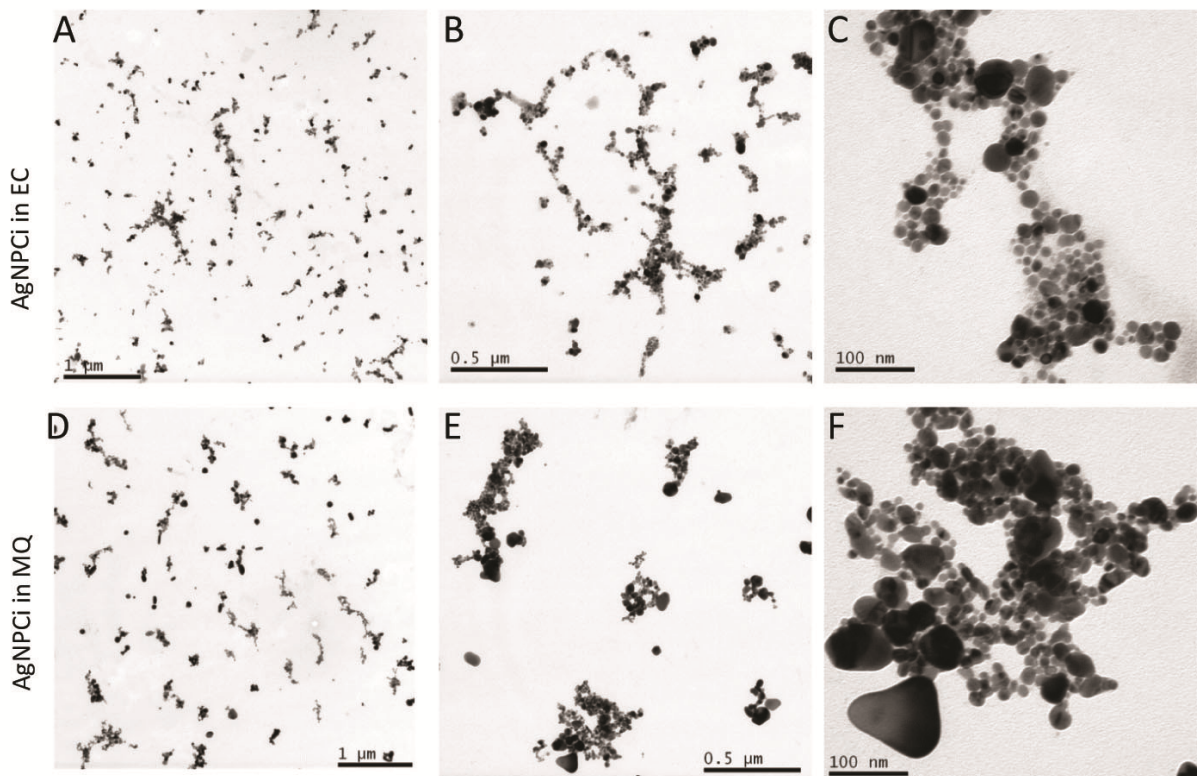
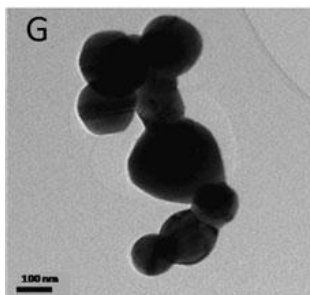


Figure S2. (G) TEM image of AgB taken from Scown et al., (2010)

Figure S2



CHAPTER 5

GENERAL DISCUSSION

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GENERAL DISCUSSION

This chapter presents a critical analysis of the studies conducted in this thesis, reviewing the key results and presenting the significance of the outcomes.

Some of the main questions being addressed in aquatic nanotoxicology are whether nano materials differ in their effects compared with bulk materials for silver nanomaterials, whether toxicity is a result of a dissolution of the silver ions, what the mechanistic pathways are for nanomaterials effects more broadly and whether exposure to nanomaterials have long term health effects, including on reproductive success. This addressed some of these questions and specifically the following objectives: (1) to establish the effects of particle size and coating of different sized Ag and TiO₂; (2) to identify some target tissues and the effect mechanism of AgNPs; (3) to establish whether Ag particles impact on the breeding of zebrafish and undergo maternal transfer with consequences for their offspring.

The following was established from this thesis work:

(1) Size plays an important role in toxicity of silver NPs and affects uptake into target organs (Chapter 2 and Chapter 4). In Chapter 2 evidence is provided that size plays a role in the degree of toxicity, and provides evidence also for a possible effect of the particle itself. Past research has shown how smaller particles sizes can result in a faster dissociation rate faster due to their larger surface area (Stebounova et al., 2011). In Chapter 4 ICP-MS revealed that Ag was only found in the liver and gonads as well as the embryos for the ciAgNP treatment only, demonstrating that the nanomaterials have different uptake dynamics in comparison to the bulk materials. Further concern that warrants further investigation with this evidence is the potential for bioaccumulation and entering food chains.

(2) The toxicity of AgNPs is largely derived from the dissolution of silver ions as demonstrated in Chapter 2 and Chapter 3. It has been widely discussed whether the main source of toxicity from AgNPs is through the dissociation of silver ions (Beer et al., 2012) and experiments were carried out also to test for this. A dissolution study on AgNPs and AgB particles established that up to 2% of silver ions can dissociate within 72hr in the embryo culture medium. An ionic AgNO₃ was run in parallel with the AgNP exposures to also test for

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silver ion effects. In Chapter 2 the morphological effects and lethality seen in the AgNPs and AgB were identical in nature to those also observed in the silver ion treatment suggesting that the toxicity of Ag particles could be explained by the dissociation of silver ions. In Chapter 3; AgNPCi, AgBCi and AgNO₃ all induced responses in the same target tissues, again supporting that the toxicity seen was mediated through silver ion availability. All this evidence supports the theory that the main source of AgNP toxicity is derived the silver ions which in nanotoxicology research is one of the main questions asked.

(3) In the natural aquatic environment, NPs will come into contact with organic matter such as humics and fulvic acids (Christian et al., 2008). We demonstrated (Chapter 2) that coating the AgNPs with fulvic acids and citrate (used in industry for stabilising the NPs) reduced toxicity, resulting in up to a 10 fold lower toxicity than for those uncoated particles (Chapter 2, Figure 1).

(4) AgNP target tissues included olfactory bulbs, lateral line neuromasts, ionocytes in the skin which are key sensory systems in fish. We determined that stress response genes *mt2*, *gstp* and *gstm1* were upregulated and genes *hmox1/fth1* were downregulated post AgNP exposure concluding how different genes respond to stimuli. Past research has shown how AgNPs can up/down regulate genes but this research established where in the body i.e which target tissues in the body of the organism these stress response genes are affected.

(5) Mechanisms of effects of AgNPs include via oxidative stress and are mediated via the Nrf2 pathway which was established in Chapter 3. Up until recently very few studies have looked at some of the mechanistic pathways involved in AgNP toxicity. By using an Nrf2 mutant fish analysis it showed that the nrf2 pathway was involved in the toxicological response to AgNPs-specifically for genes *mt2* and *gstp* (Chapter 3, Figure 5).

(6) The work presented also provides evidence for maternal transfer as Ag was detected in the embryos derived from the adults exposed to ciAgNP and transgenerational effects of AgNPs were observed (Chapter 4, Figure 3 and Figure 4). Past research has used other organisms such as *C.elegans* (Roh et al., 2009) to show fecundity effects for AgNP exposure. This research is one of the first in showing transgenerational effects from a dietary exposure route using zebrafish. *WISH* analysis on embryos from treated adults revealed upregulation

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in both treatments for genes *mt2* and *gstp* in for exposure to both NP and their bulk counterpart (Chapter 4, Figure 3). Challenging the embryos to silver materials for 24 hours from treated adults showed significant gene (*mt2*) desensitisation measured by *WISH* analysis (Chapter 4, Figure 4). The desensitisation to silver materials is hypothesised to be a result of DNA methylation as a consequence of the constant Ag exposure during embryogenesis/early life stage. Similar effects have been seen with exposure to heavy metals where sensitisation (avoidance preference) occurred in rainbow trout (*Oncorhynchus mykiss*) who were exposed from juvenile stage (Svecevicus, 2003). The transgenerational effects seen in Chapter 4 should be considered for further investigation as fundamentally it could change the way toxicology is measured and assess its implications.

The challenges and limitations of the experimental studies conducted in this thesis are also discussed and evaluated. A short discussion on the future prospects for nanotoxicology research is presented, with a foray into new promising methods that will aid scientific research in this area. Finally, a closing summary provides some overall concluding thoughts on nanotoxicology as it currently stands.

5.1 CHALLENGES AND LIMITATIONS OF THE WORK UNDERTAKEN

Whole mount in-situ hybridisation

Whole mount *in-situ* hybridisation (*WISH*) was the method applied to examine the tissue specific effects of Ag materials on stress response genes zebrafish embryos. In the preparation of the embryos for *WISH*, proteinase K is needed to soften the skin and enable penetration of the probe for embryos at 24hpf onwards as the skin becomes tougher (Le Guellec et al., 2004) and less permeable. Appropriate time periods and concentrations for proteinase K treatment for *WISH* were already known for life stages between 2-5 dpf; however, protocols had to be developed for the life periods between 5 dpf up to 12 dpf and for the different probes. This included testing a variation of temperatures and different concentrations of proteinase K along with measuring how long to leave the probe staining. Therefore optimising the right conditions took a considerable amount of time. The limitation of *WISH* is the limited sensitivity. Unlike qPCR; whereby a AgNP toxicological response

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detection of genes such as MT expression can go down to 0.16 µg/L in oyster embryos (Ringwood et al., 2010), *WISH* often requires higher concentrations.

ICP-MS analysis

With regard to determining the uptake of silver in target organs and the possibility of maternal transfer to offspring ICP-MS analysis were carried on gonads, liver and embryos that of adults fed AgNPs through diet. Preparation of samples uses acid digestion of tissues and embryos. In the past, protocols have stated how tissues have been digested but no literature stated how to digest embryos. Some length of time was spent to establish the adequate amount of nitric acid and hydrogen peroxide in combination with different temperatures to digest the embryos. A limitation of using ICP-MS is that it doesn't give you the measurement of NPs but rather the metal present in sample. As a result, in the dietary exposure it was not possible to determine exactly how much nano was present or distinguish between ions and NPs in the organism. Therefore the measurement is a combination of Ag from AgNPs and dissociation of silver ions from the AgNPs. However, considering the toxicity of Ag ion, it is likely that the majority of the Ag contained in the liver and gonad are still in NP form otherwise the cells in these tissues would not survive and significant effects on fish health would have been observed. This assumption is also supported by the fact that tissues from the fish fed with AgB did not show any detectable Ag level but their eggs still had effect of Ag exposure (elevated *mt2/gstp* and also desensitisation to Ag in embryonic exposure). These results suggest that the level of Ag ion required for change of these expression markers is at a low level, almost under detection by ICP-MS, and in turn suggest that majority of the Ag in the tissue from AgNP fed fish are still in AgNP form.

Testing of NPs at environmentally relevant concentrations

A major knowledge gap is determining environmentally relevant concentrations of NPs. It is hard to quantify NPs in the environment due to the limitations available technology to do so (Tiede et al., 2008). However; new technologies with better detection capabilities are

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working towards giving us a better idea of these concentrations in the environment to be able to test and foresee any possible effects. These new techniques include combining methods (ICP-MS, hydrodynamic chromatography (HDC) and field flow fraction (FFF)) which are already being developed (Farkas et al., 2011) to achieve single particle detection (Lorenz et al., 2012).

Therefore, at the start of conducting this thesis it was hard to test environmentally relevant concentrations. Nevertheless; some empirical data are now available, but it is still uncertain how much NP material will reach certain surface waters. Factors that will determine this include the rate and amount of NPs leaching from materials disposed on the environment facades may differ due to weather; water levels due to seasonal changes can affect the concentration of the pollutant. Therefore, sometimes predictive modelling estimates were used to model experimental doses accordingly and sometimes empirical data (where available) was applied (Chapter 3 and 4).

Stability and dispersion of the NPs

Dispersion is a major factor in establishing the toxicity of a particle, as it can change dramatically the bioavailability to an organism. It is known that different coatings can change toxicity of an NP. In the first study, NPs were used in their dry form and one could visibly see aggregates forming within your medium. Sonication in water baths were used to help prevent aggregation; but even then aggregation proved to be a problem. Coating the AgNPs with fulvic acid was done to mimic humics that NPs would come into contact within the environment. As the project progressed, better dispersion methods via sonication probes were adopted and better dispersion was achieved.

Additionally, it is known that the longevity of an experiment can in turn change the toxicity of a NP. This is crucial in measuring toxicity as some coated NPs show slower dissolution rates which in turn would show less toxicity. Therefore, in short term acute toxicity tests NPs could show minimal toxicity on organism, and not foresee the true long term toxicological effects when maximum dissolution is achieved. Hence it is fundamental to

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carry out dissolution studies in parallel to be able to assess how much silver ion dissociates within the medium and test NP toxicity.

5.2 FUTURE PROSPECT FOR NANOTOXICOLOGY RESEARCH

Nanotoxicology is still relatively new (Donaldson et al., 2004) and there are significant knowledge gaps and challenges. The high complexity and different structures of NPs makes them unpredictable and hard to predict potential hazard.

Various organisations e.g. International Organization for Standardisation (ISO) are developing guidelines and working towards a standardised approach for testing materials. This will minimise variation in NP toxicological data resulting from different lab approaches and via the adoption of different preparation procedures. These organisations are also suggesting testing different NPs (size, surface properties and charge) in different mediums to explore the biological activity and behaviour of these NPs. Until recently, NP characterisation was performed on either the dry material or in the solution in which it was produced, with no consideration for the nature of the material in the relevant biological medium for testing. Now, knowing that NPs in different mediums greatly changes the behaviour of the NP, there has been an initiative in recent reviews, to push for studies to carry out chemical characterisation in the medium tested as well in their original form (Johnston et al., 2013). In the second experimental chapter of this thesis (Chapter 3) citrate coated NPs were characterised in their original form as well as in the embryo culture medium tested. The data demonstrated the NPs formed aggregates in the embryo culture medium, in turn creating larger sized NPs. This further supports the above theory and in turn could decrease the toxicity of the NPs as a result of slower dissolution of silver ions due to smaller surface area. Characterisation of materials in the relevant exposure matrix will aid us in understanding how NPs behave in relevant environments and hopefully help us predict the toxicity of them.

Quantifying NPs within the exposed organism has been challenging so far, but new methodologies using isotope labelling looks promising and already studies have been done

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(Larner et al., 2012). Some of the benefits of using isotopically labelled NPs are being able to distinguish background metal concentration found in the environment such as Zn and new accumulated metals from the NP exposure. Additionally, by using this approach, environmentally relevant concentrations can be applied.

Surface-enhanced Raman Scattering is a non-invasive *in vivo* monitoring system and allows for single particle sensitivity by using Raman Scattering but enhances sensitivity by surface-sensitive technique; this uses molecules adsorbed onto the surface you are testing. This method has recently been done using AuNPs (40nm) to assess the distribution of NPs within various cells in organism such as the zebrafish (*Danio rerio*) (Wang et al., 2010). The study demonstrated that the NPs microinjected at the 1 cell stage can distribute into cells and organs of the developing embryo. This method proved to have better detection capabilities than CARS (used in Chapter 2) as it could detect single particles, and therefore, future nanotoxicology biodistribution studies using this new imaging technique will benefit enormously.

New nanotoxicology testing methods and assessing NPs risks are currently being evaluated (Shatkin and North, 2010, Arora et al., 2012). Recent reviews collectively suggest that more experiments should have a series of doses from low to high in the experiments. The results generated in these *in vivo* and *in vitro* studies will be used in conjunction with *in silico* studies to predict and establish nano safety. *In silico* toxicity testing uses computer simulation to predict chemical toxicity by using laboratory generated data, categorising physical properties and toxicity effects observed *in vitro* / *in vivo* studies. By mathematical modelling, they establish relationships between particles and the toxicity effects observed with potential to be able to predict hazard (Sayes and Ivanov, 2010). An example of the new mathematical methodologies for anticipating effect is through Quantitative Structure-Activity Relationship (QSAR) (Puzyn et al., 2009). Modelling strategies have shown predicted striking results on metal oxides by using nano QSAR equation. One study used *E.coli* cells to test cytotoxicity for metal oxides comparing with the predicted EC₅₀ (the effective concentration of a compound that brings about a 50% reduction in bacteria viability); ZnO EC₅₀ predicted concentration was 3.30 mol⁻¹ and ZnO EC₅₀ observed concentration was 3.45 mol⁻¹ (Puzyn et

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al., 2011), demonstrating how accurate some modelling studies can be. However, scientists have worded their concern as usually these models use very small data sets and the validity of them can be at times questionable. It is essential to use and combine *in vivo* as well as *in vitro* results to be able to develop this new type of modelling.

Another new type of modelling is called Quantitative Nanostructure-Activity Relationship (QNAR) (Fourches et al., 2010); the strategy is to be able to combine characterisation features with biological effects observed. QNAR hopes to be able to predict in a quick and efficient way the potential effect on newly commercially available NPs. Xia and colleagues proposed a biological surface absorption index (BSAI) to characterise interactions by quantifying the competitive adsorption of a set of small molecular probes onto the nanoparticles by simulating molecular interactions of the NP with amino acid residues of the proteins (Xia et al., 2010). This approach is mainly being used in developing nanomedicines to predict cellular uptake but could be used in risk assessment.

Another important step in progress of nanotoxicology is promoting data sharing (Clark et al., 2011). An open system which would have non-published as well as published data to aid scientists in their experiments, as often negative results are not published and it would be an opportunity to share difficulties within experimentation.

It was recently reviewed that within a 5 year time frame reliable *in vitro* and *in vivo* endpoints need to be established, more research into the mechanistic of how NPs interact with biology and more predictive models for biologically relevant NPs species (Winkler et al., 2012). In addition, this study established that high throughput technologies will also benefit the understanding of those mechanisms and be able to measure the effects observed. In the long-term 10 year plan, it hopes to use new modelling systems such as the ones mentioned above and QNTR which explores the interaction between structural properties and toxicity to be able to predict environmental effects by grouping biological profiles with certain NPs, as well as NP fingerprinting which will group together NPs with similar *in vivo* effects in a physiochemical, genomic and biological profile.

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5.3 SOME CONCLUDING THOUGHTS

The research conducted in this thesis has provided knowledge on a few metal/metal oxide NPs in terms of their biological effects on zebrafish from early life stages to adulthood. It has provided data on how size and coating play important roles in the magnitude of toxicity. We show that the toxicity from AgNPs is mainly due to the bioavailability of silver ions and this corresponds with the latest research. This thesis established AgNP target tissues, as well as determining some toxicological pathways, which can hopefully initiate and further explore different toxicological pathways as well as mechanisms of AgNPs. Through establishing target tissues it was concluded that sensory systems in fish are affected by AgNPs-this data is of concern for aquatic species specifically fish when exposed to AgNPs. Furthermore; it showed Ag uptake into target organs is highly dependable on size proving accumulation in organisms; therefore more research into bioaccumulation should be carried out in future to assess this. Additionally; it confirmed maternal transfer with transgenerational effects on embryos, confirming AgNPs require further investigation as a potential threat to fish, as well as an environmental issue.

I believe that in order for nanotoxicology research to move forward, more emphasis on the behaviour of these particles and mechanisms behind toxicity will be crucial. This will better enable us to predict the fate of these NPs also. Finally, the future key to nanotoxicology research is making sure the industry, policy makers and research collaborate with each other to create a network of people that each can put forth ideas and data. Progress of NP research and understanding implications for the environment requires collaboration with industry and policy makers. Future advancement for the comparison of studies and data between laboratories requires a framework that standardises tests to better compare toxicity tests and thresholds in a manner with greater precision. Programs like Horizon 2020 will help achieve this. It aims to combine research, knowledge and innovation to create guidelines, which will benefit nanotoxicology research. If there is a mutual correspondence between research and industry through a lifecycle assessment before/in the process of making NPs this will aid the process in predicting and preventing any irreversible damage to our ecosystems. In addition, funding bodies need to keep financing research to create

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laboratory data for computational predictive models. Ultimately, we need try to find a harmony between the nano industry growing and protecting the environment in a manageable way.

CHAPTER 6

REFERENCES

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REFERENCES

- Adams, L. K., Lyon, D. Y. & Alvarez, P. J. 2006. Comparative eco-toxicity of nanoscale TiO₂, SiO₂, and ZnO water suspensions. *Water research*, 40, 3527-3532.
- Aitken, R., Chaudhry, M., Boxall, A. & Hull, M. 2006. Manufacture and use of nanomaterials: current status in the UK and global trends. *Occupational medicine*, 56, 300-306.
- Allen, H. J., Impellitteri, C. A., Macke, D. A., Heckman, J. L., Poynton, H. C., Lazorchak, J. M., Govindaswamy, S., Roose, D. L. & Nadagouda, M. N. 2010. Effects from filtration, capping agents, and presence/absence of food on the toxicity of silver nanoparticles to *Daphnia magna*. *Environmental Toxicology and Chemistry*, 29, 2742-2750.
- Anselmann, R., Knapp, M. & Maurer, U. 1998. Pigment mit hoher Lichtdiffusion. DE Patent App. 1,998,142,134.
- Armelaio, L., Barreca, D., Bottaro, G., Gasparotto, A., Maccato, C., Maragno, C., Tondello, E., Štanger, U. L., Bergant, M. & Mahne, D. 2007. Photocatalytic and antibacterial activity of TiO₂ and Au/TiO₂ nanosystems. *Nanotechnology*, 18, 375709.
- Arora, S., Rajwade, J. M. & Paknikar, K. M. 2012. Nanotoxicology and *in vitro* studies: The need of the hour. *Toxicology and applied pharmacology*, 258, 151-165.
- AshaRani, P., Low Kah Mun, G., Hande, M. P. & Valiyaveetil, S. 2008a. Cytotoxicity and genotoxicity of silver nanoparticles in human cells. *ACS Nano*, 3, 279-290.
- Asharani, P., Wu, Y. L., Gong, Z. & Valiyaveetil, S. 2008b. Toxicity of silver nanoparticles in zebrafish models. *Nanotechnology*, 19, 255102.
- Awwad, A. M., Salem, N. M. & Abdeen, A. O. 2013. Green synthesis of silver nanoparticles using carob leaf extract and its antibacterial activity. *International Journal of Industrial Chemistry*, 4, 1-6.
- Baggs, R., Ferin, J. & Oberdörster, G. 1997. Regression of pulmonary lesions produced by inhaled titanium dioxide in rats. *Veterinary Pathology Online*, 34, 592-597.
- Baker, G. L., Gupta, A., Clark, M. L., Valenzuela, B. R., Staska, L. M., Harbo, S. J., Pierce, J. T. & Dill, J. A. 2008. Inhalation toxicity and lung toxicokinetics of C₆₀ fullerene nanoparticles and microparticles. *Toxicological Sciences*, 101, 122-131.
- Banfield, J. F. & Zhang, H. 2001. Nanoparticles in the environment. *Reviews in mineralogy and geochemistry*, 44, 1-58.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Bar-Ilan, O., Louis, K. M., Yang, S. P., Pedersen, J. A., Hamers, R. J., Peterson, R. E. & Heideman, W. 2012. Titanium dioxide nanoparticles produce phototoxicity in the developing zebrafish. *Nanotoxicology*, 6, 670-679.
- Battin, T. J., Kammer, F. v., Weilhartner, A., Ottofuelling, S. & Hofmann, T. 2009. Nanostructured TiO₂: transport behavior and effects on aquatic microbial communities under environmental conditions. *Environmental Science & Technology*, 43, 8098-8104.
- Becheri, A., Dürr, M., Nostro, P. L. & Baglioni, P. 2008. Synthesis and characterization of zinc oxide nanoparticles: application to textiles as UV-absorbers. *Journal of Nanoparticle Research*, 10, 679-689.
- Becker, L. & Bunch, T. 1997. Fullerenes, fulleranes and polycyclic aromatic hydrocarbons in the Allende meteorite. *Meteoritics & planetary science*, 32, 479-487.
- Beek, W. J., Wienk, M. M. & Janssen, R. A. 2004. Efficient hybrid solar cells from zinc oxide nanoparticles and a conjugated polymer. *Advanced Materials*, 16, 1009-1013.
- Beer, C., Foldbjerg, R., Hayashi, Y., Sutherland, D. S. & Autrup, H. 2012. Toxicity of silver nanoparticles—nanoparticle or silver ion? *Toxicology letters*, 208, 286-292.
- Benn, T., Cavanagh, B., Hristovski, K., Posner, J. D. & Westerhoff, P. 2010. The release of nanosilver from consumer products used in the home. *Journal of environmental quality*, 39, 1875-1882.
- Benn, T. M. & Westerhoff, P. 2008. Nanoparticle silver released into water from commercially available sock fabrics. *Environmental Science & Technology*, 42, 4133-4139.
- Bilberg, K., Hovgaard, M. B., Besenbacher, F. & Baatrup, E. 2011. In vivo toxicity of silver nanoparticles and silver ions in Zebrafish (*Danio rerio*). *Journal of toxicology*, 2012.
- Bilberg, K., Malte, H., Wang, T. & Baatrup, E. 2010. Silver nanoparticles and silver nitrate cause respiratory stress in Eurasian perch (*Perca fluviatilis*). *Aquatic Toxicology*, 96, 159-165.
- Birge, W. & Zuiderveen, J. 1995. The comparative toxicity of silver to aquatic biota. University of Wisconsin System
- Blaise, C. & Vasseur, P. 2005. Algal microplate toxicity test. *Small-scale freshwater toxicity investigations*. Springer.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Blake, D. M., Maness, P.-C., Huang, Z., Wolfrum, E. J., Huang, J. & Jacoby, W. A. 1999. Application of the photocatalytic chemistry of titanium dioxide to disinfection and the killing of cancer cells. *Separation and Purification Methods*, 28, 1-50.
- Blaser, S. A., Scheringer, M., MacLeod, M. & Hungerbühler, K. 2008. Estimation of cumulative aquatic exposure and risk due to silver: Contribution of nano-functionalized plastics and textiles. *Science of the Total Environment*, 390, 396-409.
- Boisson, F., Hartl, M. G., Fowler, S. W. & Amiard-Triquet, C. 1998. Influence of chronic exposure to silver and mercury in the field on the bioaccumulation potential of the bivalve *Macoma balthica*. *Marine environmental research*, 45, 325-340.
- Boutorine, A. S., Takasugi, M., Hélène, C., Tokuyama, H., Isobe, H. & Nakamura, E. 1995. Fullerene–Oligonucleotide Conjugates: Photoinduced Sequence-Specific DNA Cleavage. *Angewandte Chemie International Edition in English*, 33, 2462-2465.
- Brown, C. L., Parchaso, F., Thompson, J. K. & Luoma, S. N. 2003. Assessing toxicant effects in a complex estuary: A case study of effects of silver on reproduction in the bivalve, *Potamocorbula amurensis*, in San Francisco Bay. *Human and Ecological Risk Assessment*, 9, 95-119.
- Brown, D. M., Wilson, M. R., MacNee, W., Stone, V. & Donaldson, K. 2001. Size-dependent proinflammatory effects of ultrafine polystyrene particles: a role for surface area and oxidative stress in the enhanced activity of ultrafines. *Toxicology and applied pharmacology*, 175, 191-199.
- Bucher, J., Masten, S., Moudgil, B., Powers, K., Roberts, S. & Walker, N. 2004. Developing experimental approaches for the evaluation of toxicological interactions of nanoscale materials. Final workshop report, 2004. Later published in 2007: *Environmental Health Perspectives*, 115 (11): 1654-1659
- Buzea, C., Pacheco, I. I. & Robbie, K. 2007. Nanomaterials and nanoparticles: sources and toxicity. *Biointerphases*, 2, MR17-MR71.
- Bystrzejewska-Piotrowska, G., Golimowski, J. & Urban, P. L. 2009. Nanoparticles: their potential toxicity, waste and environmental management. *Waste Management*, 29, 2587-2595.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Cain, D. & Luoma, S. 1985. Copper and silver accumulation in transplanted and resident clams (*Macoma balthica*) in South San Francisco Bay. *Marine environmental research*, 15, 115-135.
- Carlson, C., Hussain, S., Schrand, A., K. Braydich-Stolle, L., Hess, K., Jones, R. & Schlager, J. 2008. Unique cellular interaction of silver nanoparticles: size-dependent generation of reactive oxygen species. *The Journal of Physical Chemistry B*, 112, 13608-13619.
- Chaudhry, Q., Boxall, A., Aitken, R. & Hull, M. 2005. A scoping study into the manufacture and use of nanomaterials in the UK. *Central Science Laboratory, Sand Hutton, York*.
- Chaudhry, Q., Scotter, M., Blackburn, J., Ross, B., Boxall, A., Castle, L., Aitken, R. & Watkins, R. 2008. Applications and implications of nanotechnologies for the food sector. *Food additives and contaminants*, 25, 241-258.
- Chen, F., Yang, X., Xu, F., Wu, Q. & Zhang, Y. 2009. Correlation of photocatalytic bactericidal effect and organic matter degradation of TiO₂ part I: observation of phenomena. *Environmental Science & Technology*, 43, 1180-1184.
- Chen, J., Dong, X., Xin, Y. & Zhao, M. 2011a. Effects of titanium dioxide nano-particles on growth and some histological parameters of zebrafish (*Danio rerio*) after a long-term exposure. *Aquatic Toxicology*, 101, 493-499.
- Chen, X., Liu, L., Peter, Y. Y. & Mao, S. S. 2011b. Increasing solar absorption for photocatalysis with black hydrogenated titanium dioxide nanocrystals. *Science*, 331, 746-750.
- Chen, W., John, J., Lin, C., Lin, H., Wu, S. & Chang, C. 2004. Expression of metallothionein gene during embryonic and early larval development in zebrafish. *Aquatic Toxicology*, 69, 215-227.
- Chinnapongse, S. L., MacCuspie, R. I. & Hackley, V. A. 2011. Persistence of singly dispersed silver nanoparticles in natural freshwaters, synthetic seawater, and simulated estuarine waters. *Science of The Total Environment*, 409, 2443-2450.
- Choi, H. S., Liu, W., Misra, P., Tanaka, E., Zimmer, J. P., Ipe, B. I., Bawendi, M. G. & Frangioni, J. V. 2007. Renal clearance of quantum dots. *Nature Biotechnology*, 25, 1165-1170.
- Choi, O. & Hu, Z. 2008. Size dependent and reactive oxygen species related nanosilver toxicity to nitrifying bacteria. *Environmental Science & Technology*, 42, 4583-4588.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Christian, P., Von der Kammer, F., Baalousha, M. & Hofmann, T. 2008. Nanoparticles: structure, properties, preparation and behaviour in environmental media. *Ecotoxicology*, 17, 326-343.
- Clark, K. A., White, R. H. & Silbergeld, E. K. 2011. Predictive models for nanotoxicology: current challenges and future opportunities. *Regulatory Toxicology and Pharmacology*, 59, 361-363.
- Coelho, S. C., Rocha, S., Carmo Pereira, M., Coelho, M. A. & Juzenas, P. 2013 Functionalized gold nanoparticles for drug delivery. *In: Bioengineering (ENBENG), IEEE 3rd Portuguese Meeting IEEE*, 1-4.
- Colvin, V. L. 2003. The potential environmental impact of engineered nanomaterials. *Nature Biotechnology*, 21, 1166-1170.
- Connor, E. E., Mwamuka, J., Gole, A., Murphy, C. J. & Wyatt, M. D. 2005. Gold nanoparticles are taken up by human cells but do not cause acute cytotoxicity. *Small*, 1, 325-327.
- Covert, D. S., Kapustin, V. N., Quinn, P. K. & Bates, T. S. 1992. New particle formation in the marine boundary layer. *Journal of Geophysical Research: Atmospheres (1984–2012)*, 97, 20581-20589.
- Daniel, M.-C. & Astruc, D. 2004. Gold nanoparticles: assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology. *Chemical reviews*, 104, 293-346.
- DEFRA 2005. Characterising the potential risks posed by engineered nanoparticles: A first UK government research report (*London: Department for Environment, Food and Rural Affairs (DEFRA)*).
- Donaldson, K., Li, X. & MacNee, W. 1998. Ultrafine (nanometre) particle mediated lung injury. *Journal of Aerosol Science*, 29, 553-560.
- Donaldson, K. & Stone, V. 2003. Current hypotheses on the mechanisms of toxicity of ultrafine particles. *Ann Ist Super Sanita* 39 (3): 405–410.
- Donaldson, K., Stone, V., Clouter, A., Renwick, L. & MacNee, W. 2001. Ultrafine particles. *Occupational and environmental medicine*, 58, 211-216.
- Donaldson, K., Stone, V., Tran, C., Kreyling, W. & Borm, P. J. 2004. Nanotoxicology. *Occupational and environmental medicine*, 61, 727-728.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Dresselhaus, M. S., Dresselhaus, G. & Eklund, P. C. 1996. *Science of fullerenes and carbon nanotubes: their properties and applications*, Academic Press.
- Drexler, K. E. 1981. Molecular engineering: An approach to the development of general capabilities for molecular manipulation. *Proceedings of the National Academy of Sciences*, 78, 5275-5278.
- Eom, H.-J. & Choi, J. 2010. p38 MAPK activation, DNA damage, cell cycle arrest and apoptosis as mechanisms of toxicity of silver nanoparticles in Jurkat T cells. *Environmental Science & Technology*, 44, 8337-8342.
- Fabrega, J., Luoma, S. N., Tyler, C. R., Galloway, T. S. & Lead, J. R. 2011a. Silver nanoparticles: Behaviour and effects in the aquatic environment. *Environment International*, 37, 517-531.
- Fabrega, J., Zhang, R., Renshaw, J. C., Liu, W.-T. & Lead, J. R. 2011b. Impact of silver nanoparticles on natural marine biofilm bacteria. *Chemosphere*, 85, 961-966.
- Farkas, J., Peter, H., Christian, P., Gallego Urrea, J. A., Hassellöv, M., Tuoriniemi, J., Gustafsson, S., Olsson, E., Hylland, K. & Thomas, K. V. 2011. Characterization of the effluent from a nanosilver producing washing machine. *Environment International*, 37, 1057-1062.
- Federici, G., Shaw, B. & Handy, R. 2007. Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): Gill injury, oxidative stress, and other physiological effects. *Aquatic Toxicology*, 84, 415-430.
- Ferin, J., Oberdorster, G. & Penney, D. 1992. Pulmonary retention of ultrafine and fine particles in rats. *Am J Respir Cell Mol Biol*, 6, 535-542.
- Fischer, H. C. & Chan, W. C. 2007. Nanotoxicity: the growing need for *in vivo* study. *Current opinion in Biotechnology*, 18, 565-571.
- Foley, S., Curtis, A. D., Hirsch, A., Brettreich, M., Pelegrin, A., Seta, P. & Larroque, C. 2002. Interaction of a water soluble fullerene derivative with reactive oxygen species and model enzymatic systems. *Fullerenes, Nanotubes and Carbon Nanostructures*, 10, 49-67.
- Fourches, D., Pu, D., Tassa, C., Weissleder, R., Shaw, S. Y., Mumper, R. J. & Tropsha, A. 2010. Quantitative Nanostructure– Activity Relationship Modeling. *ACS Nano*, 4, 5703-5712.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Gao, X., Yang, L., Petros, J. A., Marshall, F. F., Simons, J. W. & Nie, S. 2005. *In vivo* molecular and cellular imaging with quantum dots. *Current opinion in Biotechnology*, 16, 63-72.
- Geffroy, B., Ladhar, C., Cambier, S., Treguer-Delapierre, M., Brèthes, D. & Bourdineaud, J.-P. 2012. Impact of dietary gold nanoparticles in zebrafish at very low contamination pressure: The role of size, concentration and exposure time. *Nanotoxicology*, 6, 144-160.
- Gilaki, M. 2010. Biosynthesis of silver nanoparticles using plant extracts. *J. Biol. Sci*, 10, 465-467.
- Gislason, S., Hassenkam, T., Nedel, S., Bovet, N., Eiriksdottir, E., Alfredsson, H., Hem, C. P., Balogh, Z. I., Dideriksen, K. & Oskarsson, N. 2011. Characterization of Eyjafjallajökull volcanic ash particles and a protocol for rapid risk assessment. *Proceedings of the National Academy of Sciences*, 108, 7307-7312.
- Goldstein, A., Echer, C. & Alivisatos, A. 1992. Melting in semiconductor nanocrystals. *Science*, 256, 1425-1427.
- Gottschalk, F., Scholz, R. W. & Nowack, B. 2010a. Probabilistic material flow modeling for assessing the environmental exposure to compounds: Methodology and an application to engineered nano-TiO₂ particles. *Environmental Modelling & Software*, 25, 320-332.
- Gottschalk, F., Sonderer, T., Scholz, R. W. & Nowack, B. 2009. Modeled environmental concentrations of engineered nanomaterials (TiO₂, ZnO, Ag, CNT, fullerenes) for different regions. *Environmental Science & Technology*, 43, 9216-9222.
- Gottschalk, F., Sonderer, T., Scholz, R. W. & Nowack, B. 2010b. Possibilities and limitations of modeling environmental exposure to engineered nanomaterials by probabilistic material flow analysis. *Environmental Toxicology and Chemistry*, 29, 1036-1048.
- Gottschalk, F., Sun, T. & Nowack, B. 2013. Environmental concentrations of engineered nanomaterials: review of modeling and analytical studies. *Environmental Pollution*, 181, 287-300.
- Griffitt, R. J., Luo, J., Gao, J., Bonzongo, J. C. & Barber, D. S. 2008. Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. *Environmental Toxicology and Chemistry*, 27, 1972-1978.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Gulson, B. & Wong, H. 2006. Stable isotopic tracing—A way forward for nanotechnology. *Environmental health perspectives*, 114, 1486.
- Gurr, J.-R., Wang, A. S., Chen, C.-H. & Jan, K.-Y. 2005. Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells. *Toxicology*, 213, 66-73.
- Haefeli, C., Franklin, C. & Hardy, K. E. 1984. Plasmid-determined silver resistance in *Pseudomonas stutzeri* isolated from a silver mine. *Journal of bacteriology*, 158, 389-392.
- Hagenaars, A., Vergauwen, L., Benoot, D., Laukens, K. & Knapen, D. 2013. Mechanistic toxicity study of perfluorooctanoic acid in zebrafish suggests mitochondrial dysfunction to play a key role in PFOA toxicity. *Chemosphere*.
- Handy, R. & Eddy, F. 1991. Effects of inorganic cations on Na⁺ adsorption to the gill and body surface of rainbow trout, *Oncorhynchus mykiss*, in dilute solutions. *Canadian Journal of Fisheries and Aquatic Sciences*, 48, 1829-1837.
- Handy, R. D., Cornelis, G., Fernandes, T., Tsyusko, O., Decho, A., Sabo-Attwood, T., Metcalfe, C., Steevens, J. A., Klaine, S. J. & Koelmans, A. A. 2012a. Ecotoxicity test methods for engineered nanomaterials: practical experiences and recommendations from the bench. *Environmental Toxicology and Chemistry*, 31, 15-31.
- Handy, R. D., Henry, T. B., Scown, T. M., Johnston, B. D. & Tyler, C. R. 2008. Manufactured nanoparticles: their uptake and effects on fish—a mechanistic analysis. *Ecotoxicology*, 17, 396-409.
- Handy, R. D., van den Brink, N., Chappell, M., Mühlhling, M., Behra, R., Dušinská, M., Simpson, P., Ahtiainen, J., Jha, A. N. & Seiter, J. 2012b. Practical considerations for conducting ecotoxicity test methods with manufactured nanomaterials: what have we learnt so far? *Ecotoxicology*, 21, 933-972.
- Hartmann, N., Von der Kammer, F., Hofmann, T., Baalousha, M., Ottofuelling, S. & Baun, A. 2010. Algal testing of titanium dioxide nanoparticles—testing considerations, inhibitory effects and modification of cadmium bioavailability. *Toxicology*, 269, 190-197.
- Hatchett, D. W. & White, H. S. 1996. Electrochemistry of sulfur adlayers on the low-index faces of silver. *The Journal of Physical Chemistry*, 100, 9854-9859.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- He, J.-H., Wan, Y.-Q. & Xu, L. 2007. Nano-effects, quantum-like properties in electrospun nanofibers. *Chaos, Solitons & Fractals*, 33, 26-37.
- He, L., Liu, Y., Mustapha, A. & Lin, M. 2011. Antifungal activity of zinc oxide nanoparticles against *Botrytis cinerea* and *Penicillium expansum*. *Microbiological Research*, 166, 207-215.
- Heinrich, U., Fuhst, R., Rittinghausen, S., Creutzenberg, O., Bellmann, B., Koch, W. & Levsen, K. 1995. Chronic inhalation exposure of Wistar rats and two different strains of mice to diesel engine exhaust, carbon black, and titanium dioxide. *Inhalation Toxicology*, 7, 533-556.
- Hogstrand, C., Galvez, F. & Wood, C. M. 1996. Toxicity, silver accumulation and metallothionein induction in freshwater rainbow trout during exposure to different silver salts. *Environmental Toxicology and Chemistry*, 15, 1102-1108.
- Hogstrand, C. & Wood, C. M. 2009. Toward a better understanding of the bioavailability, physiology, and toxicity of silver in fish: Implications for water quality criteria. *Environmental Toxicology and Chemistry*, 17, 547-561.
- Hong, R., Han, G., Fernández, J. M., Kim, B.-j., Forbes, N. S. & Rotello, V. M. 2006. Glutathione-mediated delivery and release using monolayer protected nanoparticle carriers. *Journal of the American Chemical Society*, 128, 1078-1079.
- Horvath, J., Birringer, R. & Gleiter, H. 1987. Diffusion in nanocrystalline material. *Solid state communications*, 62, 319-322.
- Hullmann, A. 2007. Measuring and assessing the development of nanotechnology. *Scientometrics*, 70, 739-758.
- Husar, R. B., Tratt, D., Schichtel, B. A., Falke, S., Li, F., Jaffe, D., Gasso, S., Gill, T., Laulainen, N. S. & Lu, F. 2001. Asian dust events of April 1998. *Journal of Geophysical Research: Atmospheres (1984–2012)*, 106, 18317-18330.
- Hwang, E. T., Lee, J. H., Chae, Y. J., Kim, Y. S., Kim, B. C., Sang, B. I. & Gu, M. B. 2008. Analysis of the Toxic Mode of Action of Silver Nanoparticles Using Stress-Specific Bioluminescent Bacteria. *Small*, 4, 746-750.
- Hyung, H., Fortner, J. D., Hughes, J. B. & Kim, J.-H. 2007. Natural organic matter stabilizes carbon nanotubes in the aqueous phase. *Environmental Science & Technology*, 41, 179-184.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Jain, P. & Pradeep, T. 2005. Potential of silver nanoparticle-coated polyurethane foam as an antibacterial water filter. *Biotechnology and bioengineering*, 90, 59-63.
- Jenne, E. A. & Luoma, S. N. 1977. Forms of trace elements in soils, sediments, and associated waters: an overview of their determination and biological availability.
- Jiang, J., Oberdörster, G. & Biswas, P. 2009. Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. *Journal of Nanoparticle Research*, 11, 77-89.
- Johnston, B., Scown, T., Moger, J., Cumberland, S., Baalousha, M., Linge, K., van Aerle, R., Jarvis, K., Lead, J. & Tyler, C. 2010. Bioavailability of Nanoscale Metal Oxides TiO₂, CeO₂, and ZnO to Fish. *Environmental Science & Technology*, 44, 1144-1151.
- Johnston, H., Pojana, G., Zuin, S., Jacobsen, N. R., Møller, P., Loft, S., Semmler-Behnke, M., McGuinness, C., Balharry, D. & Marcomini, A. 2013. Engineered nanomaterial risk. Lessons learnt from completed nanotoxicology studies: potential solutions to current and future challenges. *Critical reviews in toxicology*, 43, 1-20.
- Judy, J. D., Unrine, J. M. & Bertsch, P. M. 2010. Evidence for biomagnification of gold nanoparticles within a terrestrial food chain. *Environmental Science & Technology*, 45, 776-781.
- Kam, N. W. S. & Dai, H. 2005. Carbon nanotubes as intracellular protein transporters: generality and biological functionality. *Journal of the American Chemical Society*, 127, 6021-6026.
- Kam, N. W. S., Liu, Z. & Dai, H. 2006. Carbon nanotubes as intracellular transporters for proteins and DNA: an investigation of the uptake mechanism and pathway. *Angewandte Chemie*, 118, 591-595.
- Karlsson, H. L., Cronholm, P., Gustafsson, J. & Möller, L. 2008. Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes. *Chemical research in toxicology*, 21, 1726-1732.
- Kawahara, T., Konishi, Y., Tada, H., Tohge, N., Nishii, J. & Ito, S. 2002. A Patterned TiO₂ (Anatase)/TiO₂ (Rutile) Bilayer-Type Photocatalyst: Effect of the Anatase/Rutile Junction on the Photocatalytic Activity. *Angewandte Chemie*, 114, 2935-2937.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Kelly, K. L., Coronado, E., Zhao, L. L. & Schatz, G. C. 2003. The optical properties of metal nanoparticles: the influence of size, shape, and dielectric environment. *The Journal of Physical Chemistry B*, 107, 668-677.
- Ketzel, M., Wåhlin, P., Berkowicz, R. & Palmgren, F. 2003. Particle and trace gas emission factors under urban driving conditions in Copenhagen based on street and roof-level observations. *Atmospheric Environment*, 37, 2735-2749.
- Kim, K.-J., Sung, W. S., Suh, B. K., Moon, S.-K., Choi, J.-S., Kim, J. G. & Lee, D. G. 2009. Antifungal activity and mode of action of silver nano-particles on *Candida albicans*. *Biometals*, 22, 235-242.
- Kim, K.-T., Truong, L., Wehmas, L. & Tanguay, R. L. 2013. Silver nanoparticle toxicity in the embryonic zebrafish is governed by particle dispersion and ionic environment. *Nanotechnology*, 24, 115101.
- Kim, S. C., Kim, D. W., Shim, Y. H., Bang, J. S., Oh, H. S., Kim, S. W. & Seo, M. H. 2001. In vivo evaluation of polymeric micellar paclitaxel formulation: toxicity and efficacy. *Journal of controlled release*, 72, 191-202.
- Kimbrell, G. A. 2009. Governance of nanotechnology and nanomaterials: principles, regulation, and renegotiating the social contract. *The Journal of Law, Medicine & Ethics*, 37, 706-723.
- Kittelson, D. B., Watts, W. & Johnson, J. 2004. Nanoparticle emissions on Minnesota highways. *Atmospheric Environment*, 38, 9-19.
- Klaine, S. J., Alvarez, P. J., Batley, G. E., Fernandes, T. F., Handy, R. D., Lyon, D. Y., Mahendra, S., McLaughlin, M. J. & Lead, J. R. 2008. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. *Environmental Toxicology and Chemistry*, 27, 1825-1851.
- Krusic, P., Wasserman, E., Keizer, P., Morton, J. & Preston, K. 1991. Radical reactions of C₆₀. *Science*, 254, 1183-1185.
- Krut'akov, Y. A., Kudrinskiy, A. A., Olenin, A. Y. & Lisichkin, G. V. 2008. Synthesis and properties of silver nanoparticles: advances and prospects. *Russian Chemical Reviews*, 77, 233-257.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Kuhlbusch, T. & Crutzen, P. 1995. Toward a global estimate of black carbon in residues of vegetation fires representing a sink of atmospheric CO₂ and a source of O₂. *Global Biogeochemical Cycles*, 9, 491-501.
- Kulthong, K., Srisung, S., Boonpavanitchakul, K., Kangwansupamonkon, W. & Maniratanachote, R. 2010. Determination of silver nanoparticle release from antibacterial fabrics into artificial sweat. *Particle and Fibre Toxicology*, 7:8
- Lacerda, L., Soundararajan, A., Singh, R., Pastorin, G., Al-Jamal, K. T., Turton, J., Frederik, P., Herrero, M. A., Li, S. & Bao, A. 2008. Dynamic imaging of functionalized multi-walled carbon nanotube systemic circulation and urinary excretion. *Advanced Materials*, 20, 225-230.
- Larner, F., Dogra, Y., Dybowska, A., Fabrega, J., Stolpe, B. r., Bridgestock, L. J., Goodhead, R., Weiss, D. J., Moger, J. & Lead, J. R. 2012. Tracing Bioavailability of ZnO Nanoparticles Using Stable Isotope Labeling. *Environmental Science & Technology*, 46, 12137-12145.
- Le Guellec, D., Morvan-Dubois, G. & Sire, J.-Y. 2004. Skin development in bony fish with particular emphasis on collagen deposition in the dermis of the zebrafish (*Danio rerio*). *International Journal of Developmental Biology*, 48, 217-232.
- Lead, J. R. & Wilkinson, K. J. 2006. Aquatic colloids and nanoparticles: current knowledge and future trends. *Environmental Chemistry*, 3, 159-171.
- Lee, K. J., Nallathamby, P. D., Browning, L. M., Osgood, C. J. & Xu, X.-H. N. 2007. In Vivo Imaging of Transport and Biocompatibility of Single Silver Nanoparticles in Early Development of Zebrafish Embryos. *ACS Nano*, 1, 133-143.
- Li, N., Sioutas, C., Cho, A., Schmitz, D., Misra, C., Sempf, J., Wang, M., Oberley, T., Froines, J. & Nel, A. 2003. Ultrafine particulate pollutants induce oxidative stress and mitochondrial damage. *Environmental health perspectives*, 111, 455.
- Liau, S., Read, D., Pugh, W., Furr, J. & Russell, A. 1997. Interaction of silver nitrate with readily identifiable groups: relationship to the antibacterial action of silver ions. *Letters in Applied Microbiology*, 25, 279-283.
- Liu, Y., He, L., Mustapha, A., Li, H., Hu, Z. & Lin, M. 2009. Antibacterial activities of zinc oxide nanoparticles against *Escherichia coli* O157: H7. *Journal of Applied Microbiology*, 107, 1193-1201.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Lorenz, C., Windler, L., von Goetz, N., Lehmann, R., Schuppler, M., Hungerbühler, K., Heuberger, M. & Nowack, B. 2012. Characterization of silver release from commercially available functional (nano) textiles. *Chemosphere*, 89, 817-824.
- Lovern, S. B., Strickler, J. R. & Klaper, R. 2007. Behavioral and physiological changes in *Daphnia magna* when exposed to nanoparticle suspensions (titanium dioxide, nano-C₆₀, and C₆₀H_xC₇₀H_x). *Environ. Sci. Technol*, 41, 4465-4470.
- Lu, Z.-X., Zhou, L., Zhang, Z.-L., Shi, W.-L., Xie, Z.-X., Xie, H.-Y., Pang, D.-W. & Shen, P. 2003. Cell damage induced by photocatalysis of TiO₂ thin films. *Langmuir*, 19, 8765-8768.
- Luoma, S. N. & Nanotechnologies, P. o. E. 2008. *Silver nanotechnologies and the environment: Old problems or new challenges*, Project on Emerging Nanotechnologies of the Woodrow Wilson International Center for Scholars.
- Mahshid, S., Askari, M. & Ghamsari, M. S. 2007. Synthesis of TiO₂ nanoparticles by hydrolysis and peptization of titanium isopropoxide solution. *Journal of Materials Processing Technology*, 189, 296-300.
- Mandal, D., Bolander, M. E., Mukhopadhyay, D., Sarkar, G. & Mukherjee, P. 2006. The use of microorganisms for the formation of metal nanoparticles and their application. *Applied Microbiology and Biotechnology*, 69, 485-492.
- Maness, P.-C., Smolinski, S., Blake, D. M., Huang, Z., Wolfrum, E. J. & Jacoby, W. A. 1999. Bactericidal activity of photocatalytic TiO₂ reaction: toward an understanding of its killing mechanism. *Applied and environmental microbiology*, 65, 4094-4098.
- Mantoura, R., Dickson, A. & Riley, J. 1978. The complexation of metals with humic materials in natural waters. *Estuarine and Coastal Marine Science*, 6, 387-408.
- Marambio-Jones, C. & Hoek, E. M. V. 2010. A review of the antibacterial effects of silver nanomaterials and potential implications for human health and the environment. *Journal of Nanoparticle Research*, 12, 1531-1551.
- Marquis, B. J., Love, S. A., Braun, K. L. & Haynes, C. L. 2009. Analytical methods to assess nanoparticle toxicity. *Analyst*, 134, 425-439.
- Maya, A.-S.-C., Claude, R. & Emilien, P. 2013. Tissue distribution and kinetics of dissolved and nanoparticulate silver in Iceland scallop (*Chlamys islandica*). *Marine environmental research*.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Menard, A., Drobne, D. & Jemec, A. 2011. Ecotoxicity of nanosized TiO₂. Review of in vivo data. *Environmental Pollution*, 159, 677-684.
- Meyer, J. N., Lord, C. A., Yang, X. Y., Turner, E. A., Badireddy, A. R., Marinakos, S. M., Chilkoti, A., Wiesner, M. R. & Auffan, M. 2010. Intracellular uptake and associated toxicity of silver nanoparticles in *Caenorhabditis elegans*. *Aquatic Toxicology*, 100, 140-150.
- Meyyappan, M. 2004. *Carbon nanotubes: science and applications*, CRC press.
- Miao, A.-J., Schwehr, K. A., Xu, C., Zhang, S.-J., Luo, Z., Quigg, A. & Santschi, P. H. 2009. The algal toxicity of silver engineered nanoparticles and detoxification by exopolymeric substances. *Environmental Pollution*, 157, 3034-3041.
- Michalet, X., Pinaud, F., Bentolila, L., Tsay, J., Doose, S., Li, J., Sundaresan, G., Wu, A., Gambhir, S. & Weiss, S. 2005. Quantum dots for live cells, in vivo imaging, and diagnostics. *Science*, 307, 538-544.
- Miller, R. J., Bennett, S., Keller, A. A., Pease, S. & Lenihan, H. S. 2012. TiO₂ nanoparticles are phototoxic to marine phytoplankton. *PloS one*, 7, e30321.
- Miura, N. & Shinohara, Y. 2009. Cytotoxic effect and apoptosis induction by silver nanoparticles in HeLa cells. *Biochemical and biophysical research communications*, 390, 733-737.
- Mu, L. & Sprando, R. L. 2010. Application of nanotechnology in cosmetics. *Pharmaceutical research*, 27, 1746-1749.
- Mueller, N. C. & Nowack, B. 2008. Exposure Modeling of Engineered Nanoparticles in the Environment. *Environmental Science & Technology*, 42, 4447-4453.
- Mueller, R., Jossen, R., Pratsinis, S. E., Watson, M. & Akhtar, M. K. 2004. Zirconia nanoparticles made in spray flames at high production rates. *Journal of the American Ceramic Society*, 87, 197-202.
- Nakamura, E., Tokuyama, H., Yamago, S., Shiraki, T. & Sugiura, Y. 1996. Biological activity of water-soluble fullerenes. Structural dependence of DNA cleavage, cytotoxicity, and enzyme inhibitory activities including HIV-protease inhibition. *Bulletin of the Chemical Society of Japan*, 69, 2143-2151.
- Navarro, E., Baun, A., Behra, R., Hartmann, N. B., Filser, J., Miao, A.-J., Quigg, A., Santschi, P. H. & Sigg, L. 2008a. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. *Ecotoxicology*, 17, 372-386.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Navarro, E., Piccapietra, F., Wagner, B., Marconi, F., Kaegi, R., Odzak, N., Sigg, L. & Behra, R. 2008b. Toxicity of silver nanoparticles to *Chlamydomonas reinhardtii*. *Environ. Sci. Technol*, 42, 8959-8964.
- Neal, C., Jarvie, H., Rowland, P., Lawler, A., Sleep, D. & Scholefield, P. 2011. Titanium in UK rural, agricultural and urban/industrial rivers: Geogenic and anthropogenic colloidal/sub-colloidal sources and the significance of within-river retention. *Science of The Total Environment*, 409, 1843-1853.
- Negahdary, M., Habibi-Tamijani, A., Asadi, A. & Ayati, S. 2012. Synthesis of Zirconia Nanoparticles and Their Ameliorative Roles as Additives Concrete Structures. *Journal of Chemistry*, 2013.
- Nel, A., Xia, T., Madler, L. & Li, N. 2006. Toxic potential of materials at the nanolevel. *Science*, 311, 622.
- Nicholson, F., Smith, S., Alloway, B., Carlton-Smith, C. & Chambers, B. 2003. An inventory of heavy metals inputs to agricultural soils in England and Wales. *Science of The Total Environment*, 311, 205-219.
- Nowack, B. 2009. Environmental Behavior and Effects of Engineered Metal and Metal Oxide Nanoparticles. *Heavy Metals in the Environment*, 63.
- Nowack, B. & Bucheli, T. D. 2007. Occurrence, behavior and effects of nanoparticles in the environment. *Environmental Pollution*, 150, 5-22.
- Nowack, B., Ranville, J. F., Diamond, S., Gallego-Urrea, J. A., Metcalfe, C., Rose, J., Horne, N., Koelmans, A. A. & Klaine, S. J. 2012. Potential scenarios for nanomaterial release and subsequent alteration in the environment. *Environmental Toxicology and Chemistry*, 31, 50-59.
- O'Loughlin, E. J., Kelly, S. D., Cook, R. E., Csencsits, R. & Kemner, K. M. 2003. Reduction of uranium (VI) by mixed iron (II)/iron (III) hydroxide (green rust): formation of UO₂ nanoparticles. *Environmental Science & Technology*, 37, 721-727.
- Oberdörster, E. 2004. Manufactured nanomaterials (fullerenes, C60) induce oxidative stress in the brain of juvenile largemouth bass. *Environmental health perspectives*, 112, 1058.
- Oberdorster, G., Elder, A. & Rinderknecht, A. 2009. Nanoparticles and the brain: cause for concern? *Journal of nanoscience and nanotechnology*, 9, 4996-5007.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Oberdörster, G., Ferin, J. & Lehnert, B. E. 1994. Correlation between particle size, *in vivo* particle persistence, and lung injury. *Environmental health perspectives*, 102, 173.
- Oberdörster, G., Finkelstein, J., Johnston, C., Gelein, R., Cox, C., Baggs, R. & Elder, A. 2000. Acute pulmonary effects of ultrafine particles in rats and mice. *Research report (Health Effects Institute)*, 5-74; disc. 75-86.
- Ostrowski, A., Martin, T., Conti, J., Hurt, I. & Harthorn, B. 2009. Nanotoxicology: characterizing the scientific literature, 2000–2007. *Journal of Nanoparticle Research*, 11, 251-257.
- Paddle-Ledinek, J. E., Nasa, Z. & Cleland, H. J. 2006. Effect of different wound dressings on cell viability and proliferation. *Plastic and reconstructive surgery*, 117, 110S-118S.
- Paerl, H. & Pinckney, J. 1996. A mini-review of microbial consortia: their roles in aquatic production and biogeochemical cycling. *Microbial Ecology*, 31, 225-247.
- Palaniappan, P. & Pramod, K. 2010. FTIR study of the effect of nTiO₂ on the biochemical constituents of gill tissues of Zebrafish (*Danio rerio*). *Food and Chemical Toxicology*, 48, 2337-2343.
- Palmgren, F., Wåhlin, P., Kildesø, J., Afshari, A. & Fogh, C. L. 2003. Characterisation of particle emissions from the driving car fleet and the contribution to ambient and indoor particle concentrations. *Physics and Chemistry of the Earth, Parts A/B/C*, 28, 327-334.
- Park, E.-J., Bae, E., Yi, J., Kim, Y., Choi, K., Lee, S. H., Yoon, J., Lee, B. C. & Park, K. 2010. Repeated-dose toxicity and inflammatory responses in mice by oral administration of silver nanoparticles. *Environmental Toxicology and Pharmacology*, 30, 162-168.
- Paull, J. 2010. Nanotechnology, No Free Lunch. Accessed online 11.09.13
<http://orgprints.org/16786/1/16786.pdf>.
- Penner, J., Eddleman, H. & Novakov, T. 1993. Towards the development of a global inventory for black carbon emissions. *Atmospheric Environment. Part A. General Topics*, 27, 1277-1295.
- Peters, A., Wichmann, H. E., Tuch, T., Heinrich, J. & Heyder, J. 1997. Respiratory effects are associated with the number of ultrafine particles. *American journal of respiratory and critical care medicine*, 155, 1376-1383.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Piccinno, F., Gottschalk, F., Seeger, S. & Nowack, B. 2012. Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. *Journal of Nanoparticle Research*, 14, 1-11.
- Pissuwan, D., Niidome, T. & Cortie, M. B. 2011. The forthcoming applications of gold nanoparticles in drug and gene delivery systems. *Journal of controlled release*, 149, 65-71.
- Polizzi, M. A., Stasko, N. A. & Schoenfisch, M. H. 2007. Water-soluble nitric oxide-releasing gold nanoparticles. *Langmuir*, 23, 4938-4943.
- Popov, A., Priezzhev, A., Lademann, J. & Myllylä, R. 2005. TiO₂ nanoparticles as an effective UV-B radiation skin-protective compound in sunscreens. *Journal of Physics D: Applied Physics*, 38, 2564.
- Powers, K. W., Brown, S. C., Krishna, V. B., Wasdo, S. C., Moudgil, B. M. & Roberts, S. M. 2006. Research strategies for safety evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation. *Toxicological Sciences*, 90, 296-303.
- Powers, K. W., Palazuelos, M., Brown, S. C. & Roberts, S. M. 2009. Characterization of nanomaterials for toxicological evaluation. *Nanotoxicology From In Vivo and In Vitro Models to Health Risks*.(S. Sahu and D. Casciano, Eds.), 1-27.
- Prabhu, S. & Poulouse, E. K. 2012. Silver nanoparticles: mechanism of antimicrobial action, synthesis, medical applications, and toxicity effects. *International Nano Letters*, 2, 1-10.
- Puzyn, T., Leszczynska, D. & Leszczynski, J. 2009. Toward the Development of "Nano-QSARs": Advances and Challenges. *Small*, 5, 2494-2509.
- Puzyn, T., Rasulev, B., Gajewicz, A., Hu, X., Dasari, T. P., Michalkova, A., Hwang, H.-M., Toropov, A., Leszczynska, D. & Leszczynski, J. 2011. Using nano-QSAR to predict the cytotoxicity of metal oxide nanoparticles. *Nature nanotechnology*, 6, 175-178.
- Rai, M., Yadav, A. & Gade, A. 2009. Silver nanoparticles as a new generation of antimicrobials. *Biotechnology advances*, 27, 76-83.
- Ramsden, C. S., Smith, T. J., Shaw, B. J. & Handy, R. D. 2009. Dietary exposure to titanium dioxide nanoparticles in rainbow trout (*Oncorhynchus mykiss*): no effect on growth, but subtle biochemical disturbances in the brain. *Ecotoxicology*, 18, 939-951.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Ren, G., Hu, D., Cheng, E. W., Vargas-Reus, M. A., Reip, P. & Allaker, R. P. 2009. Characterisation of copper oxide nanoparticles for antimicrobial applications. *International journal of antimicrobial agents*, 33, 587-590.
- Ribeiro, J., Flores, D., Ward, C. R. & Silva, L. F. 2010. Identification of nanominerals and nanoparticles in burning coal waste piles from Portugal. *Science of The Total Environment*, 408, 6032-6041.
- Rickerby, D. & Morrison, M. 2007. Nanotechnology and the environment: A European perspective. *Science and Technology of Advanced Materials*, 8, 19-24.
- Rico, C. M., Majumdar, S., Duarte-Gardea, M., Peralta-Videa, J. R. & Gardea-Torresdey, J. L. 2011. Interaction of nanoparticles with edible plants and their possible implications in the food chain. *Journal of agricultural and food chemistry*, 59, 3485-3498.
- Ringwood, A. H., McCarthy, M., Bates, T. C. & Carroll, D. L. 2010. The effects of silver nanoparticles on oyster embryos. *Marine environmental research*, 69, S49-S51.
- Robichaud, C. O., Uyar, A. E., Darby, M. R., Zucker, L. G. & Wiesner, M. R. 2009. Estimates of upper bounds and trends in nano-TiO₂ production as a basis for exposure assessment. *Environmental Science & Technology*, 43, 4227-4233.
- Roh, J.-y., Sim, S. J., Yi, J., Park, K., Chung, K. H., Ryu, D.-y. & Choi, J. 2009. Ecotoxicity of silver nanoparticles on the soil nematode *Caenorhabditis elegans* using functional ecotoxicogenomics. *Environmental Science & Technology*, 43, 3933-3940.
- Royal Society. 2004. Nanoscience and nanotechnologies: Opportunities and uncertainties. *Report by the Royal Society and The Royal Academy of Engineering*.
- Sadrieh, N., Wokovich, A. M., Gopee, N. V., Zheng, J., Haines, D., Parmiter, D., Siitonen, P. H., Cozart, C. R., Patri, A. K. & McNeil, S. E. 2010. Lack of significant dermal penetration of titanium dioxide from sunscreen formulations containing nano- and submicron-size TiO₂ particles. *Toxicological Sciences*, 115, 156-166.
- Saito, T., Iwase, T., Horie, J. & Morioka, T. 1992. Mode of photocatalytic bactericidal action of powdered semiconductor TiO₂ on mutants streptococci. *Journal of Photochemistry and Photobiology B: Biology*, 14, 369-379.
- Sayes, C. & Ivanov, I. 2010. Comparative Study of Predictive Computational Models for Nanoparticle-Induced Cytotoxicity. *Risk Analysis*, 30, 1723-1734.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Schneider, J., Hock, N., Weimer, S., Borrmann, S., Kirchner, U., Vogt, R. & Scheer, V. 2005. Nucleation particles in diesel exhaust: Composition inferred from in situ mass spectrometric analysis. *Environmental Science & Technology*, 39, 6153-6161.
- Scown, T., Santos, E., Johnston, B., Gaiser, B., Baalousha, M., Mitov, S., Lead, J., Stone, V., Fernandes, T. & Jepson, M. 2010. Effects of aqueous exposure to silver nanoparticles of different sizes in rainbow trout. *Toxicological Sciences*, 115, 521.
- Scown, T., Van Aerle, R., Johnston, B., Cumberland, S., Lead, J., Owen, R. & Tyler, C. 2009. High doses of intravenously administered titanium dioxide nanoparticles accumulate in the kidneys of rainbow trout but with no observable impairment of renal function. *Toxicological Sciences*, 109, 372.
- Shatkin, J. A. & North, W. 2010. Perspectives on Risks of Nanomaterials and Nanotechnologies: Advancing the Science. *Risk Analysis*, 30, 1627-1633.
- Shiraishi, K., Koseki, H., Tsurumoto, T., Baba, K., Naito, M., Nakayama, K. & Shindo, H. 2009. Antibacterial metal implant with a TiO₂-conferred photocatalytic bactericidal effect against *Staphylococcus aureus*. *Surface and Interface Analysis*, 41, 17-22.
- Shoults-Wilson, W. A., Reinsch, B. C., Tsyusko, O. V., Bertsch, P. M., Lowry, G. V. & Unrine, J. M. 2011. Role of particle size and soil type in toxicity of silver nanoparticles to earthworms. *Soil Science Society of America Journal*, 75, 365-377.
- Simonet, B. M. & Valcárcel, M. 2009. Monitoring nanoparticles in the environment. *Analytical and Bioanalytical Chemistry*, 393, 17-21.
- Singh, R. P. & Ramarao, P. 2012. Cellular uptake, intracellular trafficking and cytotoxicity of silver nanoparticles. *Toxicology letters*.
- Smith, D. S., Bell, R. A. & Kramer, J. R. 2002. Metal speciation in natural waters with emphasis on reduced sulfur groups as strong metal binding sites. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 133, 65-74.
- Solomatin, S. V., Bronich, T. K., Bargar, T. W., Eisenberg, A., Kabanov, V. A. & Kabanov, A. V. 2003. Environmentally responsive nanoparticles from block ionomer complexes: Effects of pH and ionic strength. *Langmuir*, 19, 8069-8076.
- Som, C., Berges, M., Chaudhry, Q., Dusinska, M., Fernandes, T. F., Olsen, S. I. & Nowack, B. 2010. The importance of life cycle concepts for the development of safe nanoproducts. *Toxicology*, 269, 160-169.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Sondi, I. & Salopek-Sondi, B. 2004. Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. *Journal of colloid and interface science*, 275, 177-182.
- Stebounova, L. V., Guio, E. & Grassian, V. H. 2011. Silver nanoparticles in simulated biological media: a study of aggregation, sedimentation, and dissolution. *Journal of Nanoparticle Research*, 13, 233-244.
- Stohs, S. & Bagchi, D. 1995. Oxidative mechanisms in the toxicity of metal ions. *Free Radical Biology and Medicine*, 18, 321-336.
- Stone, V., Nowack, B., Baun, A., van den Brink, N., von der Kammer, F., Dusinska, M., Handy, R., Hankin, S., Hassellöv, M. & Jøner, E. 2010. Nanomaterials for environmental studies: classification, reference material issues, and strategies for physico-chemical characterisation. *Science of The Total Environment*, 408, 1745-1754.
- Sunada, K., Kikuchi, Y., Hashimoto, K. & Fujishima, A. 1998. Bactericidal and detoxification effects of TiO₂ thin film photocatalysts. *Environmental Science & Technology*, 32, 726-728.
- Svecevicus, G. 2003. Avoidance response of rainbow trout *Oncorhynchus mykiss* to heavy metal model mixture after long-term exposure in early development. *Bulletin of environmental contamination and toxicology*, 71, 0226-0233.
- Tagmatarchis, N. & Shinohara, H. 2001. Fullerenes in medicinal chemistry and their biological applications. *Mini reviews in medicinal chemistry*, 1, 339-348.
- Takahashi, H., Niidome, T., Kawano, T., Yamada, S. & Niidome, Y. 2008. Surface modification of gold nanorods using layer-by-layer technique for cellular uptake. *Journal of Nanoparticle Research*, 10, 221-228.
- Taniguchi, N. 1974. On the Basic Concept of Nanotechnology. *Japanese Society of Precision Engineering*, p18-23.
- Taylor, D. A. 2002. Dust in the wind. *Environmental health perspectives*, 110, A80.
- Tiede, K., Boxall, A. B., Tear, S. P., Lewis, J., David, H. & Hassellöv, M. 2008. Detection and characterization of engineered nanoparticles in food and the environment. *Food additives and contaminants*, 25, 795-821.
- Tiwari, D. K., Jin, T. & Behari, J. 2011. Dose-dependent in-vivo toxicity assessment of silver nanoparticle in Wistar rats. *Toxicology mechanisms and methods*, 21, 13-24.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Tokuyama, H., Yamago, S., Nakamura, E., Shiraki, T. & Sugiura, Y. 1993. Photoinduced biochemical activity of fullerene carboxylic acid. *Journal of the American Chemical Society*, 115, 7918-7919.
- Tong, T., Binh, C. T. T., Kelly, J. J., Gaillard, J.-F. & Gray, K. A. 2013. Cytotoxicity of commercial nano-TiO₂ to *Escherichia coli* assessed by high-throughput screening: Effects of environmental factors. *Water research*.
- Trouiller, B., Reliene, R., Westbrook, A., Solaimani, P. & Schiestl, R. H. 2009. Titanium dioxide nanoparticles induce DNA damage and genetic instability *in vivo* in mice. *Cancer Research*, 69, 8784-8789.
- van Aerle, R., Lange, A., Moorhouse, A., Paszkiewicz, K. H., Ball, K., Johnston, B. D., de Bastos, E., Booth, T., Tyler, C. R. & Santos, E. M. 2013. Molecular Mechanisms of Toxicity of Silver Nanoparticles in Zebrafish Embryos. *Environmental Science & Technology*.
- Velzeboer, I., Hendriks, A. J., Ragas, A. M. & Van de Meent, D. 2008. Nanomaterials in the environment aquatic ecotoxicity tests of some nanomaterials. *Environmental Toxicology and Chemistry*, 27, 1942-1947.
- Von der Kammer, F., Ferguson, P. L., Holden, P. A., Masion, A., Rogers, K. R., Klaine, S. J., Koelmans, A. A., Horne, N. & Unrine, J. M. 2012. Analysis of engineered nanomaterials in complex matrices (environment and biota): General considerations and conceptual case studies. *Environmental Toxicology and Chemistry*, 31, 32-49.
- Wang, J., Zhang, X., Chen, Y., Sommerfeld, M. & Hu, Q. 2008. Toxicity assessment of manufactured nanomaterials using the unicellular green alga *Chlamydomonas reinhardtii*. *Chemosphere*, 73, 1121-1128.
- Wang, Y., Seebald, J. L., Szeto, D. P. & Irudayaraj, J. 2010. Biocompatibility and biodistribution of surface-enhanced Raman scattering nanoprobe in zebrafish embryos: *in vivo* and multiplex imaging. *ACS Nano*, 4, 4039-4053.
- Ward, J. E. & Kach, D. J. 2009. Marine aggregates facilitate ingestion of nanoparticles by suspension-feeding bivalves. *Marine environmental research*, 68, 137-142.
- Weir, A., Westerhoff, P., Fabricius, L., Hristovski, K. & von Goetz, N. 2012. Titanium dioxide nanoparticles in food and personal care products. *Environmental Science & Technology*, 46, 2242-2250.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Westerhoff, P., Song, G., Hristovski, K. & Kiser, M. A. 2011. Occurrence and removal of titanium at full scale wastewater treatment plants: implications for TiO₂ nanomaterials. *Journal of environmental monitoring*, 13, 1195-1203.
- Wiesenthal, A., Hunter, L., Wang, S., Wickliffe, J. & Wilkerson, M. 2011. Nanoparticles: small and mighty. *International journal of dermatology*, 50, 247-254.
- Wigginton, N. S., Haus, K. L. & Hochella Jr, M. F. 2007. Aquatic environmental nanoparticles. *Journal of environmental monitoring*, 9, 1306-1316.
- Winkler, D. A., Mombelli, E., Pietroiusti, A., Tran, L., Worth, A., Fadeel, B. & McCall, M. J. 2012. Applying quantitative structure-activity relationship approaches to nanotoxicology: current status and future potential. *Toxicology*.
- Wise, J. P., Goodale, B. C., Wise, S. S., Craig, G. A., Pongan, A. F., Walter, R. B., Thompson, W. D., Ng, A.-K., Aboueissa, A.-M. & Mitani, H. 2010. Silver nanospheres are cytotoxic and genotoxic to fish cells. *Aquatic Toxicology*, 97, 34-41.
- Wood, C. M., Playle, R. C. & Hogstrand, C. 1999. Physiology and modeling of mechanisms of silver uptake and toxicity in fish. *Environmental Toxicology and Chemistry*, 18, 71-83.
- Wu, J., Bai, G.-R., Eastman, J., Zhou, G. & Vasudevan, V. Synthesis of TiO₂ Nanoparticles Using Chemical Vapor Condensation. *In: Materials Research Society Symposia Proceedings*, 2005. Cambridge Univ Press.
- Wu, J., Liu, W., Xue, C., Zhou, S., Lan, F., Bi, L., Xu, H., Yang, X. & Zeng, F.-D. 2009. Toxicity and penetration of TiO₂ nanoparticles in hairless mice and porcine skin after subchronic dermal exposure. *Toxicology letters*, 191, 1-8.
- Wu, X.-N., Zhao, Y.-X., Xue, W., Wang, Z.-C., He, S.-G. & Ding, X.-L. 2010a. Active sites of stoichiometric cerium oxide cations (CeO₂m⁺) probed by reactions with carbon monoxide and small hydrocarbon molecules. *Physical Chemistry Chemical Physics*, 12, 3984-3997.
- Wu, Y. & Zhou, Q. 2012. Dose-and Time-Related Changes in Aerobic Metabolism, Chorionic Disruption, and Oxidative Stress in Embryonic Medaka (*Oryzias latipes*): Underlying Mechanisms for Silver Nanoparticle Developmental Toxicity. *Aquatic Toxicology*.
- Wu, Y., Zhou, Q., Li, H., Liu, W., Wang, T. & Jiang, G. 2010b. Effects of silver nanoparticles on the development and histopathology biomarkers of Japanese medaka (*Oryzias latipes*) using the partial-life test. *Aquatic Toxicology*, 100, 160-167.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Xia, X.-R., Monteiro-Riviere, N. A. & Riviere, J. E. 2010. An index for characterization of nanomaterials in biological systems. *Nature nanotechnology*, 5, 671-675.
- Xiong, D., Fang, T., Yu, L., Sima, X. & Zhu, W. 2011. Effects of nano-scale TiO₂, ZnO and their bulk counterparts on zebrafish: Acute toxicity, oxidative stress and oxidative damage. *Science of The Total Environment*, 409, 1444-1452.
- Yadav, A., Prasad, V., Kathe, A., Raj, S., Yadav, D., Sundaramoorthy, C. & Vigneshwaran, N. 2006. Functional finishing in cotton fabrics using zinc oxide nanoparticles. *Bulletin of materials Science*, 29, 641-645.
- Yang, W., Shen, C., Ji, Q., An, H., Wang, J., Liu, Q. & Zhang, Z. 2009. Food storage material silver nanoparticles interfere with DNA replication fidelity and bind with DNA. *Nanotechnology*, 20, 085102.
- Zhang, T., Stilwell, J. L., Gerion, D., Ding, L., Elboudwarej, O., Cooke, P. A., Gray, J. W., Alivisatos, A. P. & Chen, F. F. 2006. Cellular effect of high doses of silica-coated quantum dot profiled with high throughput gene expression analysis and high content cellomics measurements. *Nano letters*, 6, 800-808.
- Zhang, X., Sun, H., Zhang, Z., Niu, Q., Chen, Y. & Crittenden, J. C. 2007. Enhanced bioaccumulation of cadmium in carp in the presence of titanium dioxide nanoparticles. *Chemosphere*, 67, 160-166.
- Zhao, C. M. & Wang, W. X. 2011. Comparison of acute and chronic toxicity of silver nanoparticles and silver nitrate to *Daphnia magna*. *Environmental Toxicology and Chemistry*, 30, 885-892.
- Zhiqiang, Q., Siegmann, K., Keller, A., Matter, U., Scherrer, L. & Siegmann, H. 2000. Nanoparticle air pollution in major cities and its origin. *Atmospheric Environment*, 34, 443-451.
- Zhu, X., Chang, Y. & Chen, Y. 2010a. Toxicity and bioaccumulation of TiO₂ nanoparticle aggregates in *Daphnia magna*. *Chemosphere*, 78, 209-215.
- Zhu, X., Wang, J., Zhang, X., Chang, Y. & Chen, Y. 2010b. Trophic transfer of TiO₂ nanoparticles from daphnia to zebrafish in a simplified freshwater food chain. *Chemosphere*, 79, 928-933.
- Zhu, X., Zhu, L., Chen, Y. & Tian, S. 2009. Acute toxicities of six manufactured nanomaterial suspensions to *Daphnia magna*. *Journal of Nanoparticle Research*, 11, 67-75.

BIOLOGICAL EFFECTS OF SELECTED METAL NANOPARTICLES IN ZEBRAFISH (*Danio rerio*)

- Zhu, X., Zhu, L., Duan, Z., Qi, R., Li, Y. & Lang, Y. 2008. Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to Zebrafish (*Danio rerio*) early developmental stage. *Journal of Environmental Science and Health Part A*, 43, 278-284.
- Zhu, Z. J., Carboni, R., Quercio, M. J., Yan, B., Miranda, O. R., Anderton, D. L., Arcaro, K. F., Rotello, V. M. & Vachet, R. W. 2010c. Surface properties dictate uptake, distribution, excretion, and toxicity of nanoparticles in fish. *Small*, 6, 2261-2265.

CHAPTER 7

APPENDIX

Effects of particle size and coating on nanoscale Ag and TiO₂ exposure in zebrafish (*Danio rerio*) embryos

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Abstract

Manufactured metal (oxide) nanoparticles are entering the aquatic environment with little understanding on their potential health impacts for exposed organisms. Adopting an integrative approach, we investigated effects of particle size and coating on biological responses for two of the most commonly used metal (oxide) nanoscale particles, silver (Ag) and titanium dioxide (TiO₂) in zebrafish embryos. Titanium dioxide nanoparticles (nominally, 4 nm, 10 nm, 30 nm and 134 nm) had little or no toxicity on the endpoints measured. Ag both in nano form (10 nm and 35 nm) and its larger counterpart (600–1600 nm) induced dose-dependent lethality and morphological defects, occurring predominantly during gastrula stage. Of the silver material tested 10 nm nanoparticles appeared to be the most toxic. Coating Ag nanoparticles with citrate or fulvic acid decreased toxicity significantly. *In situ* hybridisation analysis identified the yolk syncytial layer (YSL) as a target tissue for Ag-nano toxicity where there was a significant induction of the heavy metal stress response gene, metallothionein 2 (Mt2) at sub-lethal exposures. Coherent Anti-stroke Raman Scattering (CARS) microscopy provided no evidence for silver particles crossing the chorionic membrane in exposed embryos. Collectively, our data suggest that silver ions play a major role in the toxicity of Ag nanoparticles.

Keywords: nanoparticle, ecotoxicology, *Danio rerio*, embryo, titanium dioxide, silver

Introduction

Nanoparticles are being introduced rapidly into the consumer market but there is still little understanding on their potential consequences for human and environmental health. Two of the first metal-based nanoparticles to gain widespread use are titanium dioxide (TiO₂) and silver (Ag). Titanium dioxide is of global importance with in excess of

4.3 million tonnes produced annually, with extensive use in sunscreen and in the pigmentation of paints. The surface reactivity and general properties of TiO₂ are well documented (Long et al. 2007). Nano-TiO₂ has been reported to cause oxidative stress effects in mammals and in fish, inducing inflammation, cell damage and genetic damage, both with and without exposure to ultraviolet A (UVA) radiation. Available data suggest sub-lethal toxicity in the concentration range of 5–50 µg L⁻¹ for exposures through the water in both invertebrates (Lovern et al. 2007; Heinlaan et al. 2008; Zhao et al. 2009) and fish (Lee et al. 2007; Scown et al. 2009). Modelled environmental concentrations indicate TiO₂ concentrations may in some circumstances reach between 0.7 and 16 µg L⁻¹ (Nowack & Bucheli 2007) and this could present a risk to aquatic organisms.

In the 1970s, 2.5 million kg of Ag was discharged into the environment in the United States (Luoma & Rainbow 2008) and its high toxicity to aquatic animals subsequently led to stringent environmental regulations by the 1980s under the Clean Water Act in the United States (Purcell & Peters 1999). Nano-silver is now used extensively in consumer products, predominantly for its effective antimicrobial properties and low production cost. In wastewater treatment works receiving influents from industries using silver nanoparticles (Ag NPs), levels of silver have been shown to reach 100 µg L⁻¹ (Hu 2010), and this exceeds tolerable limits for some bacteria, which may therefore impact adversely on bacterial communities (Marambio-Jones & Hoek 2010). Of particular concern is the potential for nano-silver to concentrate in sewage sludge as in some countries (including the United Kingdom) this can be subsequently applied to land as fertilizer. Several studies have indicated that Ag NPs have the potential to induce toxic effects in a range of species, including fish (Skebo et al. 2007; Braydich-Stolle et al. 2005; Hussain et al. 2005; Scown et al. 2009; AshaRani et al. 2009). One study exposing zebrafish embryos to an extremely high level of Ag NPs (100 mg L⁻¹), that were stabilised

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with citrate or fulvic acid, showed silver penetrated into various body tissues, including brain, heart and skin (AshaRani et al. 2009). This toxicity for exposure to Ag NPs in fish may, in part, relate to an enhanced dissociation in the exposure water, and thus bioavailability, of free silver ions (Jin et al. 2010).

The purpose of this study was to adopt an integrative approach to determine potential toxicity to zebrafish embryos of well-characterised Ag and TiO₂, of various sizes both as unmodified nanoparticles and dispersed with citrate or fulvic acid, and across a range of exposure concentrations. Zebrafish embryos offer a wide range of experimental conveniences including the ease for observing developmental effects through a transparent chorion. Mortality rates, developmental abnormalities, apoptosis and targeted (*in situ*) gene expression were used as effects assessment endpoints. Advanced imaging techniques, including Coherent Anti-stokes Raman Scattering (CARS) were employed to investigate for uptake and distribution of nanoparticles in the tissues of the exposed embryos.

Materials and methods

Fish source, culture and husbandry

Wild-type WIK (Wild-Type India Calcutta) strain zebrafish embryos were obtained from the Max Planck Institute, Tübingen, Germany, and maintained at University of Exeter as described in the supplementary material (S1).

Nanoparticle source and characterisation

Ag NPs (nominal sizes 10 nm and 35 nm) and Ag bulk (nominal size 600–1600 nm) were acquired from Nanostructured and Amorphous Materials Inc. Houston, USA. Titanium dioxide nanoparticles (TiO₂ NPs) (nominal sizes 3 nm, 10 nm and 35 nm) and 134 nm particles were acquired from Alfa Aesar - A Johnson Matthey Company, Lancashire, United Kingdom.

Physicochemical characterisation

A number of techniques were carried out to characterise and quantify the particles. The techniques applied included: nanoparticle tracking analysis (NTA), Braun Emmett Teller (BET) method of specific surface area analysis, X-ray diffraction (XRD), atomic force microscopy (AFM) and high-resolution transmission electron microscopy (HR-TEM) with associated spectroscopy - X-ray electron dispersive spectroscopy (X-EDS). A full detailing of the methods applied to Ag and TiO₂ particles can be found in (Scown et al. 2009); and details of data analysis in (Ju-Nam et al. 2012).

Silver dissolution

Samples of silver nitrate (Perkin Elmer) were made up in milli Q water and embryo culture water (0.60 mg of marine salts [Tropic Marin] per litre of deionised water) as test standards for analysis by the ICP-MS. Sample concentrations were 0, 15, 30, 60, 120 and 260 µg L⁻¹. Dissolution rates were determined for 35 nm silver and bulk silver particles. For this, duplicate 1 litre solutions containing 50 µg L⁻¹ Ag NPs were made up in embryo culture water and mixed constantly

using magnetic stirrers at a temperature of 21°C. For each treatment, 8 Spectra/por dialysis membranes MWCO 1000 (1 KDa) (prewashed in 0.05% sodium azide in Milli Q water) were set up containing 10 mL of Milli Q water, which were then clip sealed at each end before being submerged into the Ag NP or Ag bulk solutions. At different time points, 4 h, 24 h, 48 h and 72 h, one sample for each treatment vessel (two per treatment) was taken, pipetted into a 15 mL falcon tube and the silver ions stabilised through the addition of 1% of HNO₃ added before analyses using ICP-MS.

Ag/TiO₂ nanoparticle exposure and effects assessments

Particles were made up in a dilution series of six stock solutions (50 µg L⁻¹, 500 µg L⁻¹, 5000 µg L⁻¹, 50,000 µg L⁻¹, 250,000 µg L⁻¹) for each particle size. Solutions were sonicated in a water bath for 30 min and placed into glass, amber, Boston round 125 mL tubes fitted with a Teflon-lined cap and kept at 4°C until required. When the solutions were required for the exposure studies, they were sonicated in a water bath for 30 min and pipetted into the exposure wells. To investigate for effects of particle coating on biological effects a further dilution series of 10 nm Ag particles was mixed with either 0.0075% sodium citrate or a 2% fulvic acid suspension prior to the exposures. For the exposures to silver ions, a stock solution of silver nitrate (Perkin Elmer 2% AgNO₃) was made and the required amount for each exposure concentration was added into the embryo culture water.

For the embryos exposures, 500 µL of the stock solutions were added to 450 mL embryo culture medium to give final exposure concentrations of 5 µg L⁻¹, 50 µg L⁻¹, 500 µg L⁻¹,

5000 µg L⁻¹ and 25,000 µg L⁻¹. Controls received 5 mL of embryo culture water only. Eggs/embryos were collected from breeding colonies transferred into a Petri dish and washed twice with embryo culture water with the addition of 15 µL of methylene blue to prevent fungal and bacteria growth. For all exposures, there were 20 embryos (at the 1–2 cell stage, 1–1.5 hpf) per treatment well, and the studies were replicated at least three times. The embryos were incubated at 28 ± 1°C up to 48 h. After 2 h in culture, the numbers of unfertilised embryos were recorded and these were removed. At 48 hpf (hours post fertilisation) survival rates and any phenotypic deformities were recorded. Any physical deformities observed were recorded and converted to percentages for each treatment. Embryos were observed and photographed using Nikon SMZ1500 microscope equipped with a digital camera. To gain an insight into the timing of mortality and developmental effects induced by the exposures to Ag, time lapse video analysis was used, as described in the supplementary material (S2).

Cell necrosis

To investigate further for silver particle toxicity, cell necrosis was recorded in embryos exposed during early life to either 35 nm Ag or 35 nm TiO₂ at concentrations of 500 µg L⁻¹ and 25,000 µg L⁻¹. Twenty embryos for each exposure concentration were incubated at 28 ± 1°C from the 1 to 2-cell stage and subsequently removed from the exposure at 7 hpf and stained with a Propidium Iodide (PI) (Sigma) at 1 mg/mL

mixed in distilled water; Fluorescein Diacetate (DAF) (Sigma) at 1.5 mg/mL mixed in DMSO; Hoechst (HO) (Sigma) at 1 mg/mL mixed in distilled water and PBS (Pinero et al. 1997). The final concentrations of materials in the necrotic staining solution were PI, 250 mg L⁻¹; DAF, 750 µg L⁻¹; and HO, 200 µg L⁻¹. Embryos were incubated in the dark for 10 min in a 24-well plate and photographed using Leica DMI 4000 B Compound Microscope equipped with a digital camera.

Metallothionein gene expression assessed through whole mount *in-situ* hybridisation

In situ hybridisation on exposed zebrafish larvae was undertaken to investigate for differential activation of gene expression for metallothionein 2 (Mt2), known to play key roles in toxicological responses to metals, including silver (Choi et al. 2009). Mt2 cDNA was obtained from Imagen/RZPD (clone No IMAGp998C0115598Q). To prepare the RNA probe, Mt2 cDNA was amplified by PCR using two primers, Mt2_F: ATC AAC TCA TTC ACA AGC TGA; T3_Mt2_R: GGA TCC ATT AAC CCT CAC TAA AGG AAA TAC CAC CAT TTA TTT TAG, and *in vitro* transcribed by using digoxigenin labelling mixture (Roche) and T3 RNA polymerase (Promega). Using a G50 column the RNA was purified and precipitated using Lithium Chloride. The probe was then diluted with hybridisation buffer at 1/200. The *in situ* hybridisations were conducted as described in the supplementary material (S3). For these studies embryos were exposed to 35 nm Ag and 35 nm TiO₂ particles at 500 µg L⁻¹ and Ag NO₃ at 12 µg L⁻¹ (to represent the maximal rate of dissolution for the silver particle exposures - see results) from 1–2 cell stage to 24 hpf, fixed with 4% PFA (S3). Embryos were observed and photographed using Nikon SMZ1500 microscope equipped with a digital camera. The expression level for Mt2 expression (localised in the YSL) was quantified using Image-J 1.44 P. The levels of expression were determined from the mean of 15–20 embryos for each treatment subtracting for the background measurement. Responses to silver treatment were then given as fold-increase above controls.

CARS microscopy

To investigate the uptake of nanoparticles into the exposed embryos, Coherent Anti-Stokes Raman Scattering (CARS) microscopy was used to provide label-free imaging with sub-cellular resolution (S5). For this imaging work, embryos were exposed at the 1-cell stage to 500 µg L⁻¹ and 5000 µg L⁻¹ 35 nm Ag and 35 nm TiO₂ and their bulk counterparts. Ten embryos were taken at random for each exposure concentration at 24 hpf, five of which were manually dechorionated and the other five the chorion left intact. Embryos were embedded in 1% low melting agarose with 0.05% of tricaine (to anaesthetise the fish) in a glass-bottomed petri dish photographed using IX71 and FV300, Olympus, UK.

Statistics

Unless stated otherwise, all data are presented as means ± S.E.M. The coefficient of variation (CV) statistic was calculated for comparisons of variation, as CV = (standard deviation/

mean)*100. All statistical analyses were performed using Sigma Stat version 12.0 (Jandel Scientific Software, USA). Differences among groups were analysed by one-way/two-way ANOVA, followed by Holm-Sidak method comparison post hoc test, where data were not normally distributed. All data were considered statistically significant at $p < 0.05$.

Results

Particle characterisation

A summary of the characterisation and physicochemical properties of the silver particles is provided in the supplementary material Table S4 and further details are reported elsewhere (in Scown et al. 2009). All particles had purity > 99% based on trace metal analysis. The measured sizes by TEM were found to be different from those reported by the manufacturer (the nominals) and were 49.0 ± 18.5 nm and 114.0 ± 65 nm for the 10 nm and 35 nm particles, respectively. In our assessments, 10 nm Ag particles had a specific surface area of 9–11 m² g⁻¹, bulk density of 2.05 g cm⁻³ and a true density of 10.5 g cm⁻³. The 35 nm Ag particles had a density of 10.5 g cm⁻³, a specific surface area of 30–50 m² g⁻¹, and a bulk density of 0.30–0.60 g cm⁻³. Ag bulk particles had a range in particle size of 0.6–1.6 µm and purity of 99.95%.

The measured physicochemical properties of TiO₂ and TEM images of the different sized materials are shown in the supplementary material Table S1 and Figure S1, respectively. When in suspension, particles formed large aggregates of several hundreds of nanometres (Supplementary material Figure S1, Table S1). The high-resolution TEM micrographs show that both the 3 nm and 35 nm were comprised of very small particles likely to be less than 10 nm, but their precise dimensions were not resolved due to the dense aggregation resulting in the formation of sheet-like structures. The 10 nm particles formed fractal (i.e., porous) aggregates of about 19.1 ± 13.8 nm, as measured by TEM. It is worth noting the relatively high polydispersity of this sample (Supplementary material Figure 1B, inset). The TEM measurement for 10 nm particles were in good agreement with the sizes calculated from the SSA measured through the BET data and crystallite size measured by XRD (Supplementary material Table S1). The crystallite sizes were slightly larger than those measured by TEM or calculated from SSA, most likely due to the high polydispersity and aggregation observed. Measured sizes of the TiO₂ were thus again different from the data supplied by the manufacturer.

Dissolution of silver

Mean recoveries of silver for the silver nitrate control standards (in Milli Q water) were between 78% and 103%, with greater recoveries at the higher concentrations (Supplementary material Table S2). In contrast, quantification of silver in embryo culture water gave measured concentrations between 9.9% and 64.2% of nominals (Supplementary material Table S3). Dissolution of silver ions over the 72 h period for 50 µg Ag NP L⁻¹, ranged between 0.1% and 2%, and for 50 µg Ag bulk L⁻¹ between 0.21% and 0.83%.

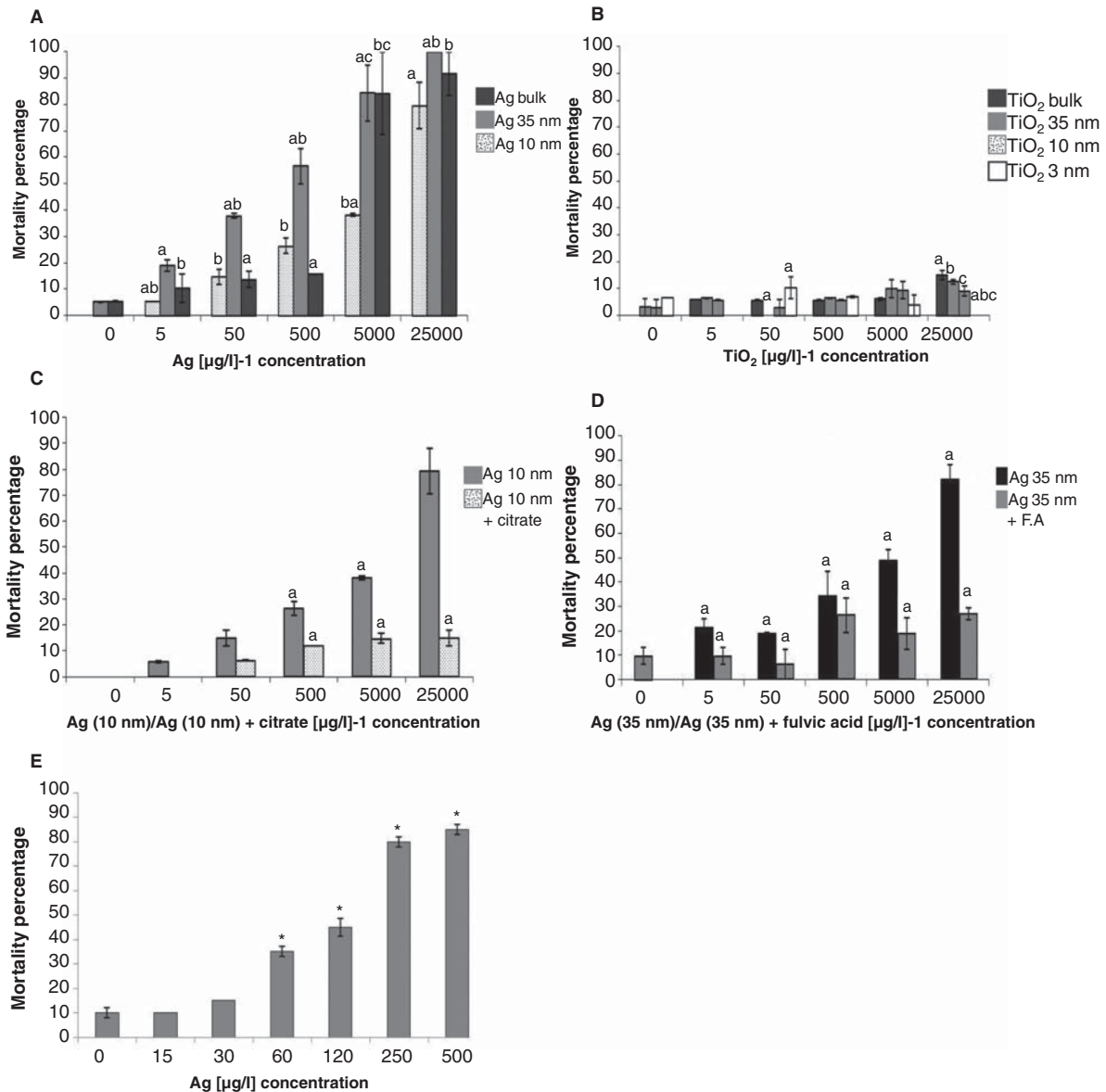


Figure 1. Mortality and morphological abnormalities at 48 hpf in zebrafish embryos exposed to Ag nanoparticles (nominal sizes), TiO₂ nanoparticles (nominal sizes), Ag coated with citrate, Ag coated with fulvic and AgNO₃. Mortality rates are shown for exposure to silver particles (A), TiO₂ particles (B), Ag (10 nm) and Ag (10 nm) citrate-coated particles (C), Ag (35 nm) and Ag (35 nm) with fulvic acid (D), silver nitrate (E). For the different exposures there were statistically significant interactions between concentration and particle size (A, B, E), citrate coating (C) or fulvic acid (D) (Two-way ANOVAs $p < 0.001$). The letters (a, b, c) indicate statistical difference ($p < 0.05$) between particles/coating for each concentration tested (All Pairwise Multiple Comparison Procedures, Holm-Sidak method). There was no statistically significant interaction between concentration and particle size for effects on heartbeat rate (Two-way ANOVA $p = 0.817$). Significant differences in mortality between the treatment groups for exposure to silver nitrate were assessed by One-way ANOVA ($*p < 0.0001$) to that of the control group.

Lethality

Overall TiO₂ had an extremely low level of toxicity: 3 nm and 35 nm TiO₂ particles showed no toxicity and the lowest effect concentration for 10 nm TiO₂ was 5000 $\mu\text{g L}^{-1}$ ($p = 0.029$) and for 134 nm TiO₂, 25,000 $\mu\text{g L}^{-1}$ ($p = 0.004$; Figure 1B). In contrast, there was a clear dose-dependent toxicity for the different sized Ag NPs and the bulk counterpart (Figure 1A). There was a statistically significant interaction between concentration and Ag particle size (Two-way ANOVA $p < 0.001$) with the following lowest effect concentrations: 5 $\mu\text{g L}^{-1}$ for 35 nm Ag ($p = 0.002$), 50 $\mu\text{g L}^{-1}$ for both 10 nm Ag ($p < 0.001$) and Ag bulk ($p = 0.003$). The 35 nm Ag was

significantly more toxic than 10 nm Ag across all concentrations and it was also significantly more toxic than Ag bulk for almost all concentrations tested (the exception was for 5 $\mu\text{g L}^{-1}$). Exposure to silver ions showed a dose-dependent toxicity with a no effect concentration (NOEC) of 30 $\mu\text{g L}^{-1}$ and a lowest effect concentration (LOEC) of 60 $\mu\text{g L}^{-1}$ ($p < 0.001$, Two-way ANOVA). At 500 $\mu\text{g L}^{-1}$ there was 85% embryo mortality (Figure 1E).

Effects of coating on silver nanoparticle toxicity

Coating the 10 nm Ag with citrate significantly reduced their toxicity across the concentration range tested

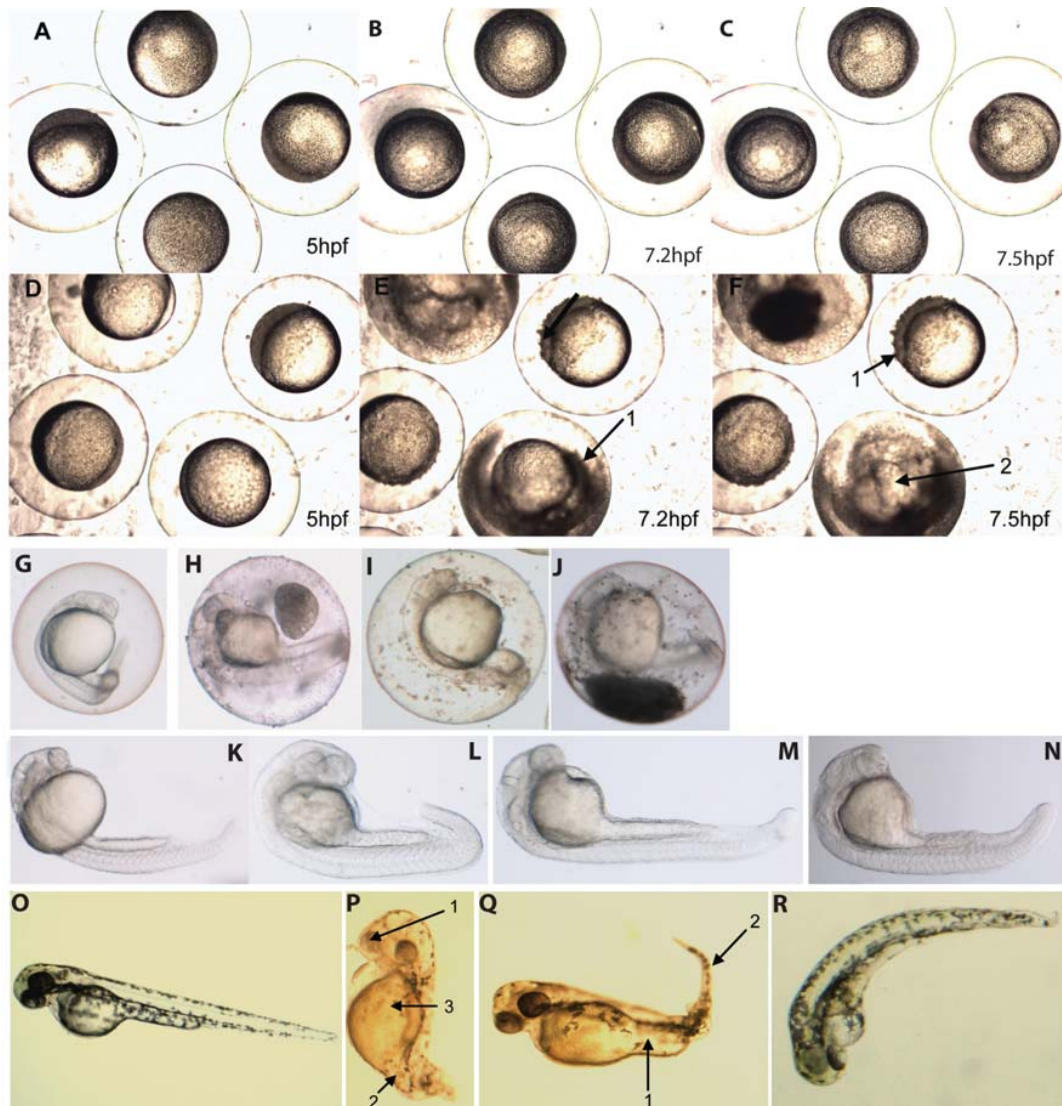


Figure 2. Images of embryos exposed to silver particles at various developmental stages. Video-captured images of zebrafish embryos in controls (A-C, 5 hpf, 7.2 hpf and 7.5 hpf, respectively) and exposed to silver particles (35 nm Ag at 25,000 µg/L) from 1-2 cell stage (D-F, 5 hpf, 7.2 hpf showing - 1. Yolk leaking out from the cells, 7.5 hpf showing - 1. Uneven surface of dividing cells, 2. Embryo bursting within the chorion membrane). G-R, microscope images of control/exposed embryos at different developmental stages. G. 24 hpf control embryo; H. 24 hpf Ag (10 nm 5000 µg/L); I. 24 hpf Ag (Bulk 5000 µg/L); J. 24 hpf AgNO₃ (120 µg/L); K. 24 hpf control embryo; L. 24 hpf Ag (35 nm 500 µg/L); M. 24 hpf Ag (Bulk 500 µg/L); N. AgNO₃ (120 µg/L); O. 48 hpf control embryo; P. 48 hpf Ag (35 nm, 5000 µg/L), showing - 1. Eyes spaced more widely on the head compared with controls, 2. absence of a tail and 3. deformed yolk sac; Q. 24 hpf Ag (Bulk, 5000 µg/L) showing - 1. bent tail, 2. reduced yolk sac; R. AgNO₃ (120 µg/L).

(Two-way ANOVA $p < 0.001$) (Figure 1C). The maximum mortality rate (exposure to 25,000 µg L⁻¹) in the citrate-coated silver particles was 14% compared with 79% for non-coated silver particles of the same size. The lowest effect concentration for 10 nm Ag was 50 µg L⁻¹ ($p < 0.001$) and for Ag 10 nm + citrate, 500 µg L⁻¹ ($p = 0.05$, Two-way ANOVA). The 10 nm Ag was more toxic than 10 nm Ag coated with citrate for all concentrations above 50 µg L⁻¹.

Similarly, the addition of fulvic acid significantly reduced the toxicity of the 35 nm Ag (Two-way ANOVA $p < 0.001$) (Figure 1D). The lowest effect concentration for 35 nm Ag was 5 µg L⁻¹ ($p < 0.001$) and for 35 nm Ag with fulvic acid, 500 µg L⁻¹ ($p < 0.001$). The 35 nm Ag was significantly more

toxic than 35 nm Ag + fulvic acid for all adopted exposure concentrations.

Ag-nano predominantly induces embryonic lethality at gastrula stage

Video analysis on the developing embryos in the controls established that at 8 hpf half had reached gastrulation stage, which is in accordance with the normal progression of expected development. In contrast, half of the embryos exposed to 25,000 µg Ag L⁻¹ had died by this stage. Time lapse analysis showed that for embryos exposed to 35 nm Ag the yolk sac membrane of the embryo became damaged, leading to the leakage of yolk and subsequently mortality (Figure 2). It was observed that the surface of the blastoderm

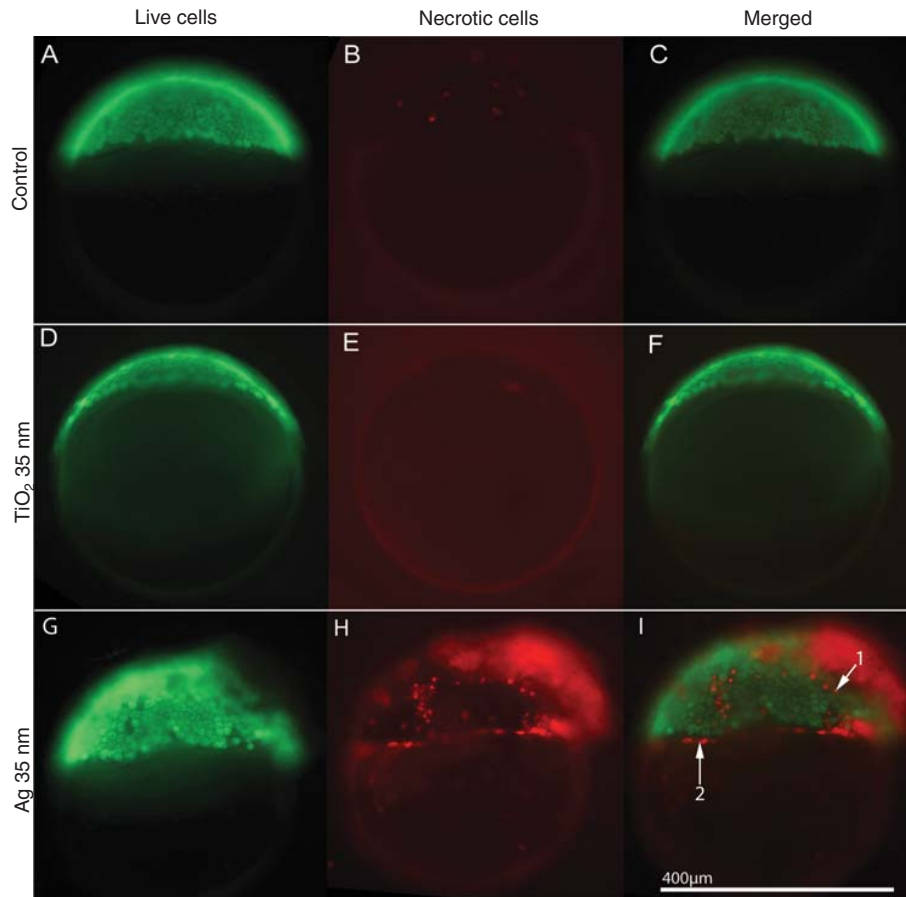


Figure 3. Images showing live (green)/necrotic (red) cells in embryos at shield stage (Lateral View). A. control-live cells; B. control-necrotic cells; C. control-merged images live/necrotic cells; D. TiO_2 (35 nm 500 $\mu\text{g/L}$) - live cells; E. TiO_2 (35 nm 500 $\mu\text{g/L}$) - necrotic cells; F. TiO_2 (35 nm 500 $\mu\text{g/L}$) - merged live/necrotic cells; G. Ag (35 nm 500 $\mu\text{g/L}$) - live cells; H. Ag (35 nm 500 $\mu\text{g/L}$) - necrotic cells; I. Ag (35 nm 500 $\mu\text{g/L}$) - merged live/necrotic cells, showing - 1. necrotic nuclei in blastoderm, 2. necrotic nuclei in yolk syncytial layer (YSL).

became rough (Figure 2E, F) and epiboly, the process where cells move and spread out into sheets of tissue that overlie or surround other groups of cells, was delayed in comparison with control embryos (normally occurring at approximately 4 hpf). The blastoderm in the surviving embryos treated with 35 nm Ag did not cover the yolk and had only reached approximately 40% epiboly in comparison with the control embryos where there was nearly 70% epiboly. Embryos that survived the exposure to silver at high exposure concentrations subsequently had morphological abnormalities including bent tails, small head and a reduced yolk sac size (Figure 2G–R).

Cell necrosis in early life stage embryos

Staining for cell necrosis during the gastrula stage (7 hpf) identified a high prevalence cell death in the exposures to 35 nm Ag (500 $\mu\text{g L}^{-1}$ and 25,000 $\mu\text{g L}^{-1}$; Figure 3H, I). In the controls and embryos exposed to TiO_2 there was a very low/no incidence of necrotic cells (Figure 3B, E). Based on a qualitative assessment only, there appeared to be similar numbers of live cells in all embryos examined in controls, TiO_2 exposures and for Ag at 500 $\mu\text{g L}^{-1}$ (Figure 3A, D, G). There was a high level of necrotic nuclei in the yolk syncytial layer (YSL) (Figure 3I).

Particle uptake (CARS)

The studies showing cell damage for the high concentration exposures suggested that material (particles and/or free silver) entered the embryo from the culture medium. CARS microscopy, however, showed no detectable particles contained within the exposed embryos (Figure 4). CARS images, including images that were focused at the outside edge of the chorion (Figure 4B, C), for exposures of embryos to both Ag NPs and TiO_2 NPs, illustrate that the particles were associated with the outer edge of the embryo and not contained with the embryo itself.

Expression of metallothionein

We conducted *in situ* hybridisation with Mt2 to identify possible tissue targets for metal responses induced by exposure to Ag-nano. For the exposures to all silver-treatment groups at sub-lethal doses, and for which no significant morphological defects were found, significant induction of Mt2 was detected in the YSL especially at the posterior extension. Exposure to 35 nm Ag (Figure 5C, E) induced a 3.9-fold increase, Ag bulk material induced a 2.7-fold increase (Figure 5B, E) and Ag ion (12 $\mu\text{g L}^{-1}$) induced a 2.8-fold increase (Figure 5D, E). TiO_2 (500 $\mu\text{g L}^{-1}$) did not show any enhanced expression of Mt2.

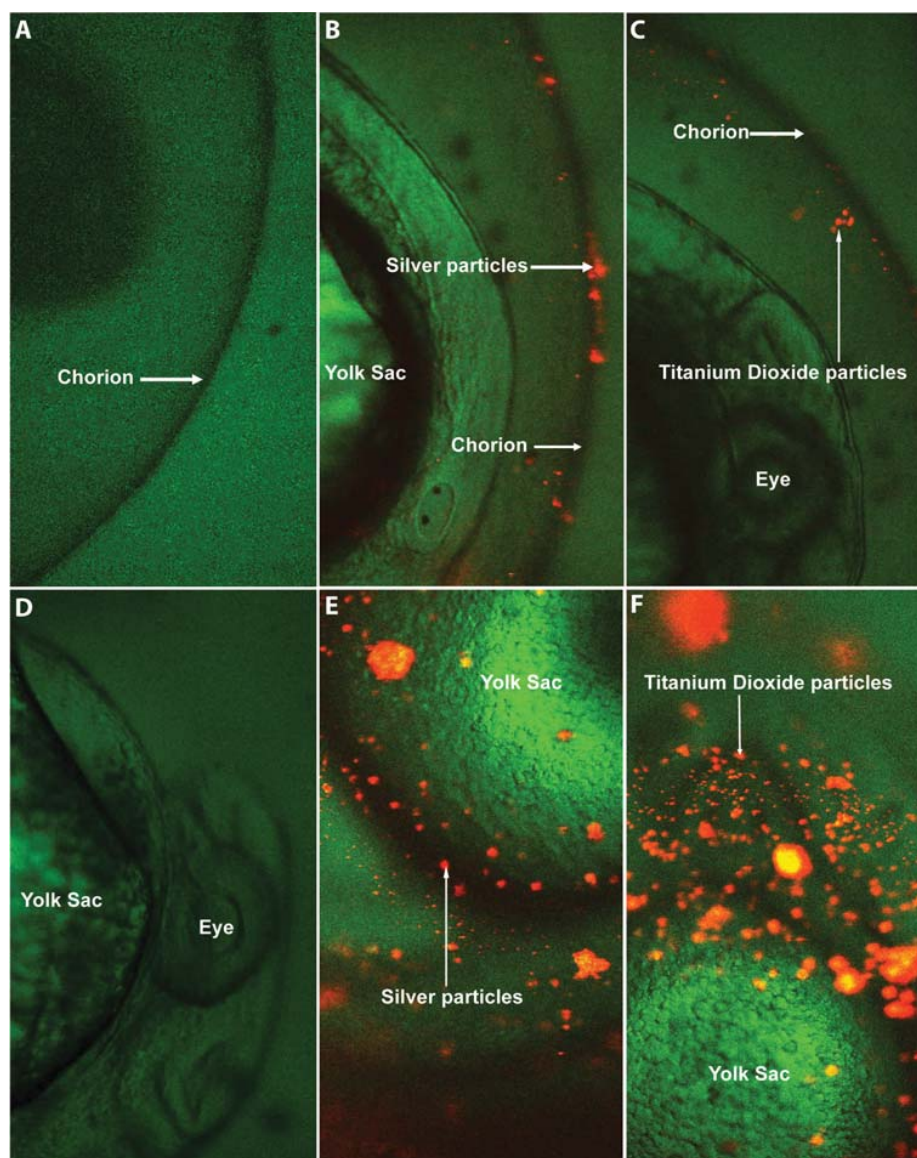


Figure 4. Coherent Anti-stokes Raman Scattering images of embryos exposed to silver and titanium nanoparticles after 24 h. Aggregates of nanoparticles appear as coloured (yellow/red) patches on the image. NPs were visible only on the outside of chorion membrane. A. Control embryo - showing chorion margin; B. Embryo exposed to Ag (35 nm, 25,000 µg/L) - showing chorion margin; C. Embryo exposed to TiO₂ (35 nm, 25,000 µg/L) - showing chorion margin; D. Control embryo showing chorion surface of embryo; E. Embryo exposed to Ag (35 nm, 500 µg/L) showing silver particles on chorion surface of embryo; F. Embryo exposed to TiO₂ (35 nm, 500 µg/L) showing titanium dioxide particles on chorion surface of embryo. CARS revealed nanomaterial on the surface of the chorion, likely as aggregates of nanoparticles B, C, but none were detected internally to the chorion within the embryo itself.

Discussion

We found TiO₂ nanoparticles had little or no toxicity in zebrafish embryos on the endpoints measured and at exposure levels far exceeding those predicted to occur in some of the most polluted environments (Colvin 2003). Our data support the majority of previous studies in this regard and would suggest, therefore, that in natural environments exposure to the TiO₂ particles tested are unlikely to pose any obvious health threat to fish embryos, which are widely accepted as highly sensitive to the effects of a wide range of toxicants.

In contrast Ag induced a dose-dependent toxicity in both nano and bulk form. One possible explanation for the

enhanced toxicity of the Ag-nano is that the particles themselves may interfere with biological processes because they have the potential to by-pass barriers which normally prevent larger molecules from entering (Scown et al. 2010). It is thought that nanoparticles can enter through pathways such as tight junctions (Luhmann et al. 2008) and, if this is the case, in turn block the channel pathways of epithelial membranes (Hunziker et al. 2009). But for these particles, this is unlikely as they were aggregated. Furthermore, an enhanced ability to cross cell membranes was not supported by the CARS imaging in this study, where at 24 hpf we found no evidence for uptake of Ag (or TiO₂) nanoparticles into the embryo (Figure 4).

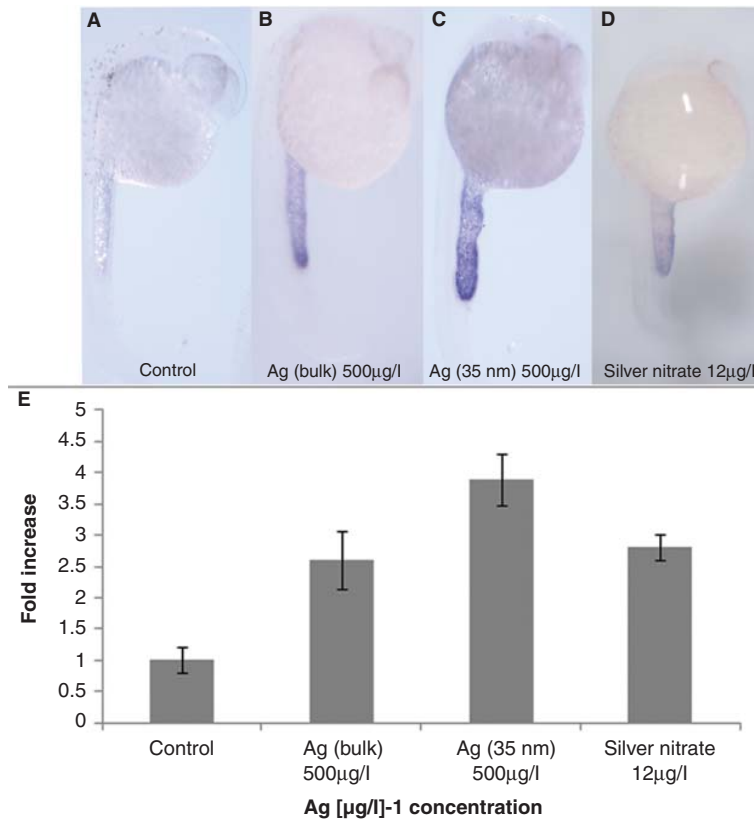


Figure 5. Embryos (24 hpf) after applying the technique *in situ* hybridisation to investigate the expression of metallothionein 2 as a measure of metal exposure/toxicity. There was a very low expression signal in the YSL especially at the posterior extension in the control embryos and in the embryos exposed to TiO_2 , but high expression in the Ag nano/bulk exposed embryos. A. Control embryo, B. Embryo exposed to Ag (Bulk 500 $\mu\text{g/L}$), C. Embryo exposed to Ag (35 nm, 500 $\mu\text{g/L}$), D. Embryo exposed to AgNO_3 (12 $\mu\text{g/L}$), E. Graphical representation of the fold-increase Mt2 expression in the different treatments.

Time lapse video analysis of the embryos exposed to nano and bulk silver established that it was during gastrulation, when the yolk sac folds in on itself over the cells, when the greatest mortality occurred. The necrosis assay confirmed a high incidence of damaged nuclei both in the blastoderm and the YSL in the Ag-exposed embryos at this development period (7 hpf). We recently reported that deformity of the YSL often results in failure of gastrulation cell movement which leads to embryonic lethality at the gastrula stage (Takesono et al. 2012). The developmental morphologies seen for exposure to silver particles (i.e., bent tails and a small head) are common for embryos exposed to xenobiotic compounds (Yeo & Kang 2008) and some abnormalities likely result from failed epiboly movement.

Citrate is used widely to stabilise NPs to prevent/reduce their aggregation (Baveye & Laba 2008), and here we found that coating 35 nm Ag particles with citrate reduced significantly rates of mortality and abnormalities in exposed embryos compared with uncoated 35 nm Ag; the LC50 of 35 nm Ag was 500 $\mu\text{g L}^{-1}$ compared with 5000 $\mu\text{g L}^{-1}$ for 35 nm Ag coated with citrate (i.e., 10-fold lower). No studies were undertaken to investigate the aggregation behaviour of the different particles in the embryo incubation water, but some other studies have shown that in high ionic strength water there can be an enhanced aggregation rate for particles coated with citrate (Christian et al. 2008). It is possible

therefore that an enhanced aggregation of the citrate-coated particles resulted in a lower bioavailability of silver particles/ions for uptake. Subtle differences in the nature of nanoparticle have also been found to profoundly affect biological effects responses (Moore 2006). An alternative hypothesis is that the toxicity of the silver nanoparticles derives from the dissolution of silver ions from the particles, and the rate of this process is much reduced in citrate-coated silver particles (Treuel et al. 2010; Studer et al. 2010; Kittler et al. 2010). Similarly, addition of fulvics to the medium also reduced the toxicity of the silver nanoparticles to the fish embryos. Such a coating could affect the particles by reducing silver particle dissolution rates, and/or complexing free Ag ions after dissolution.

It is well established that fish and many other aquatic animals are sensitive to the toxic effects of silver ions, with LC10 concentrations reported for rainbow trout (*Oncorhynchus mykiss*) between 0.7 $\mu\text{g L}^{-1}$ and 0.8 $\mu\text{g L}^{-1}$ and LC50 between 10 $\mu\text{g L}^{-1}$ and 240 $\mu\text{g L}^{-1}$ for freshwater fish species (Birge & Zulderveen 1996). The degree of dissolution (up to 2%) we found for Ag 35 nm equates well with previous literature (Kittler et al. 2010; Fabrega et al. 2009). Based on the amount of silver ions in solution, it appears that they do not explain all of the toxicity observed. However; the silver particles settle on the embryo surface (as evidenced by the CARS imaging), and therefore the local concentration of

dissolved Ag ions is likely to be higher at the membrane surface compared with the surrounding medium and therefore may explain all toxicity observed in our experiments. Nevertheless, it is still possible that the NP is having an effect directly on toxicity. These discrepancies further highlight the need for stringent reporting on the physiochemical characterisation of materials used. A further difficulty in relating the toxicity effects with the measured Ag⁺ is that the embryo medium contained relatively high levels of chloride ion (626.2 μmol L⁻¹), and this can complex Ag⁺ and in turn reduce its toxicity.

CARS images (Figure 4) illustrated that the nanomaterials, generally appearing as aggregates, were associated with the outer edge of the embryo (panels B and C) with no evidence for penetration of the embryo itself. This was supported by CARS images of embryos that were dechorionated and showed an absence of any nanomaterial at the embryo surface and again no evidence of body penetration. Contrasting with this, however, expression of Mt2, that plays a central role in metal transport, storage and detoxification (Ngu & Stillman 2006), strongly supports an intracellular presence of silver ions in exposed embryos/larvae.

We identified Mt2 expression in the (YSL) of the embryo, a body region where processing of xenobiotic compounds is known to occur in zebrafish embryos (Chen et al. 2004). The YSL was both the target for a toxicology response (cell necrosis) to Ag nanoparticles at the gastrula stage of development and location of Mt2 expression later in development (24 hpf), for exposure to the lower Ag exposure concentration. We found low level and more diffuse expression of Mt2 at 7.2 hpf compared with at 24 hpf, and this may confer a lower resistance of earlier life stage embryos to the toxic effects of Ag, but this would need further investigation to confirm this hypothesis. No such gene upregulation was seen in TiO₂-exposed embryos. These findings provide further evidence that at least some of the Ag toxicity relates to the bioavailability of silver ions that may be more readily released from nanoform silver. This would indicate the possibility for greater health effects associated with silver for Ag nanoparticle exposure. Our data further show Mt2 as an effective biomarker for exposure to silver nanoparticles in fish embryos. Where the release of silver ions occurs to induce the response in Mt2 is not known, it may potentially occur outside of the embryo from where the silver ions are then transported into the embryo or be released from Ag NPs that have penetrated the embryo, or a combination of both.

Conclusion

Our findings indicate that TiO₂ nanoparticles are not likely to have adverse biological effects in fish in the natural environment. In contrast, Ag NPs at sub-lethal exposure concentrations have the potential to induce harmful effects, disrupting embryo development predominantly at gastrula stage, inducing embryonic deformity at 1–2 cell stage and inducing the heavy metal stress response gene Mt2 in the (YSL). These reported effects occur predominantly at exposure levels exceeding those currently found (or estimated) in the most aquatic environments but with the rapid expansion

in the use and discharge of Ag NPs, concentrations in the aquatic environment are likely to rise in the near future (Simonet & Valcárcel 2009); reviewed in (Fabrega et al. 2011) heightening potential health concerns. Collectively, our data would suggest that silver ions play a major role in the toxicity of Ag NPs and furthermore we show that coating of the particles, here with citrate or natural organic matter (here fulvics) can reduce significantly associated toxicity with major implications for understanding toxicity of metal NPs in the natural environment.

Supportive Information

The supportive information contains extra information on the materials and methods. In addition it contains data for the characterisation of the titanium dioxide particles.

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Declaration of interest

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References

- AshaRani PV, Hande MP, Valiyaveetil S. 2009. Anti-proliferative activity of silver nanoparticles. *BMC Cell Biol* 10:65.
- Baveye P, Laba M. 2008. Aggregation and toxicology of titanium dioxide nanoparticles. *Environ Health Perspect* 116:A152.
- Birge W, Zulderveen J. 1996. The Comparative Toxicity of Silver to Aquatic Biota. University of Wisconsin System, Sea Grant Institute. Available at <http://images.library.wisc.edu/EcoNatRes/EFacs/Argentum/Argentumv03/reference/econatres.argentumv03.birgetoxicity.pdf>. Accessed October 2011.
- Braydich-Stolle L, Hussain S, Schlager J, Hofmann M. 2005. In vitro cytotoxicity of nanoparticles in mammalian germline stem cells. *Toxicol Sci* 88:412.
- Chen W, John J, Lin C, Lin H, Wu S, Chang C. 2004. Expression of metallothionein gene during embryonic and early larval development in zebrafish. *Aquat Toxicol* 69:215–227.
- Choi JE, Kim S, Ahn JH, Youn P, Kang JS, Park K, et al. 2009. Induction of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. *Aquat Toxicol* 100:151–159.
- Christian P, Von der Kammer F, Baalousha M, Hofmann T. 2008. Nanoparticles: structure, properties, preparation and behaviour in environmental media. *Ecotoxicology* 17:326–343.
- Colvin VL. 2003. The potential environmental impact of engineered nanomaterials. *Nat Biotechnol* 21:1166–1170.
- Fabrega J, Fawcett SR, Renshaw JC, Lead JR. 2009. Silver nanoparticle impact on bacterial growth: effect of pH, concentration, and organic matter. *Environ Sci Technol* 43:7285–7290.
- Fabrega J, Luoma SN, Tyler CR, Galloway TS, Lead JR. 2011. Silver nanoparticles: behaviour and effects in the aquatic environment. *Environ Int* 37:517–531.
- Heinlaan M, Ivask A, Blinova I, Dubourguier HC, Kahru A. 2008. Toxicity of nanosized and bulk ZnO, CuO and TiO₂ to bacteria *vibrio fischeri* and crustaceans *daphnia magna* and *thamnocephalus platyurus*. *Chemosphere* 71:1308–1316.
- Hu Z. 2010. Impact of Silver Nanoparticles on Wastewater Treatment. *Water Intelligence Online* 9:58. Available at <http://www.mendeley.com/research/impact-silver-nanoparticles-wastewater-treatment/>. Accessed November 2011.
- Hunziker W, Kiener T, Xu J. 2009. Vertebrate animal models unravel physiological roles for zonula occludens tight junction adaptor proteins. *Ann N Y Acad Sci* 1165:28–33.

- Hussain S, Hess K, Gearhart J, Geiss K, Schlager J. 2005. In vitro toxicity of nanoparticles in BRL 3A rat liver cells. *Toxicol In Vitro* 19:975-983.
- Jin X, Li M, Wang J, Marambio-Jones C, Peng F, Huang X, et al. 2010. High-throughput screening of silver nanoparticle stability and bacterial inactivation in aquatic media: influence of specific ions. *Environ Sci Technol* 44:7321-7328.
- Ju-Nam Y, Baalousha M, Cole P, Tyler C, Stone V, Fernandes T, et al. 2012. Characterisation of cerium dioxide NPs. Part I: size measurements. *Environ Toxicol Chem* in press.
- Kittler S, Greulich C, Diendorf J, Koller M, Epple M. 2010. Toxicity of silver nanoparticles increases during storage because of slow dissolution under release of silver ions. *Chem Mater* 22:4548-4554.
- Lee KJ, Nallathambi PD, Browning LM, Osgood CJ, Xu XHN. 2007. In vivo imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. *ACS Nano* 1:133-143.
- Long T, Tajuba J, Sama P, Saleh N, Swartz C, Parker J, et al. 2007. Nanosize titanium dioxide stimulates reactive oxygen species in brain microglia and damages neurons in vitro. *Environ Health Perspect* 115:1631.
- Lovern SB, Strickler JR, Klaper R. 2007. Behavioral and physiological changes in *Daphnia magna* when exposed to nanoparticle suspensions (titanium dioxide, nano-C60, and C60HxC70Hx). *Environ Sci Technol* 41:4465-4470.
- Luhmann C, Chun M, Yi D, Lee D, Wang X. 2008. Neural dissociation of delay and uncertainty in intertemporal choice. *J Neurosci* 28:14459.
- Luoma SN, Rainbow P. 2008. Metal contamination in aquatic environments: science and lateral management. Cambridge: Cambridge University Press.
- Marambio-Jones C, Hoek EMV. 2010. A review of the antibacterial effects of silver nanomaterials and potential implications for human health and the environment. *J Nanopart Res* 12:1531-1551.
- Moore M. 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? *Environ Int* 32:967-976.
- Ngu T, Stillman M. 2006. Arsenic binding to human metallothionein. *J Am Chem Soc* 128:12473-12483.
- Nowack B, Bucheli TD. 2007. Occurrence, behavior and effects of nanoparticles in the environment. *Environ Pollut* 150:5-22.
- Pinero J, Lopez-Baena M, Ortiz T, Cortes F. 1997. Apoptotic and necrotic cell death are both induced by electroporation in HL60 human promyeloid leukaemia cells. *Apoptosis* 2:330-336.
- Purcell TW, Peters JJ. 1999. Historical impacts of environmental regulation of silver. *Environ Toxicol Chem* 18:3-8.
- Scown T, Van Aerle R, Johnston B, Cumberland S, Lead J, Owen R, et al. 2009. High doses of intravenously administered titanium dioxide nanoparticles accumulate in the kidneys of rainbow trout but with no observable impairment of renal function. *Toxicol Sci* 109:372.
- Scown T, Van Aerle R, Tyler C. 2010. Review: do engineered nanoparticles pose a significant threat to the aquatic environment? *Crit Rev Toxicol* 40:653-670.
- Simonet B, Valcárcel M. 2009. Monitoring nanoparticles in the environment. *Anal Bioanal Chem* 393:17-21.
- Skebo J, Grabinski C, Schrand A, Schlager J, Hussain S. 2007. Assessment of metal nanoparticle agglomeration, uptake, and interaction using high-illuminating system. *Int J Toxicol* 26:135.
- Studer AM, Limbach LK, Van Duc L, Krumeich F, Athanassiou EK, Gerber LC, et al. 2010. Nanoparticle cytotoxicity depends on intracellular solubility: Comparison of stabilized copper metal and degradable copper oxide nanoparticles. *Toxicol Lett* 197:169-174.
- Takesono A, Moger J, Farooq S, Cartwright E, Dawid IB, Wilson SW, et al. 2012. Solute carrier family 3 member 2 (Slc3a2) controls yolk syncytial layer (YSL) formation by regulating microtubule networks in the zebrafish embryo. *Proc Natl Acad Sci* 109:3371-3376.
- Treuel L, Malissek M, Gebauer J, Zellner R. 2010. The influence of surface composition of nanoparticles on their interactions with serum albumin. *ChemPhysChem* 11:3093-3099.
- Yeo M, Kang M. 2008. Effects of nanometer sized silver materials on biological toxicity during zebrafish embryogenesis. *Bull Korean Chem Soc* 29:1179-1184.
- Zhao J, Bowman L, Zhang X, Vallyathan V, Young S, Castranova V, et al. 2009. Titanium dioxide (TiO₂) nanoparticles induce JB6 cell apoptosis through activation of the caspase-8/Bid and mitochondrial pathways. *J Toxicol Environment Health A* 72:1141-1149.

Supplementary material available online

Supplementary Tables SI–SIV.

Supplementary Figures S1, S2.