THE ECOTOXICOLOGY OF ENGINEERED NANOPARTICLES TO FRESHWATER FISH

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THE ECOTOXICOLOGY OF ENGINEERED NANOPARTICLES TO FRESHWATER FISH

by

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The ecotoxicology of engineered nanoparticles to freshwater fish

Abstract

The use of nanoscale materials is growing exponentially, but there are also concerns about the environmental hazard to aquatic biota. Metal-containing engineered nanoparticles (NPs) are an important group of these new materials, and whilst there are undoubtedly a plethora of beneficial uses for these NPs, it is essential that an appropriate risk assessment is carried out in order to protect the environment and human health, with the consumption of contaminated fish a distinct possibility. The current study aimed to assess the bioavailability, uptake and toxicological effects of two metal-NPs (TiO_2 NPs and Cu-NPs) to fish from both dietary and waterborne exposure routes and where appropriate compare them to their bulk counterpart. Whole body system effects were assessed along with the influences of the life stage of exposed fish and abiotic factors on toxicity. A technique to improve the quantification of Ti from TiO_2 NPs in fish tissue was also developed. Effects from exposure to dietary TiO_2 NPs manifested similarly to traditional dietary metal exposure, with no reduction in growth, but some sublethal affects. Exposure to waterborne Cu-NPs showed that rainbow trout were more acutely sensitive to $CuSO_4$ than the NPs, but that despite limited uptake several body systems were affected (most notably ionoregulation). Larvae were more sensitive to $CuSO_4$ than Cu-NPs, but no differences were seen with embryos, whilst larvae were more sensitive than embryos. Abiotic factors did have an effect on acute Cu-NP toxicity, though not always in a predictable manner, with some effects more pronounced than with $CuSO_4$. Overall, it appears that metal-NPs are not as acutely toxic as their bulk counterparts, but sublethal effects, were routinely observed. As TiO_2 NPs appear more toxic than its bulk counterpart, current legislation governing safe environmental limits may have to be adjusted, though the situation with Cu-NPs isn't as clear and further investigation is required. However, the risk of human exposure via the consumption of NP contaminated fish fillets is extremely low.

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From PhD thesis:

Shaw, B.J., Handy, R.D., 2011. The toxicological effects of waterborne copper nanoparticles versus copper sulphate on rainbow trout, (Oncorhynchus mykiss). *Submitted.* (Chapter 5).

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Platform presentation:

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Poster presentations:

Shaw, B., Al-Bairuty, G., Handy, R. 2010. The effects of nanocopper compared to aqueous copper sulphate on juvenile rainbow trout (*Oncorhynchus mykiss*). Marine Institute Conference, University of Plymouth, 20th December 2010, Plymouth, UK.

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(I apologise if I have missed anyone!)

AUTHOR'S DECLARATION

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Signed .....

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For Sophie and the children

Chapter 1. General Introduction.

1.1 Introduction

The ecophysiology and toxicology of trace metals in fish has been the focus of extensive research for many years (Henry and Atchison, 1991; Spry and Weiner, 1991; Wendelaar Bonga and Lock, 1992; Handy, 1996; Clearwater et al., 2002; Kamunde and Wood, 2004; Handy et al., 2005). This has led to a consensus view on the main effects of metals on fish, as well as the associated environmental hazards and risks (review, Campbell et al., 2006). It is well established that fish have essential nutrition requirements for trace metals (e.g. copper, zinc, iron, Bury et al., 2003), and that some trace metals are "non-essential" with no known or important biological role (e.g. mercury, silver). The bioavailability and biological uptake of trace metals by fish has been given special attention by researchers. These studies include detailed models of trace element speciation in water (e.g., free ion activity models, van Leeuwen et al., 2005) and investigations on the uptake of particular trace metal species by fish (e.g., Playle et al., 1993). The effects of abiotic factors in water chemistry, such as pH and water hardness, on metal uptake and toxicity are well known (e.g., Cusimano et al., 1986; Erickson et al., 2008) and models have been constructed to predict metal toxicity (e.g., biotic ligand models; Paquin et al., 2002; Niyogi and Wood, 2004). The target organs, mechanisms of sequestration and storage of metals in the biological systems of fish, and excretion have also been studied (e.g., Grosell et al., 1997), as well as the effects upon fish behaviour (review, Scott and Sloman, 2004).

In recent years, novel chemical engineering techniques have produced nanomaterials (NMs), and the application of these materials in products and processes is known as nanotechnology. Engineered nanomaterials comprise of numerous different physical forms and some of these materials have adverse effects on fish including; carbon nanotubes (e.g., Smith et al., 2007), carbon spheres called fullerenes (e.g., C_{60}, Zhu et al., 2007) and

nanoparticles (NPs) made from metals (e.g., Cu, Griffitt et al., 2007), metal oxides (e.g., TiO_2, Federici et al., 2007), or composites made of several metals (e.g., quantum dots, King-Heiden et al., 2009). Engineered NPs can also be made into many different shapes (e.g., wires, rods or spherical particles), and can be functionalised with almost any type of surface chemistry. Thus even carbon-based nanomaterials can be functionalised with metals, for example; metal-coated carbon nanotubes (Meng et al., 2009a) and metal encapsulated fullerenes, known as endohedral metallofullerenes, (Śliwa, 1996). Also in development are hybrid versions of (nano)materials containing more than one NP or metal (e.g., ZnS:Cu, ZnS nanoparticles co-doped with Pb^{2+} and Cu^{2+}, Yang et al., 2001; Cu-doped CdSe nanocrystals, Meulenberg et al., 2004; CdS–TiO_2 nano-composite, So et al., 2004; Ag-Cu nanoparticle-doped dental amalgams, Chung et al., 2008; Cu-TiO_2 nanocomposite coatings, Ramalingam et al., 2009). This has led to considerable debate on how to classify these new materials, but engineered nanomaterials are generally considered to be materials with at least one dimension of less than 100 nm (Roco, 2003). This definition has been adopted by the British Standards Institution, the American Society for Testing Materials, and the Scientific Committee on Emerging and Newly-Identified Health Risks (see reviews on NPs, Handy et al., 2008a; Klaine et al., 2008). However, it is important to note that this is a somewhat arbitrary size cut off from the view point of ecotoxicity, and it may be prudent to also consider aggregates of NPs that can be a few hundred nanometers wide (Handy and Shaw, 2007a) or that have a distribution of particles around the nanoscale; but may have some primary particles larger than 100 nm (Handy et al, 2008a).

The focus of this review is on nanomaterials that contain metals, e.g., nanoparticles made of metal or metal oxides (hereafter referred to as nanometals). Some of the types of nanometals and their product applications are outlined in Table 1.1, and although these uses will likely be continuously changing and evolving with commercial markets.

Table 1.1. Nanoparticles in production and examples of current or proposed use.

Nanoparticle or nanomaterial	**Application**
Aluminium oxide	Optical polishing, cosmetics, clothing
C_{60} Fullerenes	Hydrogen storage, drug delivery, therapeutics, coatings & pigments, lubrication, cosmetics
Carbon nanotubes (single or multi walled)	Hydrogen storage, drug delivery, textiles, electronics, water purification, sporting equipment
Ceramics	Electronics, anti-oxidants, car polish
Copper or copper oxide	Lubrication oil additive, electronics & computer processors, conductive coatings, printer inks, sintering additives, 'anti-aging' cream & skin conditioner, mineral supplements
Gold	Drug delivery, labels for immunocytochemistry, biological hazard detection e.g. ricin, E. Coli, mineral supplements
Iron oxide	Ultrafiltration, oxidation reduction catalyst
Iron sulphide	Removal of organochlorine pesticides from drinking water
Nanocrystals	Insulators, drug delivery
Nano-rods	Electronics, sensors & sensing devices
Polymers	Therapeutics, coatings & pigments, lubrication, absorbents

Quantum dots	Medical imaging, photonics
Silica	Photovaltaics, optics & optical devices, anti-graffiti paint, cosmetics
Silver	Antibacterial uses in water treatment, fabric softener, clothing, soft toys, wound dressing, kitchen utensils and appliances, computer keyboards, food storage containers, and baby products (e.g. cups) and uses in contraception and toothpaste
Titanium dioxide	Paint, sunscreen, cosmetics, capacitors, building materials, catalyst, air clearance, anti: bacterial, viral, algal, fungus & mould coating for domestic baths, sporting equipment
Nano-vitamins (some vitamins encapsulated in nano-delivery vehicles)	Vitamin E; Food, beverages & cosmetics, vitamins B12 & E; cosmetics
Zinc oxide	Sunscreen, cosmetics, cosmetic remover, foot deodorant, car polish

Data source: The Project on Emerging Nanotechnologies, The Woodrow Wilson International Center for Scholars; http://www.nanotechproject.org/inventories/consumer/.

It is clear that these new materials can include particles made from different types of metals, including metals that are consider to be toxic in the traditional dissolved form (e.g., Ag, Hogstrand and Wood, 1998; Cu, Handy, 2003; Zn, Hogstrand and Wood, 1996). It is therefore important to determine whether or not the alternative "nano" forms of metals are also toxic. Moreover, nanotechnology is a rapidly expanding industry. In 2006 the nanotechnology sector had achieved a multibillion dollar market, with predictions that it will grow to a trillion US dollars by 2015 (Aitken et al., 2006). Lux business forecast also suggests that nanotechnology products are worth about 1,500 billion dollars in 2010 (http://www.nanowerk.com/spotlight/spotid=1792.php). The Woodrow Wilson International Centre for Scholars product inventory of nanomaterials, indicted that 1015 different nano-containing products were on the market in 2009, and these products mostly involved metal-based nanomaterials (http://www.nanotechproject.org/inventories/consumer/analysis_draft). Peer reviewed scientific reports also show that products containing nanometals are well represented (Hansen et al., 2008). It is therefore likely that nanomaterials are already being released into the environment (Owen and Handy, 2007; Ju-Nam and Lead, 2008; Gottschalk et al., 2009). Modelling techniques have predicted low $\mu g\ l^{-1}$ concentrations of NPs in the environment over the long term (Boxall et al., 2007), and this has beem confirmed by field measurements for at least one nanometal (TiO_2, Kaegi et al., 2008). It is clear that the environmental hazard of NPs, and especially nanometals, needs to be addressed. The overall aim of this review is therefore to use our existing conceptual framework of metal toxicity in fish, and to compare and contrast this with emerging information on nano forms of metals. The issue of hazard is approached from the view point of body systems physiology of the fish, starting with water chemistry and uptake routes, and consideration of the toxicological principles of absorption, distribution, metabolism, excretion (ADME). Lethal toxicity and sublethal toxic effects are described with some speculation on toxic mechanisms likely to be

of concern for nanometals, as well as the likely effects on the stress response of fish. Finally, consideration is given on the interpretation of physiological effects data on nanomaterials in the context of hazard assessment and whether or not the current approaches used for dissolved metals would also be "fit for purpose" for nanometals.

1.2 Water chemistry and bioavailability

The importance of water chemistry on the bioavailability of metals has been extensively investigated over many years. The main paradigm is that the free metal ion is the bioavailable chemical species, and therefore research has focused on identifying metal speciation in water and how this is influenced by abiotic factors such as pH, and the presence of ligands in the water that may remove free ions (e.g., dissolved organic matter), anions that can complex metals or form anionic metal species (e.g., chloride, hydroxyl ions), or cations (e.g., H^+, Ca^{2+}, Mg^{2+}, Na^+) that may be competing for biological uptake with trace metals (e.g., Cusimano et al., 1986; Campbell, 1995; Bervoets and Blust, 2000; Wood, 2001; Pyle et al., 2002; van Leeuwen and Galceran, 2004; van Leeuwen et al., 2005; Erickson et al., 2008). For elements that have different redox states, the level of oxygenation and/or redox potential of the natural water may also be a consideration in calculating the metal speciation (e.g., the metalloid arsenic, Cheng et al., 2009). However for most of the simple equilibrium models (e.g., WHAM speciation model, MINEQL), the main factors are the total concentration of the metal, pH, concentrations of divalent cations (or total hardness of the water), and the electrolytes contributing to ionic strength (e.g., bulk NaCl concentration in freshwater or seawater; review, Wilkinson and Buffle, 2004). These simplified models are intended for practical use in predicting free metal ion concentrations, and have been applied successfully in hazard assessment for many years (e.g., corrections for water hardness or pH

in metals legislation) and to predictive models of acute metal toxicity (e.g., biotic ligand models, BLM, Playle et al., 1993; Hollis et al., 1997; Paquin et al., 2002).

The environmental chemistry and ecotoxicity of engineered NPs has recently been reviewed (see Handy et al., 2008a). This chemistry shares some superficial similarities with metal chemistry in that abiotic factors such as pH, the presence of divalent ions, and ionic strength can influence the colloidal behaviour (aggregation) of NPs. However, the reasons for these interactions are often fundamentally different to those for dissolved metals. Current metal speciation models are equilibrium models, whereas the behaviour of NPs is described in a very different way by dynamic process where the system is dependent on the amount of energy added to the NP dispersion and the physico-chemical properties of the particles (called Derjaguin, Landau, Verwey and Overbeek (DLVO) theory, see Handy et al., 2008a for discussion). These physico-chemical properties include particle size, shape (aspect ratio), and surface charge (often measured as zeta potentials). The ability of a particle to colloid and aggregate with another particle will depend on these properties, as well as the kinetic energy of the particles, viscosity of the water and any drag on the particles, and the presence of other materials in natural waters such as small peptides (e.g., bacterial exudates) or macromolecules (e.g., humic acids) that may provide steric hindrance of particle-particle interactions. Clearly, this is a fundamentally different chemistry to the metal speciation models that are more familiar to the fish ecotoxicologists.

However, there is also some common ground. One concept in NP toxicity is that the toxicity may be driven by the surface chemistry of the particles. The aggregation chemistry will be useful in predicting the effective surface area of the material in the context of potential interaction with the organism, but the surface chemistry (reactivity) may inform on toxicity. These ideas are not yet proven by experimental data, but for example, one might expect a particle with an oxidising metal surface to cause oxidative stress on/in the organism.

This is certainly the case for at least one type of TiO_2 NPs which caused oxidative stress in trout (Federici et al., 2007). So, perhaps metal reactivity rather than the concentration of the free metal ion will be more important in the case of NPs. Nonetheless there is a scenario where existing free metal ion models may apply, with some modifications. Some types of nanometals will eventually dissolve (albeit slowly over several hours or days) by dissolution of metal ions from the surface of the particle. This is probably the case for Ag-NPs and Cu-NPs in freshwater. In a study comparing the effects of three nanometals (Cu-NPs, Ag-NPs, and TiO_2 NPs) with dissolved metals (salts of Cu and Ag at concentrations to match estimated dissolved ions from each NP), Griffitt et al. (2009) found that both dissolved and nanoparticulate forms of Cu and Ag increased metal levels in the gill tissue after 48 h. Copper accumulation in the gill was similar following exposure to both soluble and nanoparticulate Cu; and the authors interpreted this as uptake of dissolved Cu from the Cu-NP. Conversely, branchial Ag levels were much greater in fish exposed to Ag-NPs compared to dissolved Ag, indicating that the NPs themselves (not dissolved Ag from the NPs) were contributing to the branchial Ag burden (Griffitt et al., 2009). As the authors do not mention rinsing excised gills in, for example, ion-free water prior to analysis, it is possible that Ag-NPs associated with the gills, but not actually taken up into the cells, contributed to total burden. Though they do acknowledge the possibility of Ag-NPs being trapped in the mucous layer, this is not gill Ag burden per se, although it does suggest that Ag-NPs could act as a respiratory irritant similar to other NPs (e.g., single-walled carbon nanotubes (SWCNT), Smith et al., 2007). Scown et al. (2010) also observed increased Ag burden in the gills and liver of fish exposed to different sized Ag-NPs (10 and 35 nm particles compared to bulk Ag and $AgNO_3$), though similarly no mention was made of rinsing tissue samples to remove associated, but not internally localised, NPs (particularly relevant to gill samples) prior to analysis. However, increased hepatic Ag (twice that of the gills) does indicate that there was

some uptake and transport of Ag from the NPs, which the author's state cannot be explained by dissolution of Ag ions alone. As gut Ag was not assessed the possibility of hepatic uptake via the gut (stress induced drinking and incidental NP ingestion) cannot be excluded.

In situations involving dissolution, the NP is acting as a "delivery vehicle" for the metal ions, and when the NP sticks to gill surface (for example) it could release locally high concentrations of metal ions, or provide a sustained slow release of metal ions onto the epithelia. In this scenario the free metal ion concentration would be driving toxicity, and it would be relatively straight forward to add an empirical correction factor (measured experimentally) for the dissolution rate of free metal ions for a known mass concentration of particles in a particular water quality.

There is also evidence of "delivery vehicle" effects when the metal is present as a co-contaminant with a NP. This may be related to the ability of metals to adsorp to the surface of some negatively charged NPs (Handy et al., 2008b). For example, Zhang et al. (2007) exposed carp to TiO_2 NPs and found that the fish accumulated 146 % more Cd in the presence of the TiO_2 NPs compared to when fish were exposed to Cd alone. Similarly, carp exposed to the metalloid, arsenic (As(V)), in the presence of TiO_2 NPs accumulated 132 % more As than fish exposed to an equal concentration of As only (Sun et al., 2007). In a follow up study, Sun et al. (2009) investigated the impact of TiO_2 NPs on arsenic speciation and the uptake in carp of the more toxic form; As (III). However, in separate speciation experiments (no fish), when introduced to TiO_2 NP containing water under natural light, As (III) rapidly oxidised to the less toxic As (V), although experiments without sunlight resulted in less than 3 % of As (III) changing to As (V). Therefore in the experimental conditions used (i.e., under sun light), fish were not exposed to great amounts of As (III), although it remains to be seen if increased toxicity would manifest in fish living in conditions with less natural light. Many

NPs have a net negative charge (anionic surface) in natural water and the possibility of NPs increasing metal toxicity via a vehicle or mixtures effect clearly requires further study.

1.3 Absorption of nanoparticles compared to metal ions

The mechanisms of metal uptake across biological membranes involves carrier-mediated transport on metal ion transporters (reviews, Bury et al., 2003; Handy and Eddy, 2004; Bury and Handy, 2010). The biological uptake and unstirred layer chemistry of NPs in fish gills has been recently reviewed in detail (see Handy et al., 2008b), and the main differences between metal ions and NPs are summarised (Fig. 1.1). The central issue is that NPs are too big to use ion transporters, or paracellular diffusion pathways, and that the most likely route of uptake is by endocytosis pathways (Fig. 1.1). If this is true, then the scientific community will need to reconsider the basic assumptions in metal uptake, and revise them for nanometals. For dissolved metals, bioavailability is assumed to be a function of metal speciation since only certain metal species will fit the relevant ion transport pathway, and diffusion of metal ions through the hydrophobic core of membranes is excluded (Campbell and Stokes, 1985; Campbell, 1995; Escher and Sigg, 2004).

For very small NPs (< 20 nm) that may obtain a hydrophilic surface coat (e.g., steric effects coating the particle with phospholipids), or achieve no net charge to become hydrophobic (e.g., positively charged nanometals that are incidentally coated with biological anions such as those found in mucus, Handy and Maunder, 2009); it remains theoretically possible for these materials to diffuse through the cell membrane. We should therefore add a diffusional component for nanometal uptake that is both a function of particle size and the hydrophobicity of the particle surface. Endocytosis should also be modelled, and here evidence may be drawn from trace metals that are known to use endocytosis pathways for

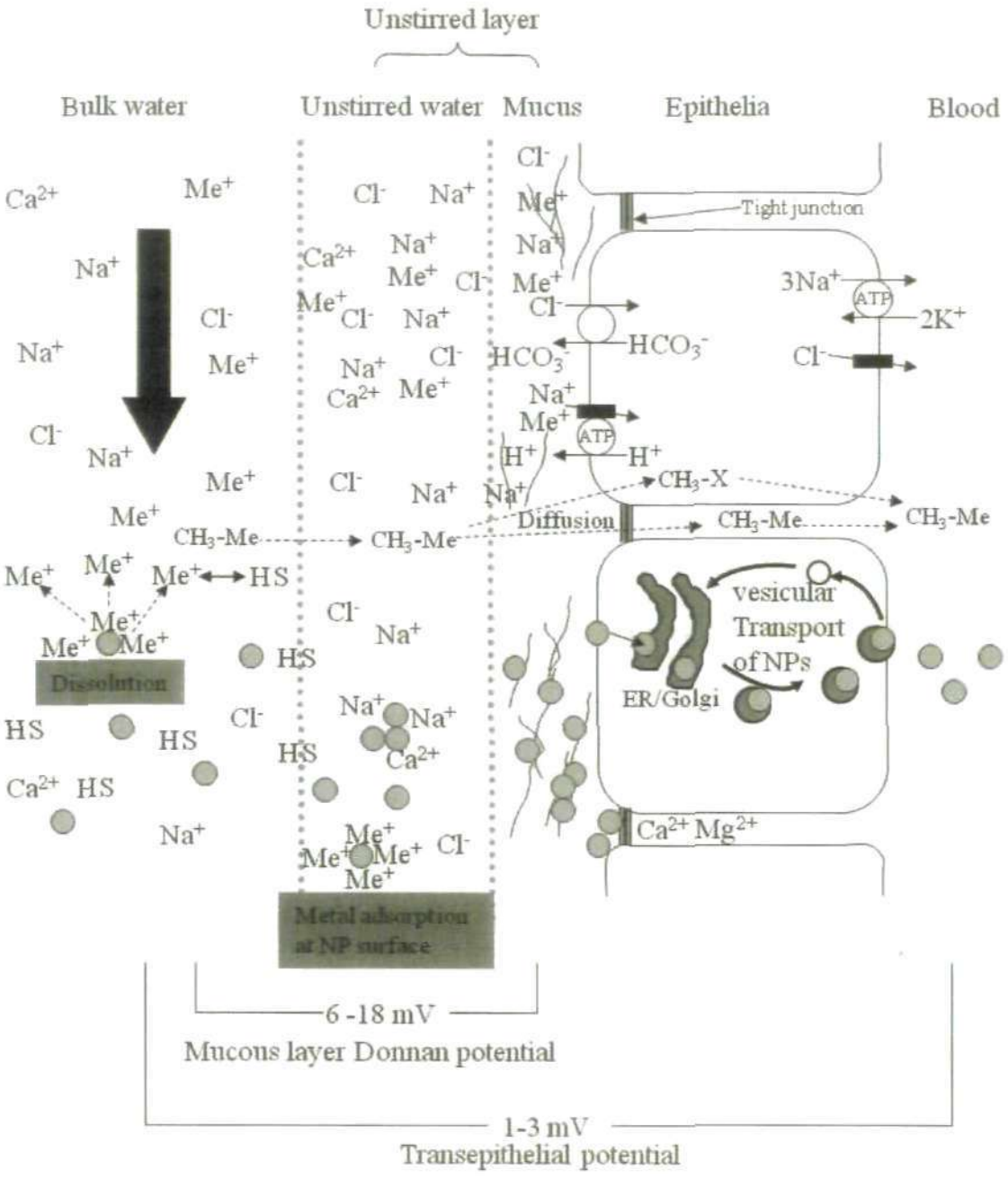


Figure. 1.1. An idealised diagram of the freshwater fish gill showing the mechanisms of uptake for electrolytes, toxic metal ions (Me^+), and electroneutral diffusion of some small organo-metals (CH_3-Me), compared to nanoparticles (NPs, filled circles). Modified from Handy et al. (2008b), and Handy and Eddy (2004). The substances in the bulk solution (the freshwater) must diffuse into an unstirred layer (USL) comprising of water/mucus secretions, prior to transfer across the gill epithelium. The upper portion of the diagram shows electrolytes and toxic metals ions which diffuse into the USL, and may bind to strands of mucus (mostly polyanionic) where the exclusion of free anions like Cl^- from the mucus layer

contributes to the Donnan potential at the apical surface. Electrolytes and toxic metal ions usually move through the cell using ion transport pathways (Na^+ transporters are illustrated here). In contrast, small electroneutral organo-metals may diffuse into the USL and then through the cells (transcellular diffusion), or between the cells via the tight junctions (paracellular diffusion). The situation for NP uptake across the gill will be a little different. NPs will diffuse into the USL, albeit at a slower rate than smaller molecules or solutes, and may be influenced by humic substances (HS). Cationic NPs will bind to strands of mucus (electrostatic attraction, fundamentally similar to other cations), but regardless of surface charge, may also become entangled in the mucoproteins (steric hindrance) to prevent uptake by the epithelial cells. NPs are too large to be taken up by ion or other transporters on the cell membranes, and although diffusion cannot be excluded for lipophilic NPs. The Ca^{2+} and Mg^{2+} rich environment in the tight junctions suggest that NPs would aggregate rather than diffuse through the paracellular route. In addition, some nanometals may release free metal ion (Me^+) by dissolution of ions into the bulk solution. In contrast, nanomaterials can also show surface adsorption of metals, and this is likely to be faster in the higher ionic strength of the USL. Diffusion of charged NPs into the USL will be affected by the Donnan and transepithelial potentials, in a similar way to other charged substances. NP uptake through vesicular transport seems likely.

uptake (e.g., Cu, Handy et al., 2000). However unlike dissolved metals, it will not be the activity of a metal ATPase controlling the loading of intracellular metal-containing vesicles (e.g., Cu ions via the Cu-ATPase), but instead, the bulk endocytosis of particles at the apical membrane. Clearly, for the latter the control of the cytoskeleton, membrane turnover, and related aspects of cell volume control will be critical, and metals are known to influence regulatory volume decrease (e.g., Cu, Kramhøft et al., 1988). The rate limiting step in NP uptake may not be the basolateral step as so often is the case for metal ions (Handy et al., 2008b). However, if the nanometal dissolves to release free ions then the current uptake models would also apply. Some of the apical events, such as the protective role of mucus, would also seem appropriate for NPs (Handy et al., 2008b). Smith et al. (2007) have already observed precipitation of carbon nanotubes in the gill mucus of trout. Similar to metal ions, the uptake site (e.g., gill, intestine) could also be a target organ for toxic effects. Some NPs are respiratory toxicants in fish and produce gill pathologies which are similar to those for metal ions (Federici et al., 2007; Smith et al., 2007). Similar to surface acting metals (e.g., Al, Handy and Eddy, 1989), it remains possible that some NPs can exert toxic effects without appreciable uptake of the metal into the internal organs (e.g., short waterborne exposures to TiO_2, Federici et al., 2007).

1.4 Distribution, metabolism and excretion of nanometals

Handy et al. (2008b) reviews the possible mechanisms of absorption, distribution, metabolism, and excretion (ADME) for NPs in fish. This review identifies numerous knowledge gaps on the ADME of NPs, and most of this is centred on a lack of routine methods for the direct measurement of NPs in tissues in order to establish body distributions and target organs. Current approaches involve electron microscopy of dissected tissues,

which is very labour intensive way of determining the presence or absence of nanometals (see Handy et al., 2008a for discussion of NP detection methods). The development of radiolabelled metal NPs is needed, but even here there are technical concerns. For example, if the exterior of the NP is labelled with a metal isotope, it may be possible for the label to become detached by dissolution of the NP in the tissue. Clearly, such methods will need thorough validation. The detection of metal in fish tissues remains possible using acid digestions followed by ICP-MS or ICP-OES for the metal of interest (e.g., Federici et al., 2007; Ramsden et al., 2009). However, nanometals do not behave the same way as metal ions in ICP-MS, and standard protocols for metal digestion and analysis may not work for NPs. Substantial modifications and revalidation of spike recovery tests, etc., are needed for nanometals. Some researchers report very poor recoveries of total tissue metal contents from nanometals (e.g., 28 %, Scown et al., 2009). Such poor recoveries typically generate large error bars on mean data values for the metal content of individual tissues, and it is likely that important differences in tissue metal content are lost due to poor resolution in the method. However careful adaptation of methods, involving correcting instrument calibrations for NPs, the addition of surfactants or dispersants, and careful stirring of samples while they are being drawn into the instrument can result in much improved metal recoveries (e.g., Ramsden et al., 2009). It is possible to identify target organs on the basis of biological effects, but as Federici et al. (2007) point out for TiO_2 NPs at the gills, surface acting toxicants can generate injury to internal organs through secondary means (e.g. diffusion of oxygen radicals). There is a concern that some oxidising nanometals could be potent surface acting toxicants, and therefore, injury to an internal organ does not necessarily confirm the presence of the nanometal in that organ. Nonetheless on the basis of biological effects, a number of target organs have been suggested for nanometals including the gills, gut, liver and brain (see below). Some of these are also well known target organs for metal ions.

Studies on the metabolism and excretion of nanometals are limited for similar technical reasons to those outlined above. Fish normally excrete trace metal ions via the liver as the central compartment in metal metabolism, with urinary losses usually being small (e.g., Grosell et al., 1998). The gills may also be able to excrete metal ions from the systemic circulation by active efflux on branchial ion transport pathways, but this would not apply to nanometals. The molecular weight cut off in the vertebrate kidney (circa 60 kDa) would suggest that NPs would not pass through the glomerular filter. This leaves the liver as the most likely route of excretion for nanometals (see discussion in Handy et al., 2008b). Fish are known to deposit metal granules in the liver (e.g. Lanno et al. 1987), and it would seem logical that nanometals could also form hepatic deposits. It remains unknown if such deposits of nanometals exist, or whether or not they would remain as inert storage granules in the liver, or be excreted via the bile in fish.

1.5 Lethal toxicity of engineered nanoparticles in fish

Only a few lethal toxicity tests have been conducted using nanometals in fish (Table 1.2). The literature on fish and invertebrates indicate that acute lethal concentrations are in the mg/l, rather than µg/l range. This suggests that metal-containing NPs, such as poorly soluble metal oxides, may have low toxicity. Concerns remain that nanometals that show dissolution of metal ions will show acute lethal toxicity in the µg/l range. This is especially worrying for toxic metals where the nano form may dissolve. Griffitt et al. (2007) reported 48-h LC_{50} concentrations of 250 µg l^{-1} and 1.56 mg l^{-1} in adult zebrafish (*Danio rerio*) exposed to dissolved Cu and Cu-NPs (80 nm primary particle size) respectively. However, following its addition to water 50-60% of added Cu-NP mass was observed to fall out of suspension due to rapid aggregation of the NPs.

Table 1.2. Summary of studies carried out into the toxicity of organic and inorganic nanoparticles to fish and fish cell lines

Nanomaterial & additional information*	**Exposure conditions**	**Fish species**	**Effects**	**Study**
Ag-NPs (5–20 nm particle size)	5-100 μg l^{-1} for 72 h	Zebrafish (*Danio rerio*) embryos	Dose-dependent increases in mortality & decreases in heart rate. Hatching only affected at highest treatment. Most NPs deposited in the cell nucleus, but uniform distribution of NPs was seen throughout the embryo. Body malformations in embryos exposed above 50 μg l^{-1}.	Asharani et al. (2008)
Ag-NPs (dry powder mean primary particle size of 26.6 ± 8.8 nm) stabilised with 0.5 % sodium citrate solution, sonicated with a probe sonicator for six 0.5 s pulses prior to dosing	0-10 mg l^{-1} followed by experiments with fish exposed to 0.6-2.78-fold the LC_{50} (both for 48 h)	Zebrafish (*Danio rerio*)	Ag-NP LC_{50} of 7.07 mg l^{-1} in adult fish and 7.20 mg l^{-1} in larvae; less toxic than soluble Ag	Griffitt et al. (2008)

Ag-NPs (particle size of 10-20 nm; stock diluted with municipal water and dechlorinated)	0-20 μg l^{-1} from 2.5 to72 hpf	Zebrafish (*Danio rerio*) embryos	Dose-dependent effects included slow hatching rates, abnormal development, and increased catalase activity suggested by the authors as possibly linked to effects on gene expression from NPs entering the cell nucleus and ROS initiated DNA damage.	Yeo and Kang, (2008)
Ag-NPs (5.9, 15.3, 51.2, and 108.9 nm measured mean particle sizes)	0-250 μM for 120 hpf	Zebrafish (*Danio rerio*) embryos	Exposure to 250 μM of each Ag-NP treatment resulted in particle size dependent mortality after 24 h (80, 64, 36, and 3 % mortality respectively) rising to 100 % at 120 hpf. Uptake of Ag into the embryo not seen in control fish.	Bar-Ilan et al. (2009)
Ag-NPs (49.6 nm mean particle size, 99 % purity), stock sonicated for 1h in 2 min bursts followed by stirring for 14 d and filtration (200 nm)	0-50 μg l^{-1} for 96 h and 0-25 μg l^{-1} for 10 d	Japanese medaka (*Oryzias latipes*) 4-5 months old	Similar 96 h LC_{50} values for Ag-NPs and $AgNO_3$ (34.6 ± 0.9 and 36.5 ± 1.8 μg l^{-1} respectively). Expression of stress related genes indicated different modes of toxicity between Ag-NPs and soluble Ag ions ($AgNO_3$).	Chae et al. (2009)

Ag-NPs (dry powder mean particle diameter 26.6 ± 8.8 nm), sonication for 6 s in 1 s bursts prior to dosing	1000 µg l^{-1} (representing the NOEC) for 48 h	Zebrafish (*Danio rerio*)	Gill filament width not affected by Ag-NPs, though dissolved Ag increased width by approximately 2-fold. Branchial Ag levels in NP exposed fish almost 20 x higher than fish exposed to a Ag dose that matched the concentration of dissolved Ag from the NPs, indicating that intact Ag-NPs were associated with the gill. Similar pattern observed in whole body Ag measurements.	Griffitt et al. (2009)
Ag-NPs (commercial name of Nanocid, particle size of 4.5 nm, data taken from manufacturers website)	0-20 µg l^{-1} for up to 96 h	Rainbow trout (*Oncorhynchus mykiss*)	48, 72, and 96 hour LC_{50} concentrations of 3.5, 3.0 and 2.3 µg l^{-1} respectively.	Shahbazzadeh et al. (2009)
Ag-NPs (spherical particles, 5–20nm in diameter), stock deionised using ion exchange resin and sonicated for 30 s prior to dosing	24 h LC_{50} (concentration range not reported, followed by 0-120 mg l^{-1} for 24 h	Zebrafish (*Danio rerio*)	24 h LC_{50} of 250 mg l^{-1}, concentration dependent increases in hepatic malondialdehyde and total glutathione, DNA damage and apoptosis in liver.	Choi et al. (2010)

Ag-NPs (two commercial Ag-NPs with nominal particle sizes of 35 nm and ≤ 100 nm, both with 99.99 % purity) sonicated at 15 kHz for 1.5 h	0-25 mg l^{-1} for 96 h	Fathead minnow (*Pimephales promelas*) embryos	96 h LC_{50} values of 9.4 and 10.6 g mg l^{-1} for the 35 and ≤ 100 nm Ag-NPs respectively. Concentration dependent increase in larval abnormalities. TEM image analysis showed that both types of Ag-NPs were present within embryos following 24 h exposure.	Laban et al. (2010)
Ag-NPs (49, 114, and 137 (bulk) nm mean particle sizes) with sonication for 30 min prior to exposure	10 or 100 µg l^{-1} for 10 d	Juvenile rainbow trout (*Oncorhynchus mykiss*)	Low uptake with the smallest particles most concentrated in gill tissue and liver (the latter along with the bulk Ag particles). No effects on lipid peroxidation amongst any treatment (TBARS assay), although possible evidence of oxidative metabolism in gills of fish exposed to 49 nm Ag-NPs.	Scown et al. (2010)
Ag-NPs (25 nm spherical particles)	0-8 mg l^{-1} for 48 h (adults) and 0-4 mg l^{-1} for 168 h (embryos), followed by 0-1000 µg l^{-1} for 60 d from embryo stage 10	Japanese medaka (*Oryzias latipes*) adults and embryos	48 h LC_{50} of 1.03 mg l^{-1} for adult fish, whilst 100 % embryo mortality was induced at 2 mg l^{-1}. Retarded development and pigmentation seen in embryos exposed to ≥ 400 µg l^{-1} Ag-NPs with morphological malformations seen all Ag-NP treatments.	Wu et al. (2010)

Al-NPs (dry powder mean primary particle size of 41.7 ± 8.1 nm) sonicated with a probe sonicator for six 0.5 s pulses prior to dosing	0-10 mg l^{-1} for 48 h	Zebrafish (*Danio rerio*)	Highest dose (10 mg l^{-1}) not elevated enough to establish an LC_{50} for adults or larvae; dissolved Al gave LC_{50} concentrations of 7.92 mg l^{-1} and over 10 mg l^{-1} in adults and larvae respectively.	Griffitt et al. (2008)
Au-nanorods (65 nm length, 15 nm diameter; purified before use & suspended in high purity water)	7.08 x 10^8 particles ml^{-1} single-dose into estuarine mesocosms for 12 days	Sheepshead Minnow (*Cyprinodon variegatus*)	Au-nanorods did not cause acute toxicology & fish did not accumulate detectable levels of Au in the gills, brain or musculoskeletal tissues (indicating low branchial & cutaneous uptake). Au was detected in combined samples of internal organs and gut content, indicating limited oral uptake/accumulation.	Ferry et al. (2009)
Au-NPs (6.5, 18, 46.1, 98.1 nm measured mean particle sizes)	0-250 μM for 120 hpf	Zebrafish (*Danio rerio*) embryos	Mortality remained below 3 % for all treatments and no evidence of morphological malformations. However, uptake of Au-NPs was observed.	Bar-Ilan et al. (2009)
CdS quantum dots coated in thiol terminated methyl polyethylene glycol (4.2 ± 1 nm) suspended in water	5, 50, or 500 μg l^{-1} for 21 d using a flow through system	Three-spined stickleback (*Gasterosteus aculeatus*)	Elevated levels of oxidised glutathione, reduced nest building activities, and 4/6 fish from the 500 μg l^{-1} treatment displayed hepatocellular nuclear pleomorphism.	Sanders et al. (2008)

Carbon NP: C_{60} fullerenes (aggregates of 30-100 µm) dispersed in tetrahydrofuran (THF) with 'overnight' stirring	0.5 or 1.0 mg l^{-1} for 48 h	Largemouth bass (*Micropterus salmoides*)	Reduced lipid peroxidation in gill & liver tissue (0.5 mg l^{-1} treatment) and increased lipid peroxidation in brain tissue (both treatments).**	Oberdörster (2004)
Carbon NP: C_{60} fullerenes (10-200 nm aggregates) dispersed in water by stirring > 2 months	0.5 mg l^{-1} for 96 h	Fathead minnow (*Pimephales promelas*)	A 42 % down regulation of the peroxisomal lipid transporter protein, PMP70.	Oberdörster et al. (2006)
Carbon NP: C_{60} fullerenes (10-200 nm aggregates) dispersed in water with THF or by stirring for 2 months	0.5 mg l^{-1} for 48 h	Fathead minnow (*Pimephales promelas*)	100 % mortality in fish exposed to C_{60} with THF between 6-18 h compared to none in the stirred C_{60} group. The latter group had elevated branchial lipid peroxidation, possibly in the brain also. Changes in P450 protein expression due to increases in hepatic CYP2-like-iso-enzymes.	Zhu et al. (2006)
Carbon NP: C_{60}, C_{70}, and $C_{60}(OH)_{24}$ fullerenes suspended in DMSO (no primary particle size given, aggregates ranged from ≈ 50-1200 nm).	100-500 µg l^{-1} for C_{60} and C_{70}, and 500-5000 µg l^{-1} for $C_{60}(OH)_{24}$ from 24 hpf to 96 hpf.	Zebrafish (*Danio rerio*) embryos (dechorionated)	Exposure to 200 mg l^{-1} C_{60} and C_{70} caused an increase in malformations, pericardial edema, and mortality, whilst higher concentrations (an order of magnitude) of $C_{60}(OH)_{24}$ produced less pronounced effects.	Usenko et al. (2007)

Carbon NP: C_{60} fullerenes (aggregates between 50-300 nm) dispersed in water by either stirring for 7 days with sonication or by use of THF	0-25 % (v/v) for 72 h	Larval Zebrafish (*Danio rerio*)	Survival reduced in THF C_{60} and THF control treatments, but not in the water stirred treatment. Minimal gene expression changes in the latter treatment compared to control, whilst changes seen in THF C_{60} and THF control fish deemed to be linked to a THF degradation product (γ-butyrolactone).	Henry et al. (2007)
Carbon NP: C_{60} fullerene aggregates in suspension	0-1.5 mg l^{-1} for 96 h	Zebrafish (*Danio rerio*) embryos (dechorionated)	Embryonic development delayed and reduced hatching success.	Zhu et al. (2007)
Carbon NP: C_{60} fullerenes suspended in DMSO (no primary particle size given, aggregates ranged from ≈ 50-1200 nm)	0-500 μg l^{-1}, with or without H_2O_2, DEM, BSO, NAC or reduced light. Exposed at 24 hpf for 5 d	Zebrafish (*Danio rerio*) embryos (dechorionated)	Embryos exposed in decreased light conditions generally experienced significantly less mortality and malformations than other treatments. A dose dependent increase in toxicity seen in embryos exposed with GSH inhibitors (i.e., DEM and BSO).	Usenko et al. (2008)
Carbon NP: CNT (single-walled, 1.1 nm mean outside diameter, 5–30 μm length) dispersed in SDS with 2 h sonication	0.1-0.5 mg l^{-1} for 10 days	Rainbow trout (*Oncorhynchus mykiss*)	Dose-dependent rise in ventilation rates, gill pathologies and mucus secretion (with CNT precipitation on gill mucus). Some dose-dependent changes in brain & gill Zn or Cu occurred along with statistically significant increases in Na^+/K^+-ATPase activity in the gills & intestine.	Smith et al. (2007)

Carbon NP: CNT (single-walled, same batch as used above in Smith et al., 2007) and C_{60} fullerenes (as used above in Henry et al., 2007)	0 or 500 mg SWCNT or C_{60} kg^{-1} diet for 6 weeks	Rainbow trout (*Oncorhynchus mykiss*)	Transient elevation (week 4 only) in brain TBARS from fish exposed to SWCNT.	Fraser et al. (2010)
Carbon NP: CNT (various forms (with or without functionalizations) of single- and multi-walled, diameters 10-20 nm, and single-walled nanohorns, diameter 2-3 nm)	*In vitro* exposure to 0-10 μg ml^{-1} for 6 or 24 h	Rainbow trout (*Oncorhynchus mykiss*) macrophage culture system	Dose dependent increases in inflammatory gene expression (IL-1β) in all treatments, with functionalized nanotubes more stimulatory than nonfunctionalized.	Klaper et al. (2010)
CeO_2-NPs (25 nm particle size)	1000 mg l^{-1} from 24-120 hpf	Zebrafish (*Danio rerio*) embryos	No acute toxicity and no intestinal or skeletal defects.	Ispas et al. (2009)
Co-NPs (dry powder mean particle size of 10.5 ± 2.3 nm), sonicated for six 0.5 s pulses prior to dosing	0-10 mg l^{-1} for 48 h	Zebrafish (*Danio rerio*)	Highest dose of 10 mg l^{-1} was not enough to establish an LC_{50} in adults or larvae exposed to both nano-form and dissolved Co.	Griffitt et al. (2008)

Cu-NPs (80 nm particles in agglomerates >1 μm in diameter)	0.1-1.5 mg l^{-1} for 48 h over two experiments	Zebrafish (*Danio rerio*)	Nanocopper found to be acutely toxic with a 48-LC_{50} of 1.5 mg l^{-1}. Dose dependent gill pathologies (proliferation of epithelial cells and oedema of primary and secondary filaments), inhibition of branchial Na^+/K^+-ATPase activity. Toxic effects suggested to be unexplained by the effects of dissolved Cu alone.	Griffitt et al. (2007)
Cu-NPs (dry powder mean primary particle size of 26.7 ± 7.1 nm) sonicated with a probe sonicator for six 0.5 s pulses prior to dosing	0-10 mg l^{-1} followed by experiments with fish exposed to 0.6-2.78-fold the LC_{50} (both for 48 h)	Zebrafish (*Danio rerio*)	48 h LC_{50} of 0.94 mg l^{-1} in adult fish and 0.71 mg l^{-1} in juveniles; the latter more toxic than soluble Cu which had a 48 h LC_{50} value of 1.78 mg l^{-1} for larvae.	Griffitt et al. (2008)
Cu-NPs (dry powder mean particle diameter 26.7 ± 7.1 nm), sonication for 6 s in 1 s bursts prior to dosing	100 μg l^{-1} (representing the NOEC) for 48 h	Zebrafish (*Danio rerio*)	Gill filament width in Cu-NP exposed fish increased by approximately 3.5-fold and 1.5-fold between 24-48 h compared to control & dissolved Cu treatments respectively. Branchial Cu levels similar in Cu-NP (likely from dissolved Cu from NPs) and dissolved Cu treatments indicating low cellular uptake of Cu-NPs.	Griffitt et al. (2009)

Fe-NPs (30 nm mean particle size)	0-50 μg l^{-1} (100 % daily renewal) for up to 8 day (embryos) and 14 days (adults)	Japanese medaka (*Oryzias latipes*), embryos and adult fish	Medaka embryos experienced dose-dependent decreases in SOD & increases in MDA (TBARS assay), whilst adult medaka showed initial decreases in liver & brain SOD and a dose dependent, though transient, decrease in reduced glutathione at the start of the experiment. Adult fish exposed to 5 & 50 μg l^{-1} Fe-NPs displayed histological changes the gill and intestine, along with large amounts of aggregated Fe on the gills and lesser accumulation within the intestine.	Li et al. (2009)
Ni-NPs (dry powder mean primary particle size of 6.1 ± 1.4 nm), sonicated with a probe sonicator for six 0.5 s pulses prior to dosing	0-10 mg l^{-1} for 48 h	Zebrafish (*Danio rerio*)	Highest dose of 10 mg l^{-1} was not enough to establish an LC_{50} in adults or larvae exposed to both nanoparticulate and dissolved Ni.	Griffitt et al. (2008)
Ni-NPs (30, 60, and 100 nm particles and dendritic clusters of 60 nm particles) sonicated for at least 30 min	10-1000 mg l^{-1} from 24-120 hpf	Zebrafish (*Danio rerio*) embryos	Similar LD_{50} concentrations between the 30, 60, and 100 nm particles and soluble Ni, with dendritic clusters more toxic. Aggregates of Ni seen in gut lumen with concomitant deleterious effects such as thinning of the intestinal epithelium & skeletal muscle fibre separation.	Ispas et al. (2009)

Se-NPs (36 nm particles)	0.2-3.2 mg l^{-1} for 48 h and 100 μg l^{-1} for 10 d followed by 7 d depuration	Japanese medaka (*Oryzias latipes*)	Se-NPs more acutely toxic than selenite (48 h LC_{50} values of 1.0 and 4.7 mg l^{-1} respectively). Exposure to 100 μg l^{-1} Se-NPs increased tissue Se burden significantly compared to fish exposed to an equal concentration of selenite. Overall trend of reduced Se after depuration, though gill burden remained persistent. Decreased hepatic SOD activity and small, though significant elevation in GSH.	Li et al. (2008)
SiO_2 NPs (fluorescent core shell, 200 and 60 nm mean particle sizes) sonicated for 90 min prior to exposure	0.0025-200 mg l^{-1} for 96 hpf	Zebrafish (*Danio rerio*) embryos < 6 hpf	Adsorption of both SiO_2 NPs to the chorion of exposed embryos, though no indication of uptake. No effects on development, survival, hatching time or hatching success.	Fent et al. (2010)
TiO_2 NPs (dry powder mean particle diameter 20.5 ± 6.7 nm), sonication for 6 s in 1 s bursts prior to dosing	1000 μg l^{-1} for 48 h	Zebrafish (*Danio rerio*)	No increase in gill filament width after 48 h compared to control. Expression of genes involved in ribosomal function altered.	Griffitt et al. (2009)

TiO_2 NPs (75 % rutile, 25 % anatase, 24.1 ± 2.8 nm mean particle size) dispersed by sonication	0.1-1.0 mg l^{-1} for 14 days	Rainbow trout (*Oncorhynchus mykiss*)	Changes in tissue Na^+/K^+-ATPase activity, TBARS, and total glutathione, the latter two indicating mild oxidative stress. Some organ pathologies, including the gut.	Federici et al. (2007)
TiO_2 NPs (average particle size of 21 nm)	10.0 ± 1.3 mg l^{-1} TiO_2 NPs in presence of 97.3 ± 6.9 μg l^{-1} Cd for up to 25 d	Common carp (*Cyprinus carpio*)	The presence of TiO_2 NPs increased uptake of Cd by 146 % after 25 d exposure.	Zhang et al. (2007)
TiO_2 NPs (dry powder mean primary particle size of 20.5 ± 6.7 nm), sonicated with a probe sonicator for six 0.5 s pulses prior to dosing	0-10 mg l^{-1} for 48 h	Zebrafish (*Danio rerio*)	Highest dose of 10 mg l^{-1} was not enough to establish an LC_{50} in adults or larvae exposed TiO_2 NPs, no bulk TiO_2 powder treatments were used.	Griffitt et al. (2008)
TiO_2 NPs (50 nm primary particle size) rutile form crystal structure, sonicated for 30 mins	0-250 mg l^{-1} for 20 d	Juvenile Common carp (*Cyprinus carpio*)	No mortalities, though fish experienced dose dependent increases in respiratory distress with changes in SOD, CAT, POD and LPO levels indicating oxidative stress effects (most evident in the liver).	Hao et al. (2009)

TiO_2 NPs (average particle size of 21 nm)	10.0 ± 1.3 mg l^{-1} TiO_2 NPs in presence of 200.0 ± 10.2 µg l^{-1} As for up to 25 d	Common carp (*Cyprinus carpio*)	Accumulation of As in viscera, gills and muscle significantly enhanced by the presence of TiO_2 NPs.	Sun et al. (2009)
TiO_2 NPs (dry powder mean particle diameter of 34.2 ± 26.1 nm); NP injection solution sonicated for 30 min	Single dose of intravenously injected NPs (mean dose 1.3 mg NPs kg^{-1} body weight) & sampled for up to 90 d	Rainbow trout (*Oncorhynchus mykiss*)	Ti accumulation in the kidney which remained for 21 d with significant clearance by day 90. TiO_2 NPs located in tissue vesicles surrounding the kidney tubules, though kidney function not compromised (as indicated by plasma protein & creatinine levels). No accumulation in brain, gills, spleen, or liver. No evidence of lipid peroxidation in blood, liver or kidney.	Scown et al. (2009)
TiO_2 NPs (24.1 ± 2.8 nm mean particle size), dispersed by sonication for 6 h prior to coating dry feed pellets	0-100 mg TiO_2 NPs kg^{-1} diet for 8 weeks followed by 2 week recovery	Rainbow trout (*Oncorhynchus mykiss*)	No effects to growth, nutritional performance or haematology. Ti accumulated in the gill, gut, liver, spleen, and brain (with Ti not clearing in some organs following recovery). Disturbances to Cu & Zn levels and a 50 % inhibition of Na^+/K^+-ATPase activity seen in the brain and a 50 % reduction in TBARS in the gill & intestine was observed during exposure, though no changes to total glutathione.	Ramsden et al. (2009)

TiO_2 NPs (anatase form, 5 nm mean particle size),	*In vitro* exposure to 0.1–1000 µg ml^{-1} for 24 h	GFSk-S1; goldfish (*Carassius auratus*) skin cells	TiO_2 NPs, in the absence of photo-activation, induced genotoxic responses most likely as a result of free radical production.	Reeves et al. (2008)
TiO_2 NPs (75 % rutile, 25 % anatase, 24.1 ± 2.8 nm mean particle size)	*In vitro* exposure to up to 50 µg l^{-1} NPs between 4-24 h depending upon assay	RTG-2 cells; rainbow trout (*Oncorhynchus mykiss*) gonadal tissue	No elevation in DNA damage in the absence of UVA irradiation, though there was a significant reduction in lysosomal integrity over 24 h exposure. UVA exposure significantly increased levels of strand breaks.	Vevers and Jha, (2008)
TiO_2 NPs (21 nm particle size), stock solution sonicated for 10 min	Fish fed *Daphnia magna* previously exposed to TiO_2 NPs for 14 d (measured Ti concentrations in 2 *D. magna* groups of 4.52 and 61.09 mg g^{-1} dry weight)	Zebrafish (*Danio rerio*)	Bioconcentration factors of less than 1 in fish fed contaminated *D. magna* show no biomagnification of TiO_2 NPs. However, dietary exposure to TIO_2 NPs resulted in higher body Ti burden than seen in aqueous exposed fish in complementary experiments.	Zhu et al. (2010)

ZnO-NPs (20 nm primary particle size, though aggregates ranged between 1037- 6823 nm depending on time), stock prepared by vigorous stirring for 2 h immediately prior to dosing	0.1-100 mg l^{-1} until 96 hpf.	Zebrafish (*Danio rerio*) embryos	Dose-dependent decrease in hatching rates with low incidence of pericardial edema observed 72 hpf in 50 & 100 mg l^{-1} treatments, affecting approximately 10 % of embryos by 96 hpf. Dissolved Zn was less toxic with no evidence of malformations. Oxidative stress suggested as one possible cause of toxicity following raised intracellular levels of ROS in ZnO-NP exposed embryos.	Zhu et al. (2009)

THF: tetrahydrofuran; Ag-NPs: silver nanoparticles; Al-NPs: aluminium nanoparticles; Au-NPs: gold nanoparticles; BSO: buthionine sulfoximine; CeO_2-NPs: cerium nanoparticles; CAT: catalase; CNT: carbon nanotubes; Cu-NPs: copper nanoparticles; Co-NPs: cobalt nanoparticles; DEM: diethyl maleate; DMSO: dimethyl sulfoxide; Fe-NPs: iron nanoparticles; GSH: reduced glutathione; H_2O_2: hydrogen peroxide; hpf: hours post fertilisation; LPO: lipid peroxidation; MDA: malondialdehyde; NAC: *N*-acetylcysteine; Ni-NPs: nickel nanoparticles; POD: peroxidase; SDS: sodium dodecyl sulphate; Se-NPs: selenium nanoparticles; SiO_2 NPs: silica nanoparticles; SOD: superoxide dismutase; TiO_2 NPs: titanium dioxide nanoparticles; ZnO-NPs: zinc oxide nanoparticles. * Additional information as provided by the authors. ** Subsequent studies have cast doubt on the source of toxicity with the dispersant THF suggested as pivotal to toxic effects seen (e.g. Henry et al., 2007).

Dissolution of Cu ions from the particles (defined as Cu present in the supernatant of samples centrifuged at 100000g for 30 min) showed that dissolution was minimal over 48 h with concentrations of dissolved Cu not exceeding 0.19 ± 0.05 mg l^{-1}, representing less than 0.1% of the initial Cu added. Notably these dissolution experiments were conducted in the absence of fish. The relatively low rate of dissolution caused the authors to conclude that observed toxicity was not due to dissolved Cu alone. Subsequent work by Griffitt et al. showed Cu-NPs to be more acutely toxic to juvenile zebrafish than dissolved Cu, although this trend was reversed in adult fish (primary particle size 26.7 nm, Griffitt et al., 2008). In the same study Ag-NPs were seen to be less toxic than dissolved Ag to adult zebrafish with a Ag-NP 48-h LC_{50} more than 300-fold higher than that of dissolved Ag (Griffitt et al., 2008). Perhaps somewhat unexpectedly, 10 mg l^{-1} dissolved Ag was not elevated enough to kill 50 % of juvenile (larval) zebrafish (compared to an LC_{50} of 0.0222 mg l^{-1} in adults and an Ag-NP LC_{50} of 7.20 mg l^{-1}). The latter data may emphasise life stage differences in the toxic responses of fish to nanometals with fathead minnow (*Pimephales promelas*) embryos proving more sensitive to Ag-NPs than zebrafish larvae (96 h LC_{50} values of 1.25 and 1.36 mg l^{-1} for two different commercially available Ag-NPs respectively (Laban et al., 2010).

1.6 Sublethal effects of nanometals compared to aqueous metal ions

The sublethal physiological effects of waterborne metal exposure are well known (reviews, several metals: Wood, 2001; Ag: Hogstrand and Wood, 1998, Wood et al., 1999; Cd: Sprague, 1987; Cu: Taylor et al., 1996; Zn: Hogstrand and Wood, 1996). In contrast, the scientific community is in the early stages of collecting data on nanometals (Table 1.2). The typical scenario for waterborne metal exposure involves some direct effects on gill functions (reviews, McDonald and Wood, 1993; Perry and Laurent, 1993). These can include

alterations to the osmoregulatory (e.g., Cd, McCarty and Houston, 1975; Giles, 1984), acid-base (e.g., Cu, Taylor et al., 1996), or respiratory functions of the gill (e.g., nickel, Pyle et al., 2002). Sublethal pathologies in the gill can be reflected by changes in mucous secretions reflecting an increase in mucous cells and chloride cells in the epithelium (e.g., Mallat, 1985), or evidence of oedema in the epithelium (e.g., Sola et al., 1995; Campbell et al., 1999). Histological changes in the gill epithelium are also associated with adaptive biochemical change in response to metal exposure (e.g., metallothionein induction in the gill, Dang et al., 1999). Metals can also inhibit the branchial Na^+/K^+-ATPase (e.g., Cu, Li et al., 1998). Following this initial disruption, the fish can then experience a general loss of branchial ionoregulatory control, efflux of electrolytes from the blood over the gill epithelium, then subsequent cardiovascular collapse and death (review, Handy, 2003 and references therein). Notably, rainbow trout display physiological responses influenced by social status. Schools of rainbow trout develop a social hierarchy where the more aggressive fish in the school are dominant over often smaller, less aggressive fish in the group. When rainbow trout are exposed to Cu, the subordinate fish accumulate more waterborne Cu (and Na) than dominant animals (Sloman et al., 2002). A similar phenomenon has also been seen in fish exposed to waterborne Ag and highlights the possibility that toxic effects seen in fish following metal exposure may differ depending upon social rank (Sloman et al., 2003).

Evidence is now also emerging that some nanometals can also affect the gill in similar ways to dissolved metals. For example, exposure to 1 mg l^{-1} TiO_2 NPs in rainbow trout caused oedema in the gills (Federici et al., 2007). Griffitt et al., (2007) found that exposure to Cu-NPs caused concentration-dependent damage to the lamellae characterised by proliferation of epithelial cells and oedema of primary and secondary filaments whilst an increase in gill filament width was observed in adult zebrafish exposed to Cu-NPs, but not TiO_2 NPs or Ag-NPs following a 48 h exposure (Griffitt et al., 2009). Notably in the later

study gills exposed to Cu-NPs showed significantly wider filaments than those from soluble Cu exposures. A concentration-dependent inhibition of branchial Na^+/K^+-ATPase activity was also observed in the Cu-NP treatments (Griffitt et al., 2007). Notably, Griffitt et al. (2007) found that the inhibition of Na^+/K^+-ATPase by Cu-NPs was not as great as dissolved Cu (inhibition in fish exposed to 0.25 mg l^{-1} $CuSO_4$ approximately 5-fold that exposed to the same concentration Cu-NPs). Other mechanisms of respiratory toxicity are also possible with metals, and for example, excess iron (irrespective of bioaccumulation) can result in iron flocs on the gills which can clog the gills resulting in respiratory distress (Peuranen et al., 1994; Dalzell and MacFarlane, 1999). This might also be highly relevant to other particles such as nanometals, but data remains to be collected on this aspect.

Toxic effects of NPs on fish blood remain to be investigated. However, for TiO_2 NPs, there seems to be no major disturbances to blood cell counts or plasma electrolytes in rainbow trout (Federici et al., 2007) and no evidence of lipid peroxidation (TBARS assay) in rainbow trout blood plasma following intravenous administration (Scown et al., 2009). The effects of other nanometals on fish haematology, immune cells, and plasma biochemistry remain to be documented. Biochemical disturbances in internal organs have been noted, and in particular, oxidative stress is emerging as a potential mechanism of NP toxicity. Oxidative stress, and the induction of enzymes involved in anti-oxidant defence is well known for dissolved metals (e.g., Cd induction of glutathione peroxidase (GPx) and superoxide dismutase (SOD) in fish muscle, Almeida et al., 2002; Cu induction of SOD and catalase (CAT), Sanchez et al., 2005). Some nanometals can also cause oxidative stress. For example, Federici et al. (2007) exposed juvenile rainbow trout 0-1.0 mg l^{-1} TiO_2 NPs for up to 14 days, which caused a rise in thiobarbituric acid reactive substances (TBARS, an indicator of lipid peroxidation) in the gills, liver and brain, along with increased total glutathione (GSH) in the gills and depleted GSH in the liver. Following exposure to 50 or 500 µg l^{-1} nano-cadmium

sulphide (quantum dots), elevated levels of oxidised glutathione were observed in the gills of three-spined sticklebacks (Sanders et al., 2008). Oxidative stress was also implicated in a study exposing Japanese medaka to Fe-NPs (Li et al., 2009). During the latter study both embryos and adult medaka experienced concentration-dependent decreases in the antioxidant; superoxide dismutase (SOD). In adult fish, decreases in hepatic and cerebral SOD during the first few days of exposure were accompanied by reduced glutathione (GSH) levels in the brain. However, levels of both SOD and GSH returned to similar to control after 3 days and no differences were seen in levels of malondialdehyde (MDA, as an indicator of lipid peroxidation) in the liver or brain throughout the exposure. This indicates that adult medaka were able to successfully protect against oxidative stress through antioxidant activity, which were then able to replenish following initial utilisation. Japanese medaka embryos experienced a different response. As with adult fish, there was a dose-dependent depletion of SOD within hours of exposure, but this did not recover throughout the 8 days of exposure (most notably in the high, 50 mg l^{-1}, treatment). Lipid peroxidation product MDA was initially depleted following exposure, presumably regulated by antioxidants such as SOD, but as the experiment progressed a dose-dependent increase in MDA was seen, particularly in the 50 mg l^{-1} treatment, which showed an almost 4-fold increase in MDA compared to control and lowest dose Fe-NP (0.5 mg l^{-1}) treatment by day 8. Adult fish also exhibited some histological and morphological alterations in the gills and intestine (cell swelling, hyperplasia and granulomas) which were deemed to be as a direct result of contact with the NPs (as evidenced for example by large aggregates of Fe-NPs on the gills and accumulation in the intestine, Li et al., 2009). Dietary Fe has previously been seen to cause lipid peroxidation in the liver and heart of African catfish (Baker et al., 1997). An increase in intracellular reactive oxygen species (ROS) was observed in zebrafish embryos exposed to ZnO-NPs and implemented in some toxic effects (see next section, Zhu et al., 2009). *In vitro* studies also

show evidence of oxidative stress from NPs in fish cell lines. Reeves et al., (2008) in a study using goldfish skin cells (GFSk-S1) demonstrated that TiO_2 NPs, in the absence of photo-activation, induced genotoxicity most likely as a result of free radical production. Vevers and Jha (2008) exposed RTG-2 cells to TiO_2 NPs with and without ultraviolet radiation (UVA) and found that whilst there was no elevation in DNA damage in the absence of UVA irradiation, there was a significant reduction in lysosomal integrity over 24 h exposure, suggesting that the nanometal was also capable of damaging the cells without UV activation.

1.7 Toxicity of nanometals to early life stages of fish

Many studies have shown that the early life stages of fish are especially sensitive to metals (reviews, e.g., Weis and Weis, 1991; Jezierska et al., 2009), and that this led to the loss of fish populations (e.g., acidic, metal-containing spring snowmelt, Havas and Rosseland, 1995). There is also some evidence that some nanometals can affect the development and survival of fish embryos. Asharani et al. (2008) exposed zebrafish embryos to silver-NPs and found the NPs distributed in the brain, heart, yolk and blood of embryos. Toxic effects included a concentration-dependent increase in mortality and delays in hatching. Developmental abnormalities consisted of twisted notochords, and slow blood flow associated with pericardial oedema and cardiac arrhythmia. Lee et al. (2007) developed techniques for imaging the transport of single silver-NPs (5–46 nm) through zebrafish embryos, and found that silver-NPs could penetrate the chorion via pore canals, with uptake kinetics characteristic of diffusion rather than active transport. In this study, concentration-dependent increases in mortality were observed as well as deformities including finfold abnormalities, tail and spinal cord flexure/truncation, cardiac malformation, also oedema in the yolk sac and head. Silver NPs were also seen to attach to the surface of the chorion and

later detected within fathead minnow embryos (though not conclusively determined to be Ag-NPs) exposed to one of two commercial Ag-NP products (see Table 1.2 for details) after just 24 h exposure (Laban et al., 2010). Yeo and Kang, (2008) saw significantly decreased hatching rates in zebrafish embryos exposed to Ag-NPs. Furthermore, hatched fish displayed abnormal notochords, weak heart beat, curved tails, and damaged or absent eyes. Catalase, an important antioxidant enzyme, was also induced in exposed embryos, possibly indicating oxidative stress as one mechanism of toxicity.

Zhu et al. (2009) conducted a study to investigate the effects of an aggregated nanometal which had settled out of the water column in an attempt to mimic realistic environmental conditions. In their study reduced hatching rates and pericardial oedema were seen in zebrafish embryos exposed to ZnO-NPs, which was not likely to be attributable to dissolved Zn from the NPs with a positive control group (exposed to dissolved Zn) experiencing far fewer toxic effects. Reactive oxygen species (ROS) generation was higher in ZnO-NPs exposed embryos compared to exposure to dissolved Zn, although notably, embryos exposed to the latter showed up regulation of genes for enzymes involved oxidative stress responses. These genes were not up regulated in ZnO-NPs exposed embryos, with levels below controls at some time points. The authors concluded that disruptions in the ability of the embryos to counteract effects from ROS cannot be discounted, with ZnO-NPs exposed animals experiencing disruptions to hatching rates and physical development that could possibly have been associated with oxidative stress. Reduced hatching rates were also seen in zebrafish embryos exposed to Zn-NPs along with decreased survival, delayed embryo and larval development and tissue damage (ulceration). However, exposure in the same study to bulk ZnO caused comparable effects and similar acute toxicity, with LD_{50} concentrations of 1.793 and 1.550 mg l^{-1} for nanoscale and bulk ZnO respectively (Zhu et al., 2008). In the same study exposure to titanium dioxide and alumina did not cause toxicity.

Size and shape effects of a nanometal on fish embryos were investigated by Ispas et al. (2009). In their study, dechorionated zebrafish embryos were exposed to spherical Ni-NPs of different sizes (30, 60, and 100 nm diameters) and a dendritic structure consisting of aggregated 60 nm particles (particle size distribution of 540 nm) with comparisons made to soluble Ni salts. Nickel NPs were equally, or less toxic than nickel salts (LD_{50}), but, dendritic clusters were markedly more toxic than both NPs and soluble Ni, (LD_{50} of the three NPs between 221-328 mg l^{-1}, soluble Ni; 221 mg l^{-1}, whilst dendritic clusters had an LD_{50} of 115 mg l^{-1}). Interestingly, although both nano- and soluble nickel had similar acute toxicity, sublethal affects manifested in different ways, with organ defects (similar in embryos exposed to Ni-NPs and dendritic clusters), absent in those exposed to soluble Ni, or at least until much higher concentrations. These effects included a thinning of the intestinal wall and skeletal muscle fibre separation. Analogous to data from Cu-NP experiments (previously detailed), the majority of toxic effects seen from Ni-NPs do not stem from dissolved Ni from the NPs, with Ispas et al. (2009) suggesting that only 2.4-3.8 % of the observed toxicity was due to soluble metal. As discussed in the previous section, oxidative stress may be one mechanism of nanotoxicity in fish at the embryonic stage (e.g., Li et al., 2009), however a comparison of these effects in embryos exposed to dissolved metals is difficult due to the sparsity of data in the literature. This is most likely related to the difficulty in making such measurements in individuals due to the small size of embryos.

Numerous studies on the lethal and developmental affects of dissolved metals to early life stage fish, have led researchers to suggest that embryos are less sensitive than larval fish (e.g., Eaton et al., 1978; McKim et al., 1978; Dave, 1985; Shazili and Pascoe, 1986; Scudder et al., 1988; Kazlauskienė and Stasiūnaitė, 1999). However, Johnson et al. (2007) argue that embryos exposed after the chorion has hardened may display reduced sensitivity compared to those exposed beforehand (i.e., < 1 h post-fertilisation) due to the protection afforded by this

membrane. Therefore, when using fish embryos in (eco)toxicology tests, including those with NPs, it may be prudent to commence exposure within an hour of the eggs being laid in order to enable a more environmentally realistic appraisal of any effects (Johnson et al., 2007), although dechorionation presents an alternative, albeit more unrealistic, option.

1.8 Dietary exposure to nanometals

The consensus view is that dietary metal exposure is the main mode of chronic exposure of wild fish to metals, and that whilst dietary metals are not acutely toxic, there can be long term sublethal effects (review, Handy et al., 2005). These sublethal effects can include dose-dependent effects on growth (Clearwater et al., 2005), although fish usually adopt a bioenergetic strategy that preserves growth at the expense of other aspects of metabolism (Handy et al., 1999; Campbell et al., 2005a). The sublethal effects include increased costs of aerobic metabolism with subsequent changes in swimming performance (Campbell et al., 2002) or the ability to compete for food (Campbell et al., 2005b). Fish can also show subtle pathological changes in the liver (e.g., fatty change, foci of necrosis, Handy et al., 1999; Shaw and Handy, 2006) and evidence of transient oxidative stress (Baker et al., 1998; Berntssen et al., 2000; Hoyle et al., 2007). The osmoregulatory effects of dietary metal exposure are much less severe than via the aqueous route, although disturbances to tissue electrolytes and Na^+/K^+-ATPase are sometimes recorded (e.g., Hoyle et al., 2007).

To the authors knowledge, apart from one report (Ramsden et al., 2009), there is very little information on the sublethal effects of dietary exposure to nanometals in fish. In mice, acute toxic effects can be observed (often after a single gavage of mg doses of the nanometal). For example, Chen et al. (2006) found that Cu-NPs caused some pathology in the kidney, spleen and liver, some of which were classified as being severe or 'deadly severe'.

Wang et al. (2006) observed severe symptoms of lethargy, with vomiting and diarrhoea for the first few days, in mice orally exposed to Zn-NPs. Two mortalities occurred after the first week, with both animals showing aggregations of Zn particles in the intestine (Wang et al., 2006). In a study by Wang et al. (2007) no acute toxicity was seen in mice exposed to either nano-sized (25 or 80 nm) or fine (155 nm) TiO_2 particles by single oral gavage. However, changes to serum biochemical parameters and cellular pathology in hepatocytes were noted. In the only detailed nutritional toxicity study of nanometals in trout, Ramsden et al. (2009) observed no impact on growth or nutritional performance, but some changes to Cu and Zn levels in the brain, along with a 50 % inhibition of brain Na^+/K^+-ATPase activity. Statistically significant decreases in TBARS in the gill and intestine compared to control were also seen during the experiment, although no changes to the total glutathione pool were observed. These data support the notion that nanometals could produce similar subtle toxic effects to metal salts in the diet, without compromising growth. Notably, Ramsden et al. (2009) compared their results for TiO_2 NPs against the known hazard from other metals, and argued that based on sublethal effects without inhibition of growth, the dietary hazard from TiO_2 NPs was similar to mercury at equivalent oral doses.

The main target organs for the dietary exposure route, and whether or not nanometals will accumulate in the edible flesh of fish to present a human health risk, remains to be established. Absorption efficiencies for essential metals ranges from around 30-70 % in the diet of fish (Handy, 1996), although values for non-essential toxic metals can be only a few percent or less of the total oral dose (e.g., Cd, Franklin et al., 2005). There are no data on dietary uptake efficiency for nanometals in fish (or rodents), but the data of Ramsden et al. (2009) suggests that the uptake of TiO_2 NPs will be a few % of the total dose and with similar target organs to other dietary metals. The effects of nanometals on luminal chemistry in the gut, and the availability of nutrients from the gut lumen are unknown; as are the effects of

nanometals on gut function. Nonetheless, the drinking of contaminated water can result in severe erosion of the gut epithelium in trout (TiO_2 NPs, Federici et al., 2007). This effect was not observed with dietary exposure via the food (Ramsden et al., 2009), suggesting the presence of food or organic matter in the gut lumen will have protective effects on the oral toxicity of some NPs in fish.

1.9 A perspective on risk assessment

A key question for ecotoxicologists and regulators is whether or not there are additional risk from the nanoform compared to the dissolved metal. This needs to be addressed on a case by case basis for each nanometal, but overall, the sublethal effects observed for NPs are (so far) comparable to existing metals. However, the mechanisms of uptake and bioavailability of nanometals are likely to be very different to dissolved metals, with the former not being able to utilise ion transporters for the uptake of NPs, but instead using other mechanisms such as endocytosis (Handy et al., 2008b). It is also clear that NP chemistry is very different to that of dissolved metals (Handy et al., 2008a), and the equilibrium models we currently use to calculate free metal ion concentrations have limited theoretical foundation for its use with nanometals. The chemistry of NPs is fundamentally different (dynamic instability, not an equilibrium process), with perhaps one very important exception; the dissolution of free metal ions from the surface of NPs. In the latter case, it may be possible to apply existing biotic ligand models to the dissolved fraction. The metals community has also been working on more sophisticated models for the behaviour of metals in natural water which account for metal binding to soft colloids in the water column (e.g., humic acids), and the compartmentalisation of metals in other colloidal fractions (Rotureau and van Leeuwen, 2008; Duval, 2009). Natural colloids operate at the nanoscale, and it may

be possible to develop bioavailability models for nanometals by incorporating knowledge on natural colloids (e.g., Lead and Wilkinson, 2006) with aspects of metal speciation, and the behaviour of engineered NPs. This would be an important step in enabling regulatory agencies to link the hazard information from physiological effects studies with the environmental chemistry of nanometals. Ideally, for the regulatory agencies it would be preferable to be able to apply corrections for water hardness, pH, ionic strength, etc., in a similar way to dissolved metals. Particle theory suggests that such abiotic factors will be important (Handy et al., 2008a), and while this may lead to empirical corrections to water quality standards that could be applied by regulators, one must not forget that the reasons behind such corrections will be fundamentally different to that for dissolved metals.

The advent of increasingly complex hybrid versions of (nano) materials containing more than one NP or doped metal (e.g., Yang et al., 2001), suggests it may be important to elucidate whether these nanohybrids exert a mixtures effect that would be equivalent to the sum of the individual metal components (or cause synergistic effects), and whether the unique properties of the material would require some novel approach to the issue of composite materials. Clearly, the stability of such composite materials in both the water and tissues may be critical in any mixture effect. This and other aspects of metal toxicity will need revisiting as new nanomaterials emerge in order to ascertain any potential hazard and risk.

1.10 Hypothesis

It is hypothesised that NPs will be both acutely and sublethally toxic to fish from waterborne exposure, but that in comparison to known metal toxicants such as copper, the 'bulk' (i.e., soluble) version will be more toxic than the NPs. Also chronic dietary exposure

will result in sublethal effects with secondary affects possibly leading to death. However, due to their small size and subsequent physico-chemical properties such as particle shape, surface area and surface charge it is hypothesised that engineered nanoparticles are able to exert effects on fish that are unique in comparison to the bulk versions of the same material. However, it is also possible that any 'nano' effect could be offset by the propensity of nanoparticles to form aggregates, thus the animal may not be exposed to the primary nanoparticle, but much larger aggregates. Using Cu^{2+} and Cu-NPs as examples Fig. 1.2 highlights some of the hypothesised differences in bioavailability of NPs to fish, whilst Fig. 1.3 illustrates the potential differences in ADME and toxicological effects.

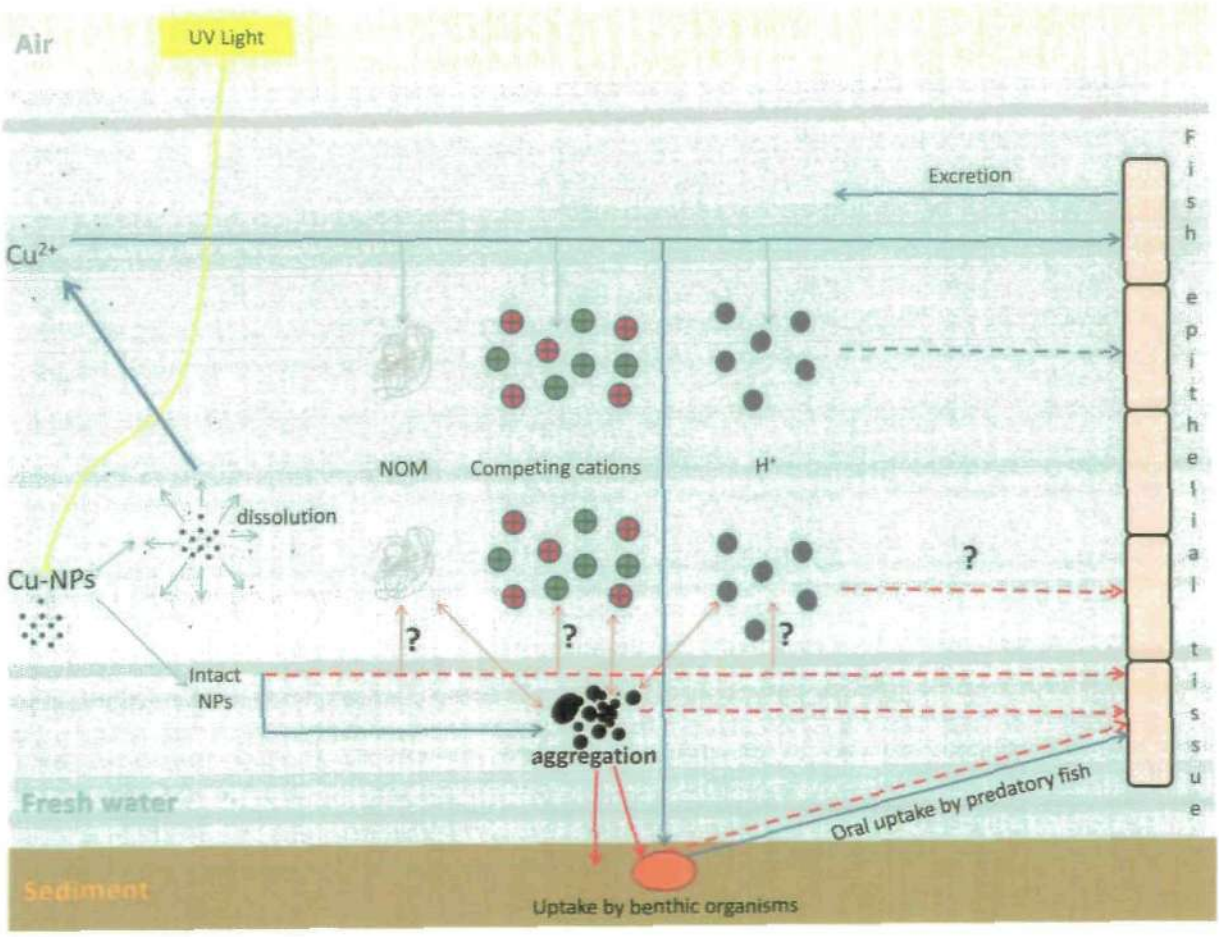


Figure 1.2. Conceptual diagram showing the differences in bioavailability of a metal salt versus the corresponding metal NP with some example abiotic factors. Copper has been used as an example. Ultraviolet light may affect some NPs, though probably not Cu-NPs. Dashed lines indicate limited bioavailability, with unbroken lines showing high bioavailability. Blue lines represent known effects, whilst red lines show possible interactions. Squiggly lines represent natural organic matter, red and green filled circles represent competing cations such as Ca^{2+}, purple filled circles represent hydrogen ions as an indicator of pH, orange filled shape represents benthic organisms, pink filled, joined rectangles represent fish epithelial tissue such as the gill or gut epithelia. Single NPs are represented by black dots with space between, whereas aggregates of NPs are represented by larger, irregular black shapes and circles.

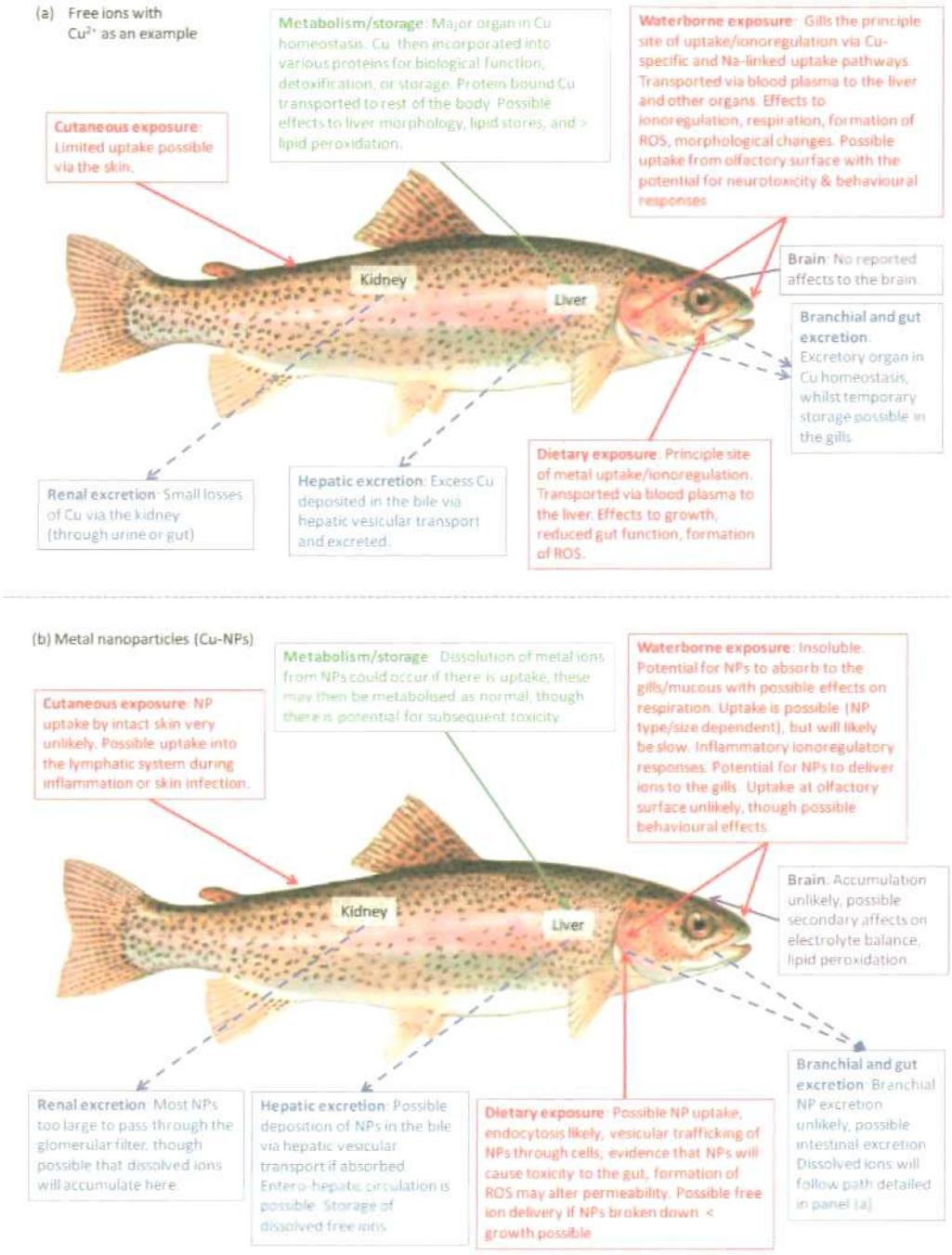


Figure 1.3. Conceptual diagrams showing the differences in absorption, distribution, metabolism, and excretion (ADME) and toxicity of a metal salt (a) versus the corresponding metal NP (b). Copper has been used as an example with empirically derived responses for panel (a) and some predicted responses for panel (b). Note that non-essential metals and metal based NPs could result in marked differences in ADME. Diagram conception based on Handy et al. (2008b).

1.11 Aims of the thesis

Nanoparticles are already in the aquatic environment (e.g., Kaegi et al., 2008) and it is therefore likely that wild fish populations are being exposed to these new materials (Handy et al., 2008b). Fish are important organisms in aquatic ecosystems and are commonly used as test animals in ecotoxicology. The use and production of metal containing NPs and NMs is expected to increase exponentially in the next decade. The thesis has several major scientific aims. These include 'proof of principal' toxicity studies to demonstrate whether or not nanometals can produce adverse effects in fish. The approach used here considers NPs consisting of both biologically essential (e.g. copper) and non-essential (e.g. titanium) metals; via both waterborne and dietary routes of exposure, and effects on different life stages or species of fish. These studies will involve detailed measurements of the tissue metal levels, and effects on the internal organs (biochemical disturbances, pathologies, metal accumulation) so that an overview of how the traditional rules of metal toxicology apply to nanomaterials, or indeed, if a new paradigm is needed to explain nanotoxicity. There are currently large knowledge gaps on ADME, the sensitivity of juvenile stages compared to adults, and species differences in toxicity. This thesis will contribute to filling these knowledge gaps for fish as well as addressing whether existing methods for metal ecotoxicology are valid for this new frontier in toxicology or whether there is a requirement to develop new methodologies or amend of existing methods. Some aspects of the work therefore also focus on the measurement of metals from nanomaterials in fish tissues.

The specific experimental objectives of the study are to:

1. Address the issue of nanometal quantification in fish tissue by assessing the efficacy of existing tissue digestion and ICP methods and developing them for NPs where required.
2. Assess the effects of dietborne TiO_2 NPs to rainbow trout to compliment a previous study on aqueous exposure to TiO_2 NPs in this species, where incidental drinking of water contaminated with these NPs resulted in gut pathologies.
3. To assess body system effects on rainbow trout from exposure to waterborne Cu-NPs and compare these effects to a similar dose (by mass) of soluble Cu.
4. Assess the lethal toxicology of aqueous Cu-NPs to early life stage zebrafish and investigate the effects of varying water quality parameters such as NOM, pH, and Ca^{2+}, along with a known Cu chelator (EDTA).
5. Reflect on the overall results in the context of the dissolved metal toxicity paradigm, and indicate if nanometals require new thinking, or present new hazards to the environment.

Chapter 2. General methodology

2.1 Risk assessment

Prior to each experiment risk assessments were conducted including generic risks (e.g. use of electrics in an aquarium environment) and those specific to certain experiments (e.g. handling of nanoparticles), and Control of Substances Hazardous to Health (COSHH) forms completed accordingly.

2.2 General chemicals

Generic chemicals and reagents used throughout (not specific to a particular experiment) are listed in Table 1. Analytical grade reagents, or higher quality, we used in all experiments.

2.3 Fish husbandry and water quality

A fish husbandry plan was devised prior to the start of each experiment. These plans set out the lower and upper limits of acceptable general water quality parameters for maintaining fish health during experiments such as pH, temperature, water hardness, and dissolved oxygen levels. The water quality considerations included meeting the husbandry requirements of the species, whilst also adhering to the specific requirements of the experimental design such as maintaining NP exposure concentrations. Consequently, different aquarium systems were employed to achieve different types of exposure including semi-static exposures in glass tanks, and the use of recirculating aquaria for dietary exposure studies (see experimental design for each experiment).

Water quality parameters were monitored throughout the experiment so that they were maintained within the limits set out in the fish husbandry plan in order to minimise any possible environmental effects upon the fish. Water samples were collected daily for pH, temperature, and oxygen saturation (all measured using a HACH HZ40d multi meter), and water samples were also collected (prior to feeding) from the waste outlet in the recirculation systems or mid water column from static tanks for total ammonia (HACH LANGE GMBH LCK kit 304 read on a HACH LANGE GmbH DR 2800 spectrophotometer). Dechlorinated Plymouth tap water was used throughout with a typical ionic composition of 0.4, 0.04, and 0.5 mmol l^{-1} for Na^+, K^+ and Ca^{2+} respectively, with background Cu levels within the 0.06-0.09 μmol Cu l^{-1} (4-6 μg Cu l^{-1}) range. Although most fish have a pH tolerance of approximately 6.0-9.5, in order to promote and maintain healthy biofilters and thus stable water quality (Timmons et al., 2002) a circumneutral pH was sought throughout. Dissolved oxygen constantly maintained above 80 % saturation and total hardness (as $CaCO_3$), was approximately 50 mg l^{-1}.

Where required, water samples were taken for Cu analysis and acidified with one drop of 6 M HNO_3.

2.4 Anaesthesia and dissection

All procedures on animals were ethically approved, and conducted under a Home Office project licence (PPL 30/2313, Animals Scientific Procedures Act, 1986). Animals were anaesthetised for all invasive procedures that involved the potential for causing pain or distress (e.g. blood collection from the caudal vein or by cardiac puncture). Typically, 25 mg l^{-1} of MS222 (tricaine methane sulphonate, MS222/1-V1, PHARMAQ, buffered to neutral pH with 200 mg l^{-1} $NaHCO_3$) was added to a bucket containing about 2-3 litres of tank water.

The fish were immersed in the buffered anaesthetic until anaesthesia was confirmed (total loss of equilibrium and the fish not responding to touch on the flank), then the relevant regulated procedure was carried out. In most experiments after blood sampling, and prior to dissection, fish were then euthanized with an overdose of 100 mg l^{-1} buffered MS222.Following termination fish were weighed, total length recorded, and any observations on gross morphology or fish health noted. Dissection was carried out using acid washed instruments, with care taken to minimise any-cross contamination between samples, and to maintain organ integrity for histological examination where appropriate.

Table 2.1. Common chemicals used throughout experiments

Chemical			**Use**
Name [CAS number]	*Other name /abbreviations*	*Purchase information*	
(4-(2-hydroxyethyl)piperazine-1-ethane sulfonic acid [7365-45-9]	HEPES	H3375, Sigma, UK	Homogenising buffer
1,1,3,3-tetraethoxypropane [122-31-6]	Malonaldehyde bis(diethyl acetal)	T9889, Sigma, UK	TBARS & protein assay
2,6-Di-*tert*-butyl-4-methylphenol [128-37-0]	Butylated hydroxytoluene / BHT	B1378, Sigma, UK	TBARS assay
2-amino-2-hydroxylmethyl-1,3-propanediol [77-861]	Tris	T1503, Sigma, UK	General solution buffering
5,5'-dithiobis-(2-nitrobenzoic acid) [69-78-3]	DTNB	D8130, Sigma, UK	Glutathione assay
Adenosine 5'-triphosphate (disodium salt) [987-65-5]	Na2ATP	A7699, Sigma, UK	Na^+/K^+-ATPase activity
Ammonium molybdate tetrahydrate [12054-85-2]	-	A1343, Sigma, UK	Na^+/K^+-ATPase activity

β-Nicotinamide adenine dinucleotide phosphate tetrasodium salt [2646-71-1]	NADPH	N1630, Sigma, UK	Glutathione assay
Bovine serum albumin [9048-46-8]	BSA	A3912, Sigma, UK	Protein assay
Copper (II) sulfate pentahydrate [7758-99-8]	Cupric sulphate	C7631, Sigma-Aldrich, UK	Protein assay
Ethylenediaminetetra-acetic acid disodium salt [6381-92-6]	Na-EDTA	E5134, Sigma, UK	Homogenising buffer
Drabkins reagent	-	D5941, Sigma, UK	Haematology
Ethanol (analytical grade) [64-17-5]	-	E/0650DF/15, Fisher Scientific, UK	TBARS assay
Ethylenediaminetetra-acetic acid dispotassium salt [25102-12-9]	K-EDTA	ED2P, Sigma, UK	Glutathione assay
Folin-Ciocalteu's Phenol Reagent	-	F9252, Sigma-Aldrich, UK	Protein assay
Glutathione reductase [9001-48-3]	-	G3664, Sigma, UK	Glutathione assay
Iron sulphate heptahydrate [7782-63-0]	Ferrous sulphate	F8633, Sigma, UK	Na^+/K^+-ATPase activity
L-Glutathione Reduced [70-18-8]	-	G4251, Sigma-Aldrich, UK	Glutathione assay
Magnesium chloride hexahydrate [7791-18-6]	-	*M2670, Sigma-Aldrich a, UK*	Na^+/K^+-ATPase activity

Nitric acid (trace analysis grade) [7697-37-2]	-	N/2272/PB17 Fisher Scientific, UK	Tissue digests
Ouabain octahydrate [11018-89-6]	G-strophanthin	03125, Sigma-Aldrich, UK	Na^+/K^+-ATPase activity
Phosphate buffered saline	PBS	P-4417, Sigma, UK	TBARS assay
Potassium chloride [7447-40-7]	-	P3911, Sigma-Aldrich, UK	Na^+/K^+-ATPase activity
Potassium hydroxide [1310-58]	-	P1767, Sigma-Aldrich, UK	Glutathione assay
Potassium phosphate monobasic [7778-77-0]	-	P5379, Sigma, UK	Na^+/K^+-ATPase & Glutathione
Potassium sodium tartrate [6381-59-5]	Sodium potassium tartrate	S2377, Sigma-Aldrich, UK	Protein assay
Rapid Decalcifier	-	CellPath Plc, UK)	Histology
Sodium carbonate anhydrous		223484, , Sigma, UK	Protein assay
Sodium chloride [7647-14-5]	-	S-7653, Sigma, UK	Na^+/K^+-ATPase activity
Sodium hydroxide [1310-73-2]	-	71689, Fluka BioChemika	TBARS & Protein
Sucrose [57-50-1]	-	S9378, Sigma, UK	Homogenising buffer
Sulphuric acid [7664-93-9]	-	S/9240?PB17, Fisher Scientific, UK	Na^+/K^+-ATPase activity
Thiobarbituric acid [504-17-6]	TBA	T5500, Sigma-Aldrich, UK	TBARS assay

Tricaine methanesulphonate [886-86-2]	3-aminobenzoic acid ethyl ester / MS222	PHARMAQ Ltd, UK	Anaesthetic
Trichloroacetic acid [76-03-9]	TCA	T/3000/53, Fisher Scientific, UK	Na^+/K^+-ATPase & TBARS

2.5 Growth and nutritional performance

For dietary studies growth and nutritional performance parameters were measured according to Handy et al. (1999) with minor modifications. Briefly, the amount of food fed to each tank was recorded at each feeding session by weighing the feed containers before and after feeding, with any uneaten food subsequently removed and deducted from the total mass added in order to accurately assess the precise mass eaten (removed pellets were counted and an equal number of dry pellets were weighed to give an approximate weight of those removed). In addition, the time delay between the introduction of feed and first feeding was recorded, along with the total time taken for the feeding event. All fish were individually weighed at the start of the experiment and at each 14 days sampling interval (a few hours prior to feeding), in order calculate net weight gains and growth rates. Individual fish weight was used for these calculations because the periodic sacrifice of fish during the experiment prevented nutritional parameters being calculated from cumulative tank biomass. These values were combined from the recorded weights of the sampled fish within each treatment and used to calculate the relevant nutritional parameters (all parameters as in Busacker et al., 1990). Specific growth rate (SGR (% day^{-1}) = ($\log_e W_2 - \log_e W_1$)/($t_2 - t_1$)×100; at time intervals t_1 and t_2, where W_1 and W_2 are the fish weights at t_1 and t_2, respectively), feed conversion ratio (FCR = feed intake (g)/weight gain (g)) and feed conversion efficiency (FCE = weight gain (g)/feed intake (g)) were calculated from mean gain in body weight for each treatment for (i) the exposure phase, (ii) recovery phase and (iii) for entire experiment (exposure and recovery combined).

2.5.1 Proximate composition of whole carcass

Proximate composition of the carcass is a routine measure of the general protein, fat, carbohydrate, and ash content (representing mostly non-combustible minerals) of the animal. Typically, 2-3 fish per tank (6-9 fish in total from a triplicate tank design) were terminally anaesthetised with MS222 and placed into a -20 °C deep freeze for storage until proximate composition analysis could be performed. Proximate composition was determined in triplicate for moisture, lipid, ash and protein content of whole carcass. For moisture content, fish were weighed then placed into a fan assisted oven and dried to a constant weight (Genlab Ltd, UK , 105 °C). Dried samples were removed and placed into a dessicator until cooled to room temperature then re-weighed. Moisture content was calculated as: ((WW – DW)/WW)×100 where WW is wet weight (g) and DW is dry weight (g).

Lipid content was determined by a solvent extraction (Soxtec) method. Briefly, dried samples were ground and weighed and (approximately 3 g) placed into a cellulose thimble. This was lightly plugged using cotton wool and inserted into a Soxtec extraction system condenser (Tecator Systems, Höganäs, Sweden; model 1043, service unit 1046). Petroleum ether (40 ml) was dispensed into pre-weighed cups and clamped onto the condenser and the extraction knobs on the instrument were raised to the boiling position for 30 mins followed by the rinsing position for 45 mins. After 30 mins in a fume cupboard, the cups were re-weighed, and lipid content determined as (LW / SW)×100, where LW is lipid weight (determined from weight increase of cup, g) and SW is the initial sample weight (g).

Ash content (total mineral or inorganic content) was determined by an adaption of the AOAC Official Method 923.03 (1995). Briefly, a sample of previously dried fish carcass (approximately 500 mg) was weighed and placed into a muffle furnace (Carbolite, Sheffield, UK) at 550 °C for 8 hours until a light grey ash resulted or samples reached a constant

weight. Samples were then weighed following cooling to room temperature in a desiccator and ash calculated as percentage of residue to sample weight; ((SR - CW)/SW)×100, where CW is crucible weight (g), SR is sample residue (including the crucible, g) and SW is the initial sample weight (g).

Protein content was determined by the Kjeldahl method to establish the total nitrogen content of samples, which was then multiplied by a factor of 6.25 to calculate crude protein content. Briefly, 100 mg of sample was transferred into a Kjeldahl digestion tube along with a catalyst tablet (3 g K_2SO_4, 105 mg $CuSO_4.5H_2O$ and 105 mg TiO_2, BDH Ltd, UK) and 10 ml of concentrated sulphuric acid. Digestion was carried out using a Gerhardt Kjeldatherm digestion block (Gerhardt Laboratory Instruments, Bonn, Germany) at 225 °C for 40 mins followed by 380 °C for 60 mins. The samples were then distilled using a Vapodest 40 automatic distillation unit (Gerhardt Laboratory Instruments, Bonn, Germany) and Crude protein determined as (((ST - BT) × 0.10×14×6.25) / SW)×100, where 0.10 = the molarity of the acid, 14 the relative atomic mass of nitrogen, 6.25 is the constant relationship between N and animal protein, ST is sample titre (ml), BT is blank titre (ml) and SW is the initial sample weight (mg).

2.6 Gross condition indices

Gross condition indices such as condition factor and organo-somatic indices may be used as a first level of screening for the effects of pollutants in animals (van der Oost et al., 2007) and as indicators of their general well-being (Schmitt and Dethloff., 2000). In turn this can offer insight into the energy reserves and possibly the ability of animals to tolerate toxicant challenges or other environmental stresses (Mayer et al., 1992). The indices used here were condition factor (*K*), hepatosomatic index (HSI) and splenosomatic index (SSI) as

described by Busacker et al. (1990). Condition factor is an indicator of an individual's health within a population with declines in *K* indicating changes in energy storage, metabolism and feeding activity. It is based upon the ratio between body weight and length, and is calculated as (K (%) = weight (g)/length^3 (cm)×100). Hepatosomatic index is the ratio between the weight of the liver and the total body weight of the fish (HSI = liver weight (g)/fish wet weight (g) x 100 % needed here). Wet liver weights are recorded following dissection and HSI calculated as (HSI (%) = liver weight (g)/wet body weight (g)×100). Changes in SSI could signal a dysfunction in the immune system capable of affecting individual health (Schmitt and Dethloff, 2000), with lesions such as necrosis or swelling due to infection affecting spleen size (Goede and Barton, 1990). Splenosomatic index is calculated as (SSI (%) = spleen wet weight (g)/body weight (g)×100).

2.7 Haematology and blood plasma analysis

Blood is the most accessible component of the vertebrate body fluid system (Houston, 1997) and is established as a useful diagnostic tool in veterinary science (Houston, 1990). Fish were randomly collected from each relevant tank and following anaesthesia whole blood collected via the caudal vein into heparinised syringes. Haematocrit (Hct) and haemoglobin (Hb) were determined immediately. Briefly, Hct was calculated in duplicate according to Handy and Depledge (1999) by centrifuging whole blood in microhaematocrit tubes (Jouan A13 haematocrit centrifuge) and calculating the ratio of packed red cells to supernatant. Haemoglobin content was determined by dispensing 20 µl of whole blood into 5 ml Drabkin reagent and the absorbance read on a spectrophotometer at 540 nm (Jenway 6300 Spectrophotometer) and haemoglobin content calculated against a standard calibration curve. Whole blood (20 µl) was fixed in Dacie's fluid (10 ml formaldehyde, 31.3 g trisodium citrate,

1.0 g brilliant cresyl blue, diluted to 1 litre with distilled water) for red and white blood cell counts. The remaining blood was centrifuged (13,000 rpm for 2 min, Micro Centaur MSE), and serum collected and stored at −20 °C until subsequent analysis of plasma ions and osmometry. Briefly, serum Na^+ and K^+ were analysed by flame photometry (Corning 480 Flame Photometer). Additionally plasma protein was analysed using the Bio-Rad (Bradford) protein assay kit II. Samples were read at 595 nm (OPTImax tunable microplate reader, Molecular Devices) against 0–1 mg ml^{-1} bovine serum albumin standards (Sigma A3912-50G). Plasma glucose was analysed in triplicate according to Sigma Diagnostics procedure No. 315 glucose (Trinder). Absorbances were read at 505 nm (Jenway 6300 spectrophotometer) and glucose determined against a standard calibration curve. Osmotic pressure was determined by the freezing-point depression method using 50 µl of plasma (Precision Systems micro osmometer, Natick, Massachusetts, USA).

2.8 Tissue ion analysis

Following blood sampling, fish were terminally anaesthetised with MS222 and dissected for tissue ion analysis. Tissues (including gill, liver, intestine, spleen, whole brain, and skinned muscle) from the flank were harvested, rinsed in deionised water and processed for ion analysis according to Handy et al. (1999) with minor variations. Samples were placed into an oven (Gallenkamp Oven BS Model OV- 160) at 100 °C for 48 h, dried to constant weight and the tissue removed into plastic polypropylene (with polyethylene cap) scintillation vials (VWR International Ltd, Poole, UK). Samples (typically 0.1-0.5 g dried tissue) were then digested in 4 ml of concentrated nitric acid (69 % analytical grade, Fisher Scientific) for 2 h at 70 °C in a water bath, cooled, and then diluted to 16 ml using ultrapure water (Milli-Q, resistivity 18.2 MΩ cm). For very small tissue samples (less than 0.1 g dry weight) the

volumes of reagents were reduced pro rata (1 ml of nitric acid, diluted to a final volume of 4 ml). Samples were then analysed for trace elements (e.g., Cu, Zn, Mn, Ca, Na and K) by inductively coupled plasma optical emission spectrometry (ICP-OES, Varian 725 ES). Instrument calibration was achieved using mixed, matrix-matched standards between 0-500 mg l^{-1} (depending upon the analyte), prepared from Aristar® plasma emission grade solutions, with accuracy checked after every 10-20 samples during the analysis by running a blank or standard as a sample. Certified fish reference tissues were used where possible along *with tissue spike recovery tests, where a recovery of* $\geq$ *90 % of the nominal spike* concentrations was considered acceptable. When computing results any values below the detection limit were set to zero.

2.9 Biochemistry

Samples of tissues were collected and stored at -80 °C in order to assay for biochemical indicators of osmoregulatory disturbances or oxidative stress.

2.9.1 Sample collection and homogenisation

Fish tissue (e.g., gill, liver, intestine, and whole brain) were removed and immediately snap frozen in liquid nitrogen and stored at -80 °C until required. Tissues (about 0.5 g, or whole brain) were weighed and homogenised (Cat X520D with a T6 shaft, medium speed, Bennett & Co., Weston-super-Mare) in five volumes (2.5 ml) of ice-cold isotonic buffer (in mmol l^{-1}; 300 sucrose, 0.1 EDTA, 20 HEPES, adjusted to pH 7.8 with a few drops of Tris hydrochloride. Crude homogenates were stored in 0.5 ml aliquots at −80 °C until required.

2.9.2 Protein assay

A semi-micro method based on the Hartree (1972) modification of the Lowry protein assay was used to determine protein content of the tissue homogenates in order to normalise enzyme activity for further biochemical analyses. Briefly, 1 ml of blank (double distilled water (DDW)), protein standard (BSA, 40-200 µl ml^{-1}), or diluted sample (10 µl of homogenate in 0.99 ml of DDW) was added to a clean test tube containing 0.9 ml of 1.0 g potassium sodium tartrate and 50 g sodium carbonate in 250 ml of 1M NaOH made up to 500 ml with DDW and incubated at 50 °C for 10 minutes. After cooling to room temperature 0.1 ml of 1.0 g potassium sodium tartrate, 0.5 g $CuSO_4$, and 5 ml of 1M NaOH made up to 50 ml with DDW was added to each tube and incubated at room temperature for 10 minutes. Following this 3 ml of 1 volume of Folin Ciocalteau reagent diluted with 15 volumes DDW was added and the samples vortexed to ensure rapid mixing and the samples incubated at 50 °C for 20 minutes. After cooling to room temperature the samples were dispensed to a 96-well microplate and read at 650 nm (Dynex MRX plate reader).

2.9.3 Sodium-potassium-ATPase (Na^+/K^+-ATPase) activity

The Na^+/K^+-ATPase assay was performed according to Bouskill et al. (2006). Briefly, each sample (15 µl, in triplicate) was dispensed into test tubes containing either 400 µl of a K^+-containing buffer (in mmol l^{-1}; 100 NaCl, 5 $MgCl_2$, 10 KCl, 1.25 Na2ATP, 30 HEPES, adjusted to pH 7.4) or a K^+-free buffer (identical, but replacing KCl with 1.0 mmol l^{-1} ouabain, a sodium pump inhibitor), then incubated at 37 °C for 10 minutes. The reaction was stopped by adding 1 ml of ice-cold trichloroacetic acid and 1 ml of colour reagent was added to each tube (9.6 %, w/v $FeSO_4 \cdot 6H_2O$, 1.15 %, w/v ammonium heptamolybdate dissolved in

0.66 M H_2SO_4), and colour allowed to develop for 20–30 minutes at room temperature. Absorbances were measured at 630 nm (Dynex MRX microplate reader) against 0–2.5 mmol l^{-1} phosphate standards. Na^+/K^+-ATPase activity is determined as the difference in inorganic phosphate released (from ATP hydrolysis by the enzyme) in the presence and absence of potassium (ouabain, a sodium pump inhibitor, used in K^+-free media, with the difference representing Na^+/K^+-ATPase activity), corrected for spontaneous nonenzymatic breakdown of ATP (in a sample blank) over 1 hour and normalised per mg protein.

2.9.4 Thiobarbituric acid reactive substances (TBARS)

Total lipid peroxidation products were measured by TBARS. A 40 µl aliquot of homogenate was added in triplicate to a well of a 96-well microplate containing 1 mmol l^{-1} butylated hydroxytoluene (2,6-di-O-tert-butyl-4-methylphenol), and the final volume was made up to 200 µl with 1 mmol l^{-1} phosphate buffered saline (adjusted to pH 7.4). Following this, 50 % (w/v) trichloroacetic acid (TCA) and 1.3 % (w/v) thiobarbituric acid (TBA) (dissolved in 0.3 % (w/v) NaOH), were added, and the plate incubated at 60 °C for 60 minutes and then cooled on ice. Absorbances were read at 530 and then 630 nm (Dynex MRX microplate reader), to normalise for any turbidity prior to reading against 1,1,3,3-tetraethoxypropane standards (0.5–25 µmol l^{-1}) and expressed per mg of protein in the initial volume of homogenate added.

2.9.5 Total glutathione

For this assay 20 µl of gill or liver homogenate or 40 µl of intestine or brain homogenate was added in triplicate to a microplate well containing 20 µl of 10 mmol l^{-1}

DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)), 260 µl of assay buffer (100 mmol l^{-1} K_2HPO_4, 5 mmol l^{-1} K-EDTA, pH 7.5), and 20 µl of 2 U ml^{-1} glutathione reductase. The reaction was commenced by the addition of 20 µl of 3.63 mmol l^{-1} NADPH, with changes in absorbance at 412 nm (OPTImax tunable microplate reader, Molecular Devices) recorded over 6 minutes, and total GSH content (µmol g^{-1} wet weight tissue) determined using a standard calibration curve (serial dilution of a 200 µmol l^{-1} reduced glutathione solution).

2.10 Routine statistics

All data were analysed by StatGraphics Plus version 5.1 and graphs drawn using SigmaPlot 10.0 or 11.0. Where no tank effects were observed throughout the experiment (i.e. between replicates of a treatment at each time point), data were pooled by treatment for statistical analysis and significance set at 95 % limits, so that *P*-values equal to or greater than 0.05 were not considered statistically significant. Data were also evaluated for kurtosis (degree of peakedness of a distribution) and skewedness (degree of asymmetry of a distribution) with deviations of ± 2 from zero considered abnormal distribution (from a Gaussian distribution). Bartlett's test was performed initially in order to check for unequal variance. Statistical tests such as analysis of variance (ANOVA) are reliant upon the assumption of equal variances across groups or samples and whilst Bartlett's test is sensitive to departures from normality, with skewed data possibly affecting the tests' robustness (Quinn and Keough, 2002), data from the current study were not skewed, therefore justifying usage of this variance check and the subsequent ANOVA.

In most instances the statistical analysis required the comparison of treatment, time, and treatment x time interactions. On parametric data his would be carried out by use of a multifactor ANOVA. If this model showed statistically significant differences then further

statistical tests could be applied for 'simple effects'. For simple two sample comparisons, the Student's *t*-test (two tailed, unpaired) was used, or the Mann Whitney test for non-parametric data. However, for comparison of multiple data points with one variable (e.g., time effects or treatment effects, but not time x treatment) one-way ANOVA was used. All ANOVAs were followed by Fisher's 95 % least-squares difference post hoc test. Fisher's test is a two-step testing procedure for pair wise comparisons of the exact means of several treatment groups (Meier, 2006); employed after the ANOVA null hypothesis of equal means has been rejected using the ANOVA *F*-test. For non-parametric data, where transformation was not effective, the Kruskal–Wallis (analysis of variance by ranks) test was used and differences located by notched box and whisker plots. Where the ANOVA test depends upon the assumption that all data are normally distributed, the Kruskal–Wallis test has no such restrictions. Box and whisker plots (also called box plots) display batches of data and the notched function provides a measure of the rough significance of differences between the values, and specifically indicates a significant difference (at a 95 % confidence level) if the notches about two medians do not overlap (McGill et al., 1978).

Where appropriate, linearity of standard calibrations was confirmed by correlation coefficients (Pearson Product Moment Correlation method), as was the correlation between two variables. In this analysis pairs of variables with positive correlation coefficients and *P* values below 0.050 tend to increase together, whilst pairs with negative correlation coefficients and *P* values below 0.050, tend to have one variable decreasing whilst the other increases. For pairs with *P* values greater than 0.050, there is no significant relationship between the two variables.

The Trimmed Spearman-Karber method (Hamilton et al., 1977), was used for estimating median lethal concentrations in toxicity bioassays (i.e., LC_{50} tests), using free software from the United States Environmental Protection Agency (US EPA;

http://www.epa.gov/eerd/stat2.htm). Statistical significance was assumed when there was no overlapping of the 95 % confidence intervals between treatments.

Chapter 3. An Improved Method for the Determination of Total Titanium from Titanium Dioxide Nanoparticles in Fish.

Abstract

The reliable detection of Ti from TiO_2 nanoparticles (NPs) is required to support ecotoxicological research on TiO_2 NP uptake, and the food safety industry also requires a method for measuring Ti from TiO_2 NPs in edible fillets (muscle) of fish. Existing analytical techniques are inadequate for some metallic NPs, yielding low spike recoveries. For example, fish intestine spiked with TiO_2 NPs or Ti metal spikes gave recoveries of 18.1 ± 9.1 and 97.5 ± 2.9 % respectively (all data, mean ± SEM, $n = 6$). Investigations of analyte loss showed no effect with the type of digestion vials used (glass versus plastic). Attempts to improve recovery by stirring or sonicating samples, or adding sodium dodecyl sulphate were not effective. However, the addition of 2 % Triton X-100 improved recovery of Ti from TiO_2 NPs by 3-10 fold. Spikes of 100 $\mu g\ l^{-1}$ TiO_2 NPs produced the highest recoveries and were 69.7 ± 8.6; 65.0 ± 1.7; 55.0 ± 5.8; 44.7 ± 5.8; and 41.3 ± 4.0 % for whole zebrafish, and trout gills, muscle, intestine, and liver respectively. Precision and accuracy were also within acceptable limits. The new method represents a marked improvement in determining Ti metal from TiO_2 NPs in tissues with potential uses in both ecotoxicology and food safety.

3.1 Introduction

Engineered nanomaterials (NMs) can be defined as novel materials with at least one dimension < 100 nm (SCENIHR, 2007). These materials can be categorised by their main chemical composition, and include nanoscale metals and metal oxides, carbon-based materials such as carbon nanotubes (CNTs) and carbon spheres (e.g., C_{60} fullerenes), as well as a range of nano-ceramics, quantum dots and nano-composites that are each made from several chemical substances (Boxall et al., 2007; Stone et al., 2010). The interest in NMs partly arises from their unique physical and chemical properties at the nanoscale (Štengl et al., 2007; Handy et al., 2008). However, some of these properties such as poor solubility in water, the colloidal behaviour of nanoparticles (NPs) in aqueous media (aggregation, agglomeration), and adsorption to surfaces, are creating new challenges for analytical chemists (see Handy et al., 2008 and Hassellöv et al., 2008 for reviews of measurement methods for NPs).

Titanium dioxide (TiO_2) NPs are used in cosmetics, sunscreen, paint, and building materials (Aitken et al., 2006), and potential applications in food have also been suggested (Chaudhry et al., 2008; Sozer and Kokini, 2009). The latter is particularly interesting with respect to food safety as the traditional pigment form of ordinary TiO_2 powder has been used for many years (e.g., in fish nutrition, Lied et al., 1982; Weatherup and McCracken, 1998; Mamun et al., 2007) and is regarded as safe at inclusion levels of a few percent in foods. However, the potential benefits of TiO_2 NPs in food production are also balanced by concerns about the ecotoxicity of these materials to fish (Federici et al., 2007; Zhang et al., 2007; Handy et al., 2008) and, more generally, to humans (Handy and Shaw, 2007b) with oral exposure to TiO_2 NPs causing lesions in the liver and kidneys of mice (Wang et al., 2007). A reliable method of detecting titanium from TiO_2 NPs in meat or fish would therefore be

valuable to the food industry as well as for the quantification of the exposure of internal organs of fish during ecotoxicity studies.

The traditional methods for determining trace metals in fish tissues rely on mineral acid digestion of the flesh, and subsequent analysis by atomic absorption spectrophotometry (AAS, e.g., Manutsewee et al., 2007), inductively coupled plasma optical emission spectrophotometry (ICP-OES; e.g., Handy et al., 1999), and sometimes inductively coupled plasma mass spectrometry (ICP-MS) for elements such as uranium where very low detection limits are required, (e.g., Bourrachot et al., 2008). However, for TiO_2 NPs, these traditional methods give variable results and very poor spike recovery tests (e.g., Scown et al., 2009). Hydrofluoric acid (HF) digestion has previously been used for determination of Ti from bulk TiO_2 in geological samples (e.g., silicate materials, Makishima and Nakamura, 2000) with Ti being a fluorophile element that will form a stable, soluble fluoro-complex (anion) in HF (Cotton and Wilkinson, 1988). However, HF is a strong, inorganic acid that has a highly caustic and corrosive effect on organic tissue (Matsuno, 1996) and even when dilute can cause deep, slow-healing, and extremely painful burns, with the vapour also seen to produce lesions on contact with skin (Dale and Chir, 1951). HF will also dissolve glassware and damage the nebuliser and spray chamber of the ICP instrument (Varian, personal communication). As such specially designed, HF resistant, apparatus are required for sample processing and subsequent storage, and for sample introduction and processing within a dedicated ICP MS instrument. Coupled with the extra health and safety precautions and measures required when working with this acid, its use for routine analysis of tissues in ecotoxicology is not practical. Researchers have therefore tried various approaches to improve digestion and quantification using more practical means. For example, Scown et al., 2009 report a "digestion efficiency" of 28.8 % after additions of the non-ionic surfactant, Triton-X 100, to improve the dispersion of NPs in the tissue digest. Instruments also respond

differently to Ti metal solutions compared to dispersions of TiO_2, and in the absence of certified standards for TiO_2 NPs in fish tissues, dual calibrations, where titanium metal (dissolved Ti) standards are run against carefully prepared TiO_2 NP dispersions have been used to correct for the calibration responses of instruments (Federici et al., 2007; Zhang et al., 2007).

However, full details of the analytical method development for measuring Ti in tissues from TiO_2 NPs have not been documented. For practical use in food safety the accuracy, precision, such as within and between sample variability (e.g., coefficient of variation for repeat analyses) need to be established. It would also be useful to establish whether the methodology works for the different types of tissues that may be studied in ecotoxicology. The overall aim of this study was to describe, test, and validate a method of quantifying total tissue Ti levels from fish tissues exposed to TiO_2 NPs, and to report the utility of the method for determining tissue Ti levels for ecotoxicology studies, as well as for measuring Ti levels in fish muscle from the view point of food safety of fish fillets. A reliable method of Ti analysis is also a pre-requisite for the development of certified reference fish tissues for TiO_2 NPs, which are currently not available.

3.2 Experimental Section

3.2.1 Titanium Dioxide Nanoparticle Stock Solution

The titanium dioxide NP powder used herein was from the same batch previously characterised and used by our laboratory (Federici et al., 2007) and stock solution preparation was identical in the present study. Briefly, dry, powdered TiO_2 NPs ("Aeroxide" P25 TiO_2, DeGussa AG, supplied via Lawrence Industries, Tamworth, UK) comprised 75 % rutile and

25 % anatase TiO_2, had a purity of at least 99 % TiO_2 (< 1 % Si), an average particle size of 21 nm and a specific surface area of 50 ± 15 $m^2 g^{-1}$. Chemical analysis of stock solutions revealed no metal impurities (data not shown), and particle analysis by transmission electron microscopy (TEM, JEOL 1200EXII) showed a mean primary particle size of 24.1 ± 2.8 nm (mean ± SEM, n = 100 electron microscope images, see Federici et al., 2007). A 10 g l^{-1} stock solution of TiO_2 NPs was generated without solvents by dispersing the NPs in ultrapure Milli-Q (Millipore) water with sonication (bath type sonicator, 35 kHz frequency, Fisherbrand FB 11010, Germany) for 6 h.

3.2.2 Stock Animals and Collection of Tissue Samples

Stock rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) were kept in stock aquaria with flowing, aerated, Plymouth tap water (dechlorinated by standing with aeration for at least 24 h prior to use in tanks). The ionic composition of the dechlorinated tap water was 0.3, 0.1, and 0.4 mmol l^{-1} of Na^+, K^+ and Ca^{2+} respectively and the photoperiod for the stock fish was set to a 12 h light: 12 h dark cycle. The rainbow trout used varied in age from fingerlings to 1 year old juveniles with a wet weight range of 20-900 g whereas zebrafish were all mature adults with a weight range of 0.3-0.9 g (wet weight). For sample collection, fish were terminally anaesthetised with MS222 and dissected to harvest target organs using acid cleaned instruments (triple washed in 5 % nitric acid and then triple washed in deionised water). Dissected tissues or whole zebrafish were thoroughly rinsed with deionised water, blotted dry and placed onto new, acid washed, slides in preparation for dehydration.

3.2.3 Established Metal Detection Method

Initially, attempts were made to utilise an existing method for trace element analysis in fish tissues in order to measure TiO_2 NPs. This method has been used many times by our laboratory (e.g., Handy et al., 1999; Handy et al. 2000; Shaw and Handy, 2006, see Chapter 2 for method details) and typically gave recoveries of 100 ± 5 % of the target value in spike recovery tests performed on tissue samples for soluble metals (e.g., Handy et al., 2000). Briefly, fish tissues were oven dried to constant weight over 48 h following sample collection and rinsing as described previously and then transferred to 20 ml plastic polypropylene (with polyethylene cap) scintillation vials (VWR International Ltd, Poole, UK). Approximately 0.1-0.3 g of dried tissue was digested in 4 ml of concentrated nitric acid at 70 °C for 7 hours and then diluted to 16 ml using Milli-Q water. Samples were analysed for Ti along with Cu, Zn, Mg, Ca, Na and K by inductively coupled plasma optical emission spectrometry (ICP-OES, Varian 725 ES, Mulgrave, Australia).

3.2.4 Instrumentation and Quality Control

During sample analysis the Varian 725 ES ICP-OES operating parameters were; power, 1.4 KW, plasma, auxiliary and nebuliser flows, 15, 1.5, and 0.68 l min^{-1}, respectively, and instrument stabilisation, time uptake delay, and replicate read time, 10, 15, and 4 s, respectively, with a wavelength of 336.122. Calibration was achieved with mixed, matrix-matched standards between 0-1000 mg l^{-1} (depending upon the analyte), prepared from Aristar® plasma emission grade solutions. The calibration blank contained 25 % nitric acid with no standards. Calibration of the ICP-OES for Ti analysis was successfully achieved using either dissolved Ti or TiO_2 NPs and for practical reasons (see discussion) the former

was used throughout. The detection limit of the instrument for dissolved Ti (3 x standard deviation of the 2 % nitric acid blank) was 7.04 μg l^{-1} (n = 18). The procedural detection limit of dissolved Ti for tissue digests going through the entire protocol (3 x standard deviation of the digestion protocol blank) was 4.58 μg l^{-1} (n = 6). Titanium dioxide values were calculated from the dissolved Ti values using stoichiometric conversion based on atomic weight. In a typical sample run, the blank or a standard was checked (run as a sample) after every 10 samples. In the absence of certified fish reference tissues for total Ti analysis, or for the TiO_2 NP content of tissues, spike recovery tests using both dissolved Ti and TiO_2 NPs were conducted using rainbow trout intestine. Briefly, samples were oven dried as described above and known concentrations of dissolved Ti or TiO_2 NPs were added to the vial prior to the addition of concentrated nitric acid and the subsequent digestion. Samples were then diluted with Milli-Q water as described above. However, following the poor recovery obtained from TiO_2 NP spiked tissue it was evident that the established method of analysis needed improving and optimising for TiO_2 NPs.

3.2.5 Method Development

A series of experimental tests were conducted to improve the methodology for detection of TiO_2 NPs in biological samples. A pilot study comparing ICP-OES (Varian 725 ES) with ICP-Mass Spectrometry (V.G. Plasmaquad PQ2 ICP-MS, Fisons Instruments, Winsford, Cheshire, UK) found that percentage recovery of Ti from fish tissue was lower when using ICP-MS compared to ICP-OES with a high level of variability between samples in the former (i.e., more than ± 20 %, data not shown). The ICP-OES showed far greater sensitivity to Ti in the form of TiO_2 NP than the ICP-MS, and so the method was optimised for ICP-OES. The optimisation trials included: (i) characterising the response of the ICP-OES

to TiO_2 NP standard solutions compared to dissolved Ti standards and optimising the instrument settings so that both responses were linear; (ii) trials to determine where any losses of TiO_2 NPs could be occurring during the digestion protocol. This involved spiking samples with known concentrations of TiO_2 NPs (see results) and investigating adsorption losses to different types of vials used in acid digestion, i.e., plastic polypropylene vials (VWR International, Poole, UK) compared to glass vials (type 1 'fiolax' borosilicate glass vials, Taab Laboratories, UK), and the effect of spiking before or after the acid digestion process; (iii) the use of surfactants, i.e., sodium dodecyl sulphate (SDS, general laboratory grade, code no. S/5200/53, Fisher Scientific, UK) and Triton X-100 (10 % in solution, laboratory grade, lot no. 126H1030, Sigma-Aldrich, UK); (iv) the effects of stirring or sonication (2 h prior to analysis) to aid the dispersion of NPs at various stages during tissue digestion; and (v) a repeat of the spike recovery tests above with different tissues (gill, liver, muscle, spleen and intestine) to demonstrate whether or not the type of tissue altered the success of the protocol. All trials were conducted using appropriate controls (negative controls; no added TiO_2 NPs or dissolved Ti), and in some of the trials tissue samples that were spiked with known amounts of dissolved Ti (positive controls) were used to compare with TiO_2 NP spikes.

Following these trials, an optimised method was established, and carried out several times with different tissue samples in order to determine the precision and accuracy of the optimised method. One set of samples were also analysed after 2 h, 3 d, and 14 d to test for changes in the measured Ti levels over time.

3.2.6 Final Optimised Method for Determining Ti from TiO_2 NPs in Fish Tissue

For the tissue digestion phase, the main modification included the addition of 2 % Triton X-100 during sample dilution. Tissue samples were processed for acid digestion as

described above in the initial method, however once samples had cooled, Triton X-100 was slowly added to each digested sample prior to dilution with Milli-Q water to achieve a final volume of 2 % Triton X-100 in each sample (3.2 or 0.8 ml of the 10 % Triton X-100 solution carefully pipetted into each digest vial for 16 or 4 ml final dilutions, respectively). Following the addition of Triton X-100 and final dilution (to 16 ml or 4 ml respectively), samples were then stored in a cool, dark place until subsequent analysis.

Prior to each analysis, samples were placed on an orbital shaker (KS501 digital orbital shaker, IKA Labortechnik) set at 145 $r.min^{-1}$ for a minimum of 30 min to ensure proper mixing of the sample. Samples were sequentially removed from the orbital shaker and immediately analysed by ICP-OES without further agitation for Ti, Cu, Zn, Mg, Ca, Na and K (ICP-OES parameters set precisely as described in Instrumentation section above), using matrix matched standards (i.e., containing 25 % nitric acid and 2 % Triton X-100).

The precision of the optimised protocol was then assessed, firstly by comparing within sample variation (the same trout muscle sample measured in triplicate) in order to ascertain the coefficient of variation of the ICP-OES, and secondly by measuring multiple muscle segments (each as individual samples) from the flesh of one fish, to get a measure of the procedural variation. Between fish variation was assessed by measuring Ti levels in tissues from different animals. To assess any differences in tissue used, trout gills were then tested for precision over a serial dilution of TiO_2 NP spikes (100-1000 $\mu g\ l^{-1}$). Data for precision are presented per g tissue.

3.2.7 Statistical Analysis

All data were analysed by StatGraphics Plus version 5.1 and graphs drawn using SigmaPlot 10.0 as described in Chapter 2.

3.3 Results

3.3.1 Quality Control and Assurance

Calibration with both dissolved Ti and TiO_2 NP standards produced r^2 values in excess of the 0.9975 value threshold that the ICP-OES requires in order to calibrate the instrument. Linearity was established for dissolved Ti and TiO_2 NP standards with both producing correlation coefficients of 1, with P values of 2.50×10^{-8} and 1.14×10^{-6} for dissolved Ti and TiO_2 NP respectively (Pearson Product Moment Correlation). Standards were ran every 10 samples in order to assess for deviation from nominal levels (i.e., > 5 % of the nominal value) or blanks for Ti peaks above background levels. However, as no drift was observed (i.e., < 5 %). The instrument did not require recalibrating and the absence of discernable Ti peaks in the blanks indicated no standard or sample carry over. Recovery of dissolved Ti (100 $\mu g\ l^{-1}$) spikes was complete using the standard metal method in fish intestine samples (data were, mean ± SEM, $n = 6$; 97.5 ± 2.9 %), but this original method gave very poor results for intestine samples spiked with 100 $\mu g\ l^{-1}$ TiO_2 NPs (recovery of 18.1 ± 9.1 %, $n = 6$).

3.3.2 Method Development

An investigation into potential variations in recovery related to the vial types (i.e., glass or plastic polypropylene) used for tissue acid digestion showed no statistically significant differences in spike recoveries between the two container materials (students *t*-test, $P > 0.05$; Fig. 3.1a). Similarly, no significant differences were seen when the stage of

spiking the sample during the process was altered (i.e., before or after acid digestion) for both dissolved Ti and TiO_2 NP spiked samples (students *t*-test, $P > 0.05$; Fig. 3.2).

Samples were then manipulated to improve the spike recovery. Samples that were stirred or sonicated immediately prior to reading on ICP-OES did not show improved Ti recovery compared with controls (Fig. 3.1b), nor did the use of the anionic surfactant, SDS, in spiked samples (recovery appeared reduced in these samples, though not significantly; ANOVA, $P > 0.05$; Fig. 3.1c). However, the addition of Triton X-100 to each spiked sample significantly improved Ti recovery compared to controls (spiked samples with no added detergent) or SDS-treated samples (ANOVA, $P < 0.05$; Fig. 3.1c). Therefore further experiments were carried out in order to ascertain the optimal concentration of Triton X-100 required and to standardise the procedure (see below).

3.3.3 Optimisation of Triton X-100 Method for Ti Determination from TiO_2 NPs in Fish Tissue

A series of spike recovery experiments (with 100 $\mu g\ l^{-1}$ TiO_2 NPs spikes) were conducted whereby increasing concentrations of Triton X-100 (i.e., 0-10 % v/v of final digest volume) were added to trout gill samples following acid digestion. Recovery was concentration dependent with values of (data mean ± SEM, $n = 6$ TiO_2 NP spikes per concentration of Triton X-100) 25 ± 3, 35 ± 9, 49 ± 8, 75 ± 8, 64 ± 9, 25 ± 8, 12 ± 4, and 0.5 ± 0.02 % for samples containing 0, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 % Triton X-100 respectively. No differences in recovery were observed when Triton X-100 was added before or after Milli-Q water dilution of the acid digest (student's *t*-test, $P > 0.05$, data not shown). Notably, subsequent experiments showed that the use of standards to calibrate the ICP-OES

that were not matrix matched to the samples (i.e., did not contain equivalent Triton X-100 (or acid concentrations)) resulted in an over reading of Ti in the spiked samples (e.g., Fig. 3.3a).

Further experiments also revealed that recovery could be further improved by agitation of the samples immediately prior to ICP analysis and so the method, vigour and time of mixing were explored further. The most effective method proved to be the swirling of samples on an orbital shaker at 145 $r.min^{-1}$ for at least 30 min prior to reading (significantly different from no active agitation and other mixing methods tested: stirring, inversion, vortexing; ANOVA, $P < 0.05$) For example, gill samples, gave recoveries of 67 ± 4 and 47 ± 7 % (mean ± SEM, $n = 6$) when measured without agitation and then with swirling on an orbital shaker as described above.

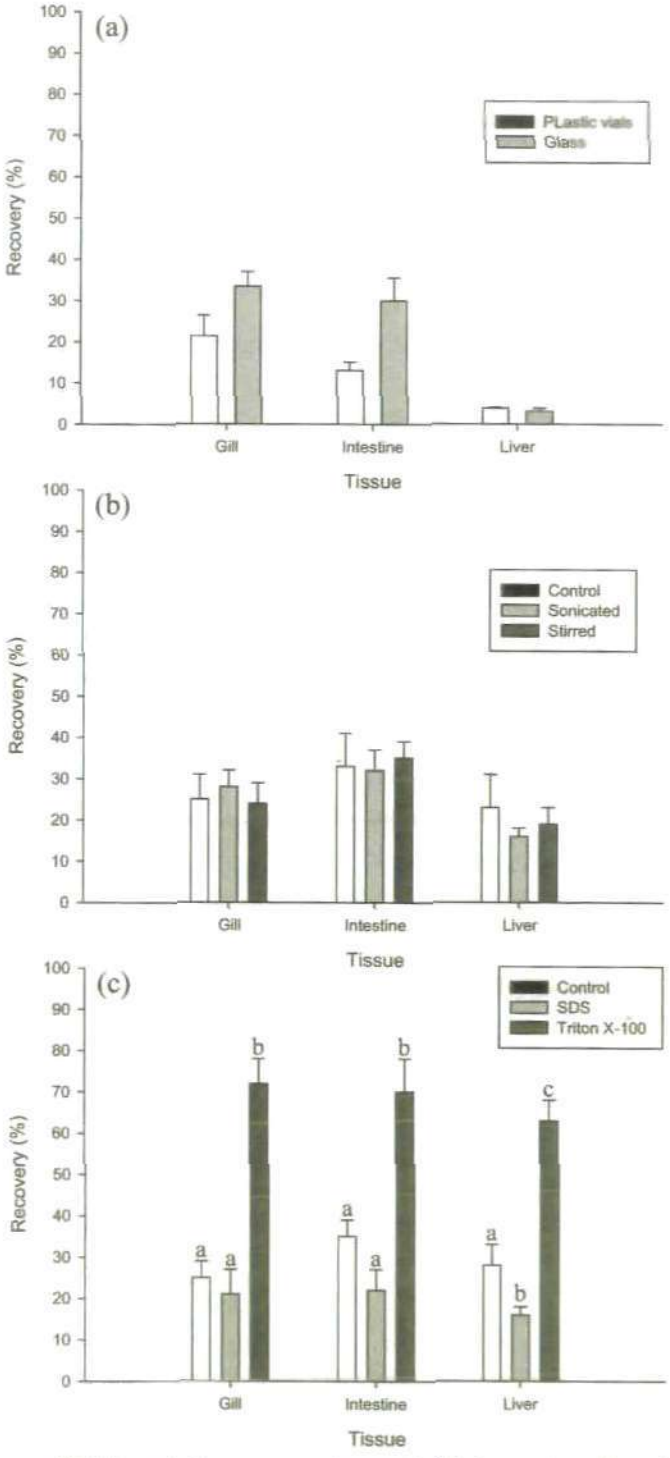


Figure 3.1. Recovery of Ti in rainbow trout tissue (gill, intestine, liver) spiked with 100 μg l^{-1} TiO_2 NPs, effects of protocol variables: (a) glass versus plastic polypropylene digestion vials, and (b) 2 h stirring or sonication of samples, and (c) the effects of SDS and Triton X-100 (both 2 % v/v of final digest). Note that the standards did not contain any surfactant and as such the samples over read. Data are mean ± SEM, % recovery of TiO_2 NP spike, $n = 6$; different letters indicates significantly different to other treatments within tissue (ANOVA, $P < 0.05$); absence of annotation on panels (a) and (b) indicates no statistically significant differences between treatments within each tissue.

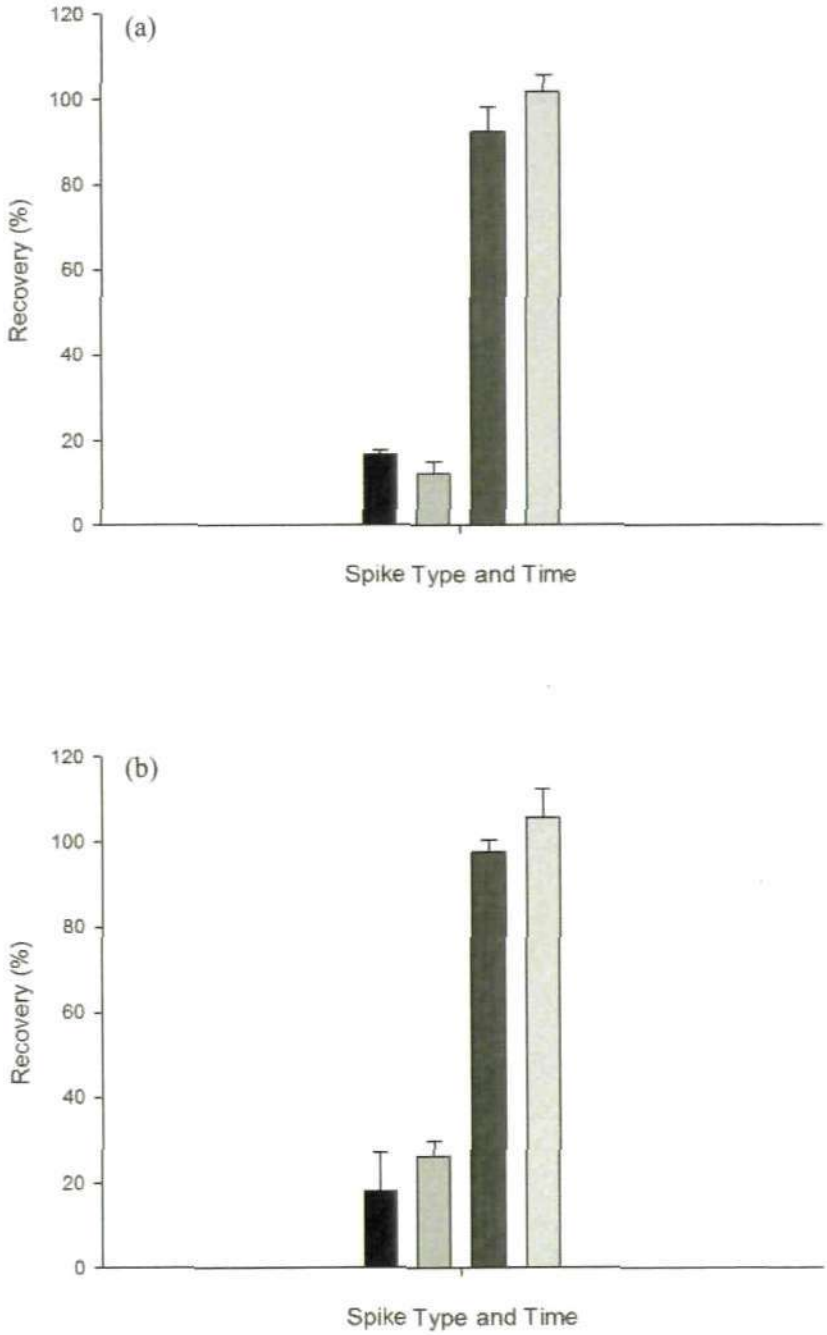


Figure. 3.2. Recovery of Ti in rainbow trout (a) gill and (b) intestine, spiked with 100 $\mu g\ l^{-1}$ Ti metal or TiO_2 NPs before and after digestion by nitric acid. Data are mean ± SEM, % recovery of Ti metal or TiO_2 NP spike, $n = 6$; the absence of annotation on both panels (a) and (b) indicates no statistically significant differences between the recovery of Ti from each treatment when spiking before or after digestion (Student's *t*-Test, $P > 0.05$).

3.3.4 Validation of Optimised Protocol

Following optimisation of the protocol a series of spike recovery tests were conducted using a serial dilution of TiO_2 NPs (0-1000 $\mu g\ l^{-1}$) in order to validate the efficacy and precision of the method. Ti recovery was tissue- and spike concentration-dependent, with the highest recovery achieved using whole zebrafish and the lowest using liver tissue. A spike concentration of 100 $\mu g\ l^{-1}$ TiO_2 NPs provided the highest recovery in each tissue type compared with more concentrated spikes (see Fig. 3.3b for % recovery data of 100 $\mu g\ l^{-1}$ TiO_2 NP spikes). The overall trend was for reduced recovery with increased spike concentration (non-significant in liver, muscle, and intestine; ANOVA, $P > 0.05$). Recovery data for trout tissues ranged from (% mean ± SEM, $n = 6$ samples for each tissue at each spike concentration); 36.2 ± 4.5 to 53.3 ± 4.4 for liver; 45.9 ± 1.7 to 55.0 ± 5.8 for muscle; and 32.5 ± 5.6 to 52.1 ± 1.1 for intestine. Spike recovery was significantly higher in zebrafish samples spiked with 100 $\mu g\ l^{-1}$ TiO_2 NPs than was achieved at higher spike concentrations (Kruskal-Wallis; $P < 0.05$) with recoveries of 69.7 ± 8.5, 53.8 ± 2.5, 54.4 ± 2.3, 42.2 ± 5.0, 45.5 ± 0.6, and 44.9 ± 2.8 from 100, 200, 300, 400, 500, and 1000 $\mu g\ l^{-1}$ TiO_2 NP spikes respectively (data mean ± SEM % recovery, $n = 6$).

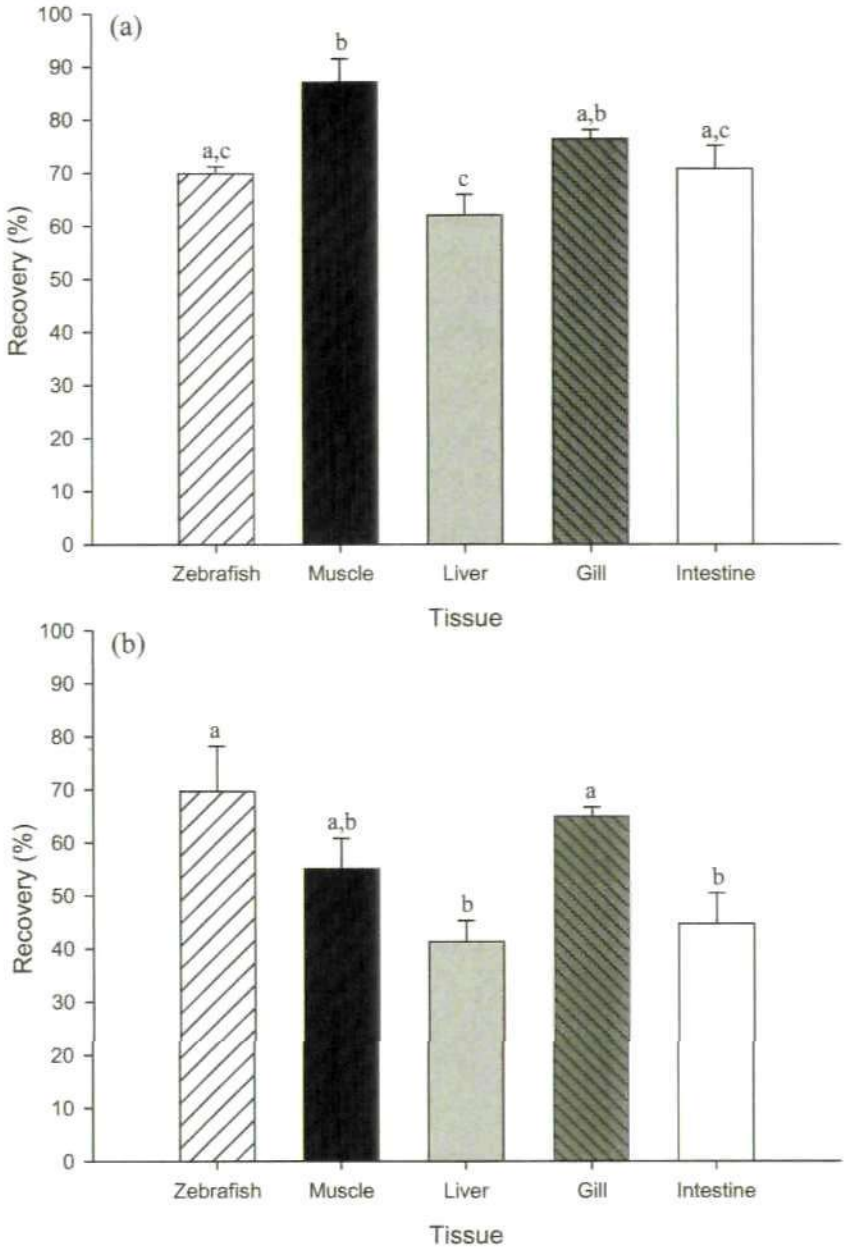


Figure 3.3. Recovery of Ti in whole zebrafish and rainbow trout tissue (muscle, liver, gill, intestine) samples spiked with 100 µg l^{-1} TiO_2 NPs with 2 % Triton X-100 added following acid digestion and measured on ICP-OES calibrated using (a) standards with no added Triton X-100 and (b) standards containing 2 % Triton X-100. Note that where calibration was carried out with standards that did not contain Triton X-100 the samples over read (i.e., panel a). Data are mean ± SEM, % recovery, $n = 6$. Different letters indicate significant differences between tissues with the same letter indicating no significant difference (ANOVA, $P < 0.05$).

Significant differences were also seen between the 200 and 300 μg l^{-1} TiO_2 NP spiked samples and the 400, 500, and 1000 μg l^{-1} spikes (Kruskal-Wallis; $P < 0.05$). Gill samples from trout showed concentration-dependent increases in percentage recovery which were statistically significant in samples spiked with 100 μg l^{-1} TiO_2 NPs compared with all other spikes (ANOVA, $P < 0.05$; data mean ± SEM % recovery (nominal TiO_2 NP spike concentration in μg l^{-1}); 65.0 ± 1.7 (100) 59.8 ± 1.8 (200); 57.8 ± 1.8 (300); 58.7 ± 1.3 (400); 56.2 ± 2.2 (500); and 54.4 ± 2.7 (1000), $n = 6$).

The effective shelf life of tissue digest samples containing 2 % Triton X-100 was investigated by measuring the samples after 2 hours, 3 d and 14 d after the addition of Triton X-100. Notably, 14 d after adding the Triton X-100, Ti recovery from the TiO_2 NP spiked samples was significantly reduced compared with the initial measurement (e.g., spike recovery of 45 % in intestine samples after 14 d compared to approximately 70 % after 2 h, ANOVA, $P < 0.05$), whilst an approximately 5 % reduction was noticed after 3 d compared to 2 h. In order to ascertain whether samples containing Triton X-100 could also be used to accurately measure other analytes, whole zebrafish samples were tested for differences in tissue Ca, Na, Cu, Zn, Mg, and K levels with and without Triton X-100. No significant differences were seen in samples or standards containing 2 % Triton X-100 compared to those without (Table 3.1; Students *t*-test, $P > 0.05$) indicating that interferences were not a concern.

The results of precision testing of the optimised protocol are presented in Tables 3.2 and 3.3. Within-sample precision using rainbow trout muscle tissue (triplicate readings from the same sample; Table 3.3) produced coefficient of variation (CV) values ranging from 5.45-12.38 %. However, within-fish variation (differences when muscle tissue from one fish was divided equally, each piece processed as individual samples and measured for Ti content) was slightly higher (14.88 %, Table 3.2) than the within-sample variation indicating a minor

Table 3.1 Effects of Triton X-100 on the analysis of trace elements in whole zebrafish using ICP-OES (data are mean ± SEM, μmol g^{-1} dry weight, $n = 6$).

Standards	**Trace element analysed**					
	Ca^{2+}	Cu	K^{+}	Mg^{2+}	Na^{+}	Zn
Without Triton X-100	219.5 ± 7.2	0.034 ± 0.003	61.2 ± 1.8	18.4 ± 0.9	45.7 ± 2.3	2.63 ± 0.7
With 2 % Triton X-100	224.9 ± 6.5	0.039 ± 0.004	64.6 ± 1.1	19.0 ± 1.2	44.5 ± 3.1	2.81 ± 0.5

Note: these were not spikes, but naturally occurring levels of each element in whole zebrafish, measured initially without and then with 2 % Triton X-100 added. Note however, that the standards for each run were matrix matched to the samples (i.e., when Triton X-100 was added to the samples it was also added to the standards at the same concentration).

Table 3.2. Instrument and procedure precision in rainbow trout muscle tissue at different TiO_2 NP spike concentrations.

TiO_2 NP spike ($\mu g\ l^{-1}$)	Fish and sample number	Ti metal ($\mu g\ g^{-1}$) Triplicate reading 1	2	3	Mean ± SD	SEM	Within-sample CV (%)
100	Fish 1 sample 1	2.88	2.67	3.09	2.88 ± 0.21	0.12	7.29
100	Fish 1 sample 2	2.13	1.92	2.45	2.17 ± 0.27	0.15	12.32
100	Fish 1 sample 3	2.03	2.13	2.45	2.20 ± 0.22	0.13	9.96
100	Fish 1 sample 4	2.03	2.03	2.45	2.17 ± 0.24	0.14	11.17
	Within-fish Ti metal mean ± SD (SEM)	2.36 ± 0.35 (0.18)					
	Within-fish CV (%)	14.88					
1000	Fish 2 sample 1	39.04	40.43	44.48	41.32 ± 2.83	1.63	6.84
1000	Fish 2 sample 2	41.49	41.71	45.65	42.95 ± 2.34	1.35	5.45
1000	Fish 2 sample 3	48.43	44.27	41.39	44.69 ± 3.54	2.04	7.92
	Within-fish Ti metal mean ± SD (SEM)	42.99 ± 1.69 (0.98)					
	Within-fish CV (%)	3.93					

Within sample coefficient of variation (CV (%)) at end of each row is the variation from repeat (x3) measurements of the same tissue sample; CV (%) at the bottom of a column is the total procedural variation from acid digestion of pieces muscle from the same fish.

Table 3.3. Procedural precision from triplicate digestions of rainbow trout gill tissue at different TiO_2 NP spike concentrations.

TiO_2 NP spike ($\mu g\ l^{-1}$)	Fish number	Ti metal ($\mu g\ g^{-1}$) Tissue replicate 1	2	3	Mean ± SD	SEM	Between-sample CV (%)
100	1	6.11	5.67	5.53	5.77 ± 0.30	0.17	5.25
200	2	11.64	9.89	10.18	10.57 ± 0.94	0.54	8.87
300	3	13.38	15.27	15.71	14.79 ± 1.24	0.71	8.37
400	4	20.51	20.65	21.53	20.90 ± 0.55	0.32	2.65
500	5	23.13	24.73	22.69	23.52 ± 1.07	0.62	4.57
1000	6	47.85	43.93	46.84	46.21 ± 2.04	1.18	4.40

Procedural precision calculated from triplicate digestions of rainbow trout gill tissue at different TiO_2 spike concentrations. Between sample coefficient of variation (CV (%)) is the total procedural variation from acid digestion of pieces gill from the same fish.

disparity between tissue sections from the same fish. Variation between triplicate samples taken from the same gill tissue was also calculated to examine any variability in the procedure (Table 3.3). Gills from six different trout were digested following spiking with a serial dilution of TiO_2 NP ($n = 3$ gill samples per fish, Table 3.3). The variation in the gill tissue was lower than muscle with all coefficients being under 10 %.

3.4 Discussion

With the expected future growth in the nanotechnology sector and predicted increase of environmental releases of engineered NPs (Boxall et al., 2007; Ju-Nam et al., 2008; Owen and Handy, 2007), it will be essential to measure NPs in biological tissues. Importantly for the food industry, food items for human consumption such as edible fish muscle, also need to be monitored for public safety. An improved method to measure Ti from TiO_2 NPs in the tissues of fish is presented here, which shows good recovery of Ti metal and a reproducible analysis of the tissue samples.

In the current study, calibration of the ICP-OES was achieved with standards made using both dissolved Ti and TiO_2 NPs, with each showing good linear correlation (correlation coefficient P values of < 0.00001). In light of the data, a decision was made to use titanium metal standards for all future calibrations for several reasons. Firstly, the instrument is designed to detect metals in their elemental form. Secondly, the chemical speciation and form of TiO_2 NPs in tissues remains unknown, but total metal in the tissues has been detected in a previous study (Federici et al., 2007).

Determining analyte recovery (spike recovery) from the protocol is an important part of method validation, and for TiO_2 NPs, the traditional trace element methods for fish tissues gave a poor recovery (e.g., < 35 %, Fig. 3.1a). Experiments were therefore conducted using

TiO_2 NP spikes in whole zebrafish and rainbow trout tissues to ascertain where losses of analyte were occurring. It is well established that without sufficient acidification significant losses of metal from aqueous samples may occur (e.g., Jones et al., 1985) and this can also be influenced by metal adsorption to the surface of the container (e.g., Parker and Bloom, 2005). All samples remain acidified at low pH in the protocol and no statistically significant differences in Ti spike recovery (from TiO_2 NPs) observed when acid digestions were performed in either plastic or glass vials (Fig. 3.1a). Clearly the pH and type of container were not responsible for the apparent loss of the spike. However, a theoretical possibility was that the NPs were not dispersing well in the digestion vials, or the NPs were altering the behaviour of the sample as it was drawn into the nebuliser in the instrument. Several dispersion methods have been employed with nanomaterials including sonication (e.g., TiO_2 NPs, Federici et al., 2007), stirring (e.g., C_{60} fullerenes, Henry et al., 2007) and the use of surfactants (SWCNT, Smith et al., 2007). However, these appeared to have no effect on spike recovery. Neither stirring nor sonication of the sample 2 h prior to measuring on ICP-OES improved spike recoveries (Fig. 3.1b). Similarly, the use of the surfactant sodium dodecyl sulphate (SDS) to disperse the NPs in solution did not improve recovery, and sometimes decreased it (e.g., liver in Fig. 3.1c).

However, the use of another surfactant, Triton X-100, successfully improved Ti recovery from TiO_2 NP spikes, with an optimum concentration of 2 % (Fig. 3.1c). The concentration of Triton X-100 used was much higher than the critical micelle concentration (CMC) as indicated by the manufacturers data sheet (CMC; 0.22 to 0.24 mM for Triton X-100 in water) and this would most likely aided the dispersion of any NPs in the sample. The CMC is the concentration above which a surfactant will form micelles following its addition to water. In the current experiment, Triton X-100 formed micelles around components of the digestion matrix, including the TiO_2 NPs, and following micellisation the NPs are less likely

to aggregate and settle out of suspension. All concentrations of Triton X-100 used here were much higher than the CMC of this surfactant in water (0.22 to 0.24 mM), although many factors can influence the CMC (Fuguet et al., 2005) and it is likely that the CMC required for Triton X-100 to form micelles with TiO_2 NP and other organic material present is greater than in pure water. Interestingly even in the presence of Triton X-100, tissues with more fat such as the liver consistently produced lower percentage recoveries than other tissues (e.g., Fig. 3.3). Although micelle formation with Triton X-100 can be affected by the viscosity of the solution or presence of organic matter (Mukerjee and Mysels, 1971), the amount of Triton X-100 in the sample was not a limiting factor in the spike recovery, and was optimal in the method. Concentrations of Triton X-100 over 2 % resulted in decreased spike recovery (e.g., recoveries of 75 ± 8, 64 ± 9, 25 ± 8, 12 ± 4, and 0.5 ± 0.02 % (mean ± SEM) for samples containing 2.0, 4.0, 6.0, 8.0, and 10.0 % Triton X-100 respectively). Triton X-100 was also much more effective than SDS at improving spike recoveries. Triton X-100 has a lower CMC than SDS (about 0.25 and 8.5 mM for Triton X-100 and SDS respectively, Mukerjee and Mysels, 1971) and therefore is likely to be more effective at lower concentrations. Triton X-100 is also a non-ionic surfactant, while SDS is anionic with a long (12 carbon) chain, suggesting that non-ionic surfactants may be better although the reasons for this require further investigation. One possible explanation could be that some non-ionic surfactants are known to have T_2 relaxation process times as long as minutes (Patist et al., 2002). It may therefore be possible that the longer the relaxation time the less chance there is of TiO_2 NPs falling out of solution, i.e., a longer relaxation time will increase stability with the NPs held in micelles for longer periods.

During the method development it also became clear that the matrix matching of standards and samples was sensitive to the amount of Triton X-100 added. Measuring samples that contained Triton X-100 against standards without, resulted in over reading (e.g.,

Figs. 3.1c and 3.3a). It is therefore prudent to also include Triton X-100 in the standards. The storage time of samples was also tested. Triton X-100 containing intestinal tissue digests had lower spike recovery after 14 d compared to samples analysed within 2 h (reducing from 70 % or more to only 45 % by day 14). Acid hydrolysis of Triton X-100 was probably occurring, although further investigation is needed to define the precise kinetics of this process. The data suggests that it would be prudent to analyse samples within 48 h of adding the Triton X-100.

The final optimised protocol yielded percentage recoveries 3-10 fold higher than the initial recoveries achieved using the established metal analysis protocol; with the highest recoveries coming from samples spiked with 100 $\mu g\ l^{-1}$ TiO_2 NPs (Fig. 3.3b). Whole zebrafish and rainbow trout gills gave the greatest spike recovery (approximately 70 and 65 % respectively). There are very few reports of spike recovery data for TiO_2 NPs, and our method is a major improvement. Scown et al. (2009) reported a TiO_2 NP digestion efficiency of 28.82 %, and the new method here more than doubles this recovery. However, in that study 200 µl of 10 % Triton-X 100 was added to a 10 ml digest solution in order to achieve a final concentration of 2 % Triton-X 100 (actually 0.2 %). Sun et al. (2007) reported Ti (from TiO_2 NPs) recovery values of 90-105 % in fish tissues, although that study used spiked samples that contained unrealistically high Ti concentrations (0.80 g Ti ml^{-1} of final analysis solution). This method and data were published in further studies by the same group, (Zhang et al., 2007; Sun et al., 2005), but attempts to reproduce the method were not successful, with spike recoveries of 5-20 % (data not shown).

For both ecotoxicology research, and especially investigations of food safety when the analytical chemist may often only have one suspect food sample to analyse, it is vitally important to demonstrate the reproducibility of the analytical method. For ecotoxicology, it is also important to determine the responses of groups of fish, and to understand the within and between fish variability in the analysis. Precision was tested by measuring one sample

multiple times, or several samples from the same piece of fish tissue. The within sample tests used a standard spike concentrations of 100 $\mu g\ l^{-1}$ TiO_2 NPs and produced coefficients of variation (CV) between 7.29 and 12.32 % for rainbow trout muscle, whilst a higher spike concentration (1000 $\mu g\ l^{-1}$ TiO_2 NPs) produced lower CV values of 5.45-7.92 % (Table 3.2). Between sample variation again showed a concentration affect in muscle tissue with CV of 14.88 and 3.93 % for 100 and 1000 $\mu g\ l^{-1}$ TiO_2 NP spikes respectively. However, no such relationship was seen in trout gills exposed to a serial dilution of spikes (100-1000 $\mu g\ l^{-1}$ TiO_2 NPs) with CV values ranging from 2.65-8.37 % (Table 3.3).With typical variation for routine metal analysis being approximately 5-10 % in fish tissues (e.g., Kamaruzzaman et al., 2010; Voegborlo and Adimado, 2010), and inter-fish variability often being greater (e.g., Rose et al., 1999) this current method is within acceptable limits when compared to other metals. That the addition of Triton X-100 to whole fish samples did not interfere with the ICP-OES analysis of other analytes (i.e., Ca^{2+}, Cu, K^{+}, Mg^{2+}, Na^{+}, and Zn, Table 3.1) is also of interest to ecotoxicologists, meaning that a single, multi-element analysis can take place as long as the standards are matrix matched to the samples.

In conclusion, an improved method is established for determining Ti metal from TiO_2 NPs in whole zebrafish and rainbow trout tissues with potential uses in ecotoxicology and food safety. The new method gives greatly improved spike recoveries compared to existing methods, and the analytical precision and accuracy are within the limits normally accepted for trace metals in fish tissues.

Chapter 4. Toxicity of dietary titanium dioxide nanoparticles to rainbow trout, (*Oncorhynchus mykiss*).

Contribution statement (of workers on this chapter).

The research carried out in Chapter 4 was conducted by Ben Shaw along with Christopher Ramsden and Timothy Smith, who were both studying for Master of Research degrees. Ben Shaw adopted both an active and a supervisory role throughout the experimental and practical work (training and instructing the students as well as taking part in the bench work), and was solely responsible for the subsequent data analysis and manuscript writing for publication (final edits by the P.I., Prof. Handy, aside). However, the histological analysis as presented in the accompany research paper (see Appendix) was not carried out by Ben Shaw and is therefore omitted from this thesis. Where referred to in this chapter this work is referenced as the research paper (Ramsden et al., 2009).

Abstract

Mammalian and *in vitro* studies have raised concerns about the potential toxicity of titanium dioxide nanoparticles (TiO_2 NPs), and a recent study reported gut pathology following the incidental ingestion of TiO_2 NPs during aqueous exposures in trout, but there are almost no data on dietary exposure to TiO_2 NPs in fish. The aim of this experiment was to observe the sublethal effects of dietary exposure to TiO_2 NPs in juvenile rainbow trout (*Oncorhynchus mykiss*). Stock solutions of dispersed TiO_2 NPs were prepared by sonication without the use of a solvent and applied to a commercial trout diet. Fish were exposed in triplicate to either, control (no added TiO_2), 10, or 100 mg kg^{-1} TiO_2 NPs (0.125 and 1.252 mmol kg^{-1} respectively) diets for 8 weeks followed by a 2 week recovery period where all fish were fed the control diet. TiO_2 NPs had no impact on growth or nutritional performance and no major haematological or blood disturbances were observed in terms of haematocrit values, red and white blood cell counts, whole blood haemoglobin, and plasma Na^+ and K^+ concentrations. Tissue Ti levels showed significant increases in the spleen and liver in the early stages of the exposure, however these changes did not persist. There were generally no effects on tissue ion levels (Na^+, K^+, Ca^{2+}, or Mn), although some small changes in tissue Cu and Zn levels were observed, most noticeably in the brain (statistically significant at weeks 4 and 6; ANOVA or Kruskal-Wallis, $P < 0.05$). Exposure to TiO_2 NPs caused a dose-dependent decrease in Na^+/K^+-ATPase activity in the gills, intestine, and brain (statistically significant in the latter; ANOVA or Kruskal-Wallis, $P < 0.05$). Total glutathione in the gills, intestine, liver and brain were not affected by dietary TiO_2 NPs, whilst levels of thiobarbituric acid reactive substances (TBARS) did not increase in the gill, intestine, or brain but did show a small rise (not statistically significant) in the liver. In conclusion, TiO_2 NPs are not acutely toxic to rainbow trout when they are exposed to dietary levels of 10 or 100 mg kg^{-1} TiO_2 NPs for 8 weeks, and that the adverse effects via the dietary route are less severe than aqueous exposure.

4.1 Introduction

Engineered nanoparticles (NPs) are novel materials with at least one dimension < 100 nm and there are concerns about the fate and ecotoxicity of these materials in the aquatic environment (reviews, Moore, 2006; Handy and Shaw, 2007a; Nowack and Bucheli, 2007; Handy et al., 2008a). Nanomaterials currently in production and use include nano-powders (metals, metal oxides, alloys), magnetic nanomaterials, carbon nanotubes, nano-ceramics, nano-silica, quantum dots, polymer nano-composites, and nano-films (Boxall et al., 2007). The potential benefits of these new materials are vast and include environmental remediation, drug delivery, electronics, optics, fuel additives, textiles, and cosmetics (Aitken et al., 2006; Boxall et al., 2007; Handy et al., 2008a). However, data are needed for environmental risk assessment of these new materials; especially on uptake and biological effects (Owen and Handy, 2007; Crane et al., 2008; Handy et al., 2008a; Handy et al., 2008b).

TiO_2 NPs are used in a range of commercially available products such as cosmetics, sunscreen, paint, and building materials (Aitken et al., 2006). The pigment form of ordinary TiO_2 powder is not considered toxic and has historical use as a negative control in dust toxicity studies (Warheit et al., 1997), and as an inert dietary marker for nutrition studies on animals including fish (e.g., Lied et al., 1982; Weatherup and McCracken, 1998; Mamun et al., 2007). However, several studies with fine and ultrafine (< 100 nm) TiO_2 have demonstrated some respiratory toxicity and epithelial inflammation of the lung in rodents (e.g., Ferin and Oberdörster, 1985; Ferin et al., 1991; Oberdörster et al., 1992; Bermudez et al., 2004; Warheit et al., 2005; 2006). Ultrafine particles are sometimes more toxic that fine particles (e.g. Ferin and Oberdörster, 1985; Ferin et al., 1991), and some researchers argue this may be a surface area effect (Oberdörster et al., 1992; 1994).

Very few ecotoxicological studies have been carried out using TiO_2 NPs, and most of these have so far used a waterborne rather than dietary exposure route. *Daphnia magna* show mortality during exposure to TiO_2 NPs, and the toxicity is dependent on sample preparation with filtered particles (single TiO_2 particles; 30 nm mean diameter) being more toxic (Lovern and Klaper, 2006). Zhang et al. (2007) showed that exposure to TiO_2 NPs can also influence the uptake of other pollutants, with carp experiencing 146 % more Cd uptake in the presence of TiO_2 NPs compared to fish exposed to an identical concentration of Cd only. Juvenile rainbow trout were exposed to 0-1.0 mg l^{-1} TiO_2 NPs for up to 14 days with a range of resulting toxic effects and organ pathologies including evidence of oxidative stress, respiratory toxicity, and erosion of the gut epithelium (Federici et al., 2007). The latter was probably caused by stress-induced drinking, and the fish showed a milky coloured mucous residue in the gut, presumably ingested TiO_2 (see discussion in Federici et al., 2007). These observations on the gut raise the possibility that TiO_2 NPs might be toxic via the oral exposure route.

To the authors knowledge, there are no reports of dietary TiO_2 NP exposure in fish and only a few reports on oral toxicity in the mammalian literature. Wang et al. (2007) exposed mice to either nano-sized (25 or 80 nm) or fine (155 nm) TiO_2 particles by single oral gavage, and whilst no acute mortality occurred, there were changes to serum biochemical parameters (e.g., alanine amino transferase activity, lactate dehydrogenase activity) and liver pathology (inflammation, hydropic degeneration and necrosis of hepatocytes) were observed. Toxic effects have also been seen in mice orally exposed to nanosized copper and zinc. Chen et al. (2006) studied the effects of Cu-NPs (108-1080 mg kg^{-1}, 23.5 nm NPs) in mice after single oral gavage and found the particles to be moderately toxic with pathological changes in the kidney, spleen and liver. Wang et al. (2006) observed severe symptoms of lethargy,

vomiting and diarrhea for the first few days, with two mortalities after the first week, in mice gastrointestinally exposed to Zn-NPs (5 g kg^{-1} body weight, 58 nm NPs). Both dead mice had severe aggregations of Zn particles in the intestine demonstrating the ability of Zn-NPs to easily aggregate within the gut, suggesting these particles could induce far more severe intestinal responses than their larger counterparts (Wang et al., 2006). Despite the fact that these oral studies on rodents have used large doses of NPs, there are some common concerns with fish nutritional toxicity. Notably, in these few studies on dietary metal NP exposure in rodents and fish, one common feature is toxicity and/or metal accumulation in the internal organs which raises the concern that the NPs may cross or compromise the gastrointestinal barrier. Consequent effects of the NPs on the nutritional performance of animals are also unknown.

The aim of the current study was to provide some of the first toxicological observations on sublethal dietary exposure to TiO_2 NPs in fish, using rainbow trout to enable some comparison with previous aqueous exposures with NPs using this species (Federici et al., 2007; Smith et al., 2007). The aim was simply to establish whether or not this material was toxic via the dietary route compared to an unexposed control, and uses a well established experimental design for nutritional studies that are identical to previous work on dietary metals (e.g., Cu, Handy et al., 1999; Shaw and Handy, 2006). A body systems approach was adopted similar to Federici et al. (2007) with measurements in key areas of physiology such as osmoregulation and haematology, as well as documenting biochemical responses and nutritional performance. In addition, during previous studies on dietary metals in fish it has been noted that toxic effects sometimes occur when switching from contaminated diets back to control diets (e.g., dietary Cu, Shaw and Handy, 2006). This experiment therefore also

includes a period on control diets at the end of the exposure to look for possible latent post-exposure effects.

4.2. Methodology

4.2.1 Experimental design

Juvenile rainbow trout (n = 400) were obtained from Exmoor Fisheries, Somerset, UK, and held for 10 days in a stock aquaria with flowing, aerated, dechlorinated Plymouth tap water (see below). Following this the fish were transferred into a recirculation system consisting of nine 120 l experimental fibre glass aquaria (40 fish/tank; identical water conditions) and acclimated for 14 days prior to experiments. A daily 10 % water renewal from the municipal water supply was employed to aid water quality, and filtration consisted of four fine mesh (400 micron) bag filters at the waste outlet and two bio-filters (bio-balls) within the sump. The bag filters were cleaned twice daily prior to each feed to minimise the amount of organic waste matter (uneaten food, faeces) entering the bio-filters. Temperature was controlled by a thermostatically controlled glycol cooling system.

Fish were individually weighed (21.63 ± 0.15 g mean ± S.E.M., n = 360) and 3 tanks per treatment were randomly allocated. Fish were exposed in triplicate to one of the following treatments for 8 weeks: control diet (no added TiO_2 NPs), 10 or 100 mg kg^{-1} dry weight feed TiO_2 NPs (0.125 and 1.252 mmol TiO_2 NPs kg^{-1} respectively, see below for diet formulation). This was followed by a 2 week recovery period where all fish were fed the control (no added TiO_2 NPs) diet. The TiO_2 NP concentration in the feed was selected after considering TiO_2 NP toxicity in a previous waterborne exposure experiment (Federici et al., 2007) and from previous experience of dietary metal toxicity in fish (e.g., Shaw and Handy,

2006). Fish were fed twice daily to satiation (10.00 and 16.00 hours) and behaviour monitored during each feeding event. Care was taken to ensure that all the feed added to the tanks was eaten and the self-cleaning design of the aquarium system ensured that faecal waste was quickly removed from the tanks. Ti analysis of the tank water before and after feeding during the experiment indicate that no TiO_2 leached from the food during the experiment and that background Ti levels in the water were low (< 25 ng l^{-1}) and leaching experiments with food pellets showed no release of Ti after 25 minutes of stirring in water (data not shown).

Water quality monitoring was carried out as described in Chapter 2. There were no differences between tanks within treatments and no treatment differences in water quality throughout (ANOVA, $P > 0.05$) with levels remaining within the limits set out in the fish husbandry plan for the experiment (Appendix 2). Values were (mean ± S.E.M., n = 68 or 30 samples) pH, 7.09 ± 0.04; temperature, 15.46 ± 0.07 °C; oxygen saturation, 90.6 ± 0.48 %; total ammonia, 0.29 ± 0.07 mg l^{-1}, nitrite, 0.35 ± 0.06 mg l^{-1}, nitrate, 1.96 ± 0.18 mg l^{-1}. Photoperiod for the experiment was 12 h light: 12 h dark. The electrolyte composition of the dechlorinated tap water used was 0.3, 0.1, and 0.4 mmol l^{-1} for Na^+, K^+ and Ca^{2+}, respectively. Fish were randomly sampled at the start of the experiment (the initial fish stock) and then every two weeks for the duration of the experiment followed by a two week recovery period with all fish fed the control diet. Fish were sampled for haematology, plasma ions, tissue electrolytes, and biochemistry. In addition nutritional performance and growth were monitored and recorded throughout, and proximate composition analysis of whole fish (ash, lipid, protein and moisture) carried out at weeks 0 and 8 (6 fish/treatment, n = 5 for initial fish).

4.2.2 Titanium dioxide nanoparticle stock solution

The titanium dioxide NPs used here was from the same batch previously characterised in Chapter 3.

4.2.3 Diet formulation

The control diet was a commercial fish feed; Advanced Fish Feed Trout Excel 18 (2 mm pellets), with fish progressing onto a mixture of this feed and Trout Excel 30 (3 mm pellets) at week 5 as their body size increased. Proximate composition of the diets was (% of dry diet from manufacturer's guidelines, Trout Excel 30 diet in brackets): lipid 18 (21); protein 50 (46); ash 8 (8); fibre 1 (1); phosphorus 1.2 (1.2). In order to obtain experimental diets, the NP stock solutions (above) were sonicated for 8 h, and then either 1 or 10 ml of stock solution was added to 49 or 40 ml of ultrapure water (for the 10 and 100 mg kg^{-1} treatments respectively) to make a 0.2 or 2.0 g l^{-1} TiO_2 NP dilution that could be sprayed on to the food. These diluted solutions were sonicated for a further 15 minutes just before spraying to ensure even delivery of the material through the spray nozzle. One kg of commercial feed was place in a commercial food mixer (Kenwood Catering Professional food mixer XKM810) and gradually sprayed with the appropriate TiO_2 NP solution. The TiO_2 NP immediately coated the feed, and was then sealed in by sprayed the food with a 10 % bovine gelatine (BDH, Poole, UK) solution. The gelatine coat was allowed to dry, after which the feed was transferred into an airtight container for storage. The control diet was formulated by precisely the same process, but with the TiO_2 solution replaced by equal volumes of ultrapure water. Titanium metal concentrations in the diets were confirmed by ICP-OES following nitric acid digestion (as in tissue ion analysis below) and were 5.4 and 53.6 mg kg^{-1}

feed weight of Ti metal respectively. Calibrations showed that Ti metal forms 59.9 % of the TiO_2 (data not shown) and equates to recoveries of 90 and 89 % of nominal TiO_2 NP concentrations in the 10 and 100 mg kg^{-1} TiO_2 NP diets respectively.

4.2.4 Growth and nutritional performance

Growth and nutritional performance were measured as detailed in Chapter 2. Briefly, food intake was calculated twice daily for each tank by weighing food containers before and after feeding. Individual fish weight was used because the periodic sacrifice of fish during the experiment prevented nutritional parameters being calculated from cumulative tank biomass. Specific growth rate, feed conversion ratio, and feed conversion efficiency were calculated from mean gain in body weight for each treatment for (i) the TiO_2 NP exposure phase (weeks 0-8), (ii) recovery phase (weeks 8-10), and (iii) for entire experiment (TiO_2 NP exposure and recovery combined; weeks 0-10). Two fish per tank (6/treatment, n = 5 for initial fish) were terminally anaesthetised with MS222 at weeks 0 and 8 and placed into -20 °C for storage until proximate composition analysis could be performed for lipid, ash, protein, and moisture.

4.2.5 Haematology and blood plasma analysis

Two fish were randomly collected from each tank (six fish/treatment and initial fish) at weeks 0, 2, 4, 6, 8, and 10 and carefully anaesthetised with buffered MS222. Whole blood was collected via the caudal vein into heparinised (lithium heparin) syringes, and the fish weighed and total length recorded. Haematology (haematocrit, haemoglobin concentration, and calculated mean erythrocyte cell volume and mean erythrocyte haemoglobin content), and plasma ions and osmometry were performed as described in Chapter 2.

4.2.6 Tissue ion analysis

Following blood sampling, fish were terminally anaesthetised with MS222 and dissected for tissue ion analysis as described in Chapter 3. Briefly, gill, liver, intestine, spleen, skinned muscle from the flank, and whole brain were harvested and processed for ion analysis. A working stock of Triton x-100 was prepared in pure water by slowly dissolving with stirring and slowly added to each digest sample to achieve a final concentration of 2 % Triton x-100 in each tube. Following this the samples were diluted to 5 or 20 ml with ultra pure (ion free) water and analysed for Ti, Cu, Zn, Mn, Ca, Na and K by inductively coupled plasma optical emission spectrometry (ICP-OES, Varian 725 ES). Analytical grade standards and reference materials were used throughout.

4.2.7 Biochemistry

Biochemical analyses were performed exactly as described in Chapter 2. Briefly an additional two fish were randomly collected from each tank (6 fish/treatment, and initial fish) at weeks 0, 2, 4, 6, 8, and 10 and gills, liver, intestine, and whole brain removed and *immediately snap frozen in liquid nitrogen and stored at −80 °C until subsequent* biochemistry (Na^+/K^+-ATPase activity, thiobarbituric acid reactive substances (TBARS), and total glutathione (GSH) content).

4.2.8 Statistical analysis

All data were analysed by StatGraphics Plus version 5.1 as described in Chapter 2.

4.3 Results

4.3.1 Dietary exposure to titanium dioxide nanoparticles

There was a small background incidence of mortality (2 % in total), typical of juvenile trout, and not associated with any one treatment. A total of nine mortalities during the experiment; 2, 4 and 3 fish from the control, 10 and 100 mg kg^{-1} TiO_2 treatments respectively. Most of these fish were small and were probably subordinate fish that died as a result of aggression. The remaining fish did not show any visible signs of ill health and retained normal swimming behaviours throughout.

Total Ti concentrations in the tissues of the fish are shown in Fig. 4.1. Elevated levels of TiO_2 (as Ti metal) were observed in fish from both TiO_2 NP treatments compared to the controls (Fig. 4.1). The gill, gut and livers from both TiO_2 NP treatments showed statistically significant increases in Ti compared to control from weeks 4 or 6 of exposure, and Ti levels generally remained elevated compared to controls in the post-exposure period especially in the fish from the highest TiO_2 NP treatment (Fig. 4.1). Notably, liver Ti levels peaked at week 4 in the TiO_2 NP treatments, and at the highest exposure concentration, showed a gradual and statistically significant decrease within the treatment over time suggesting some partial elimination of Ti from the liver (Fig. 4.1). There was also a time effect in the intestine of control fish, with Ti decreasing over time (Fig. 4.1), and reflects a reduction in background dietary Ti intake associated with switching from farm food to the experimental feeds at the start of the experiment. Apart from some background noise there were no treatment or time related changes in Ti levels in the muscle. The brain of exposed fish showed transient increases of Ti in the both TiO_2 NP treatments at week 4 and 10 compared to controls (Kruskal-Wallis, $P < 0.05$, Fig. 4.1). The spleen showed the highest Ti levels of any tissue,

concentrations increasing earlier than in other tissues (week 2 instead of week 4 or 6) in the treatments compared to controls (statistically significant, Kruskal-Wallis, $P < 0.05$), but then decreased sharply following this, suggesting the fish were able to regulate excess TiO_2 in the spleen (Fig. 4.1).

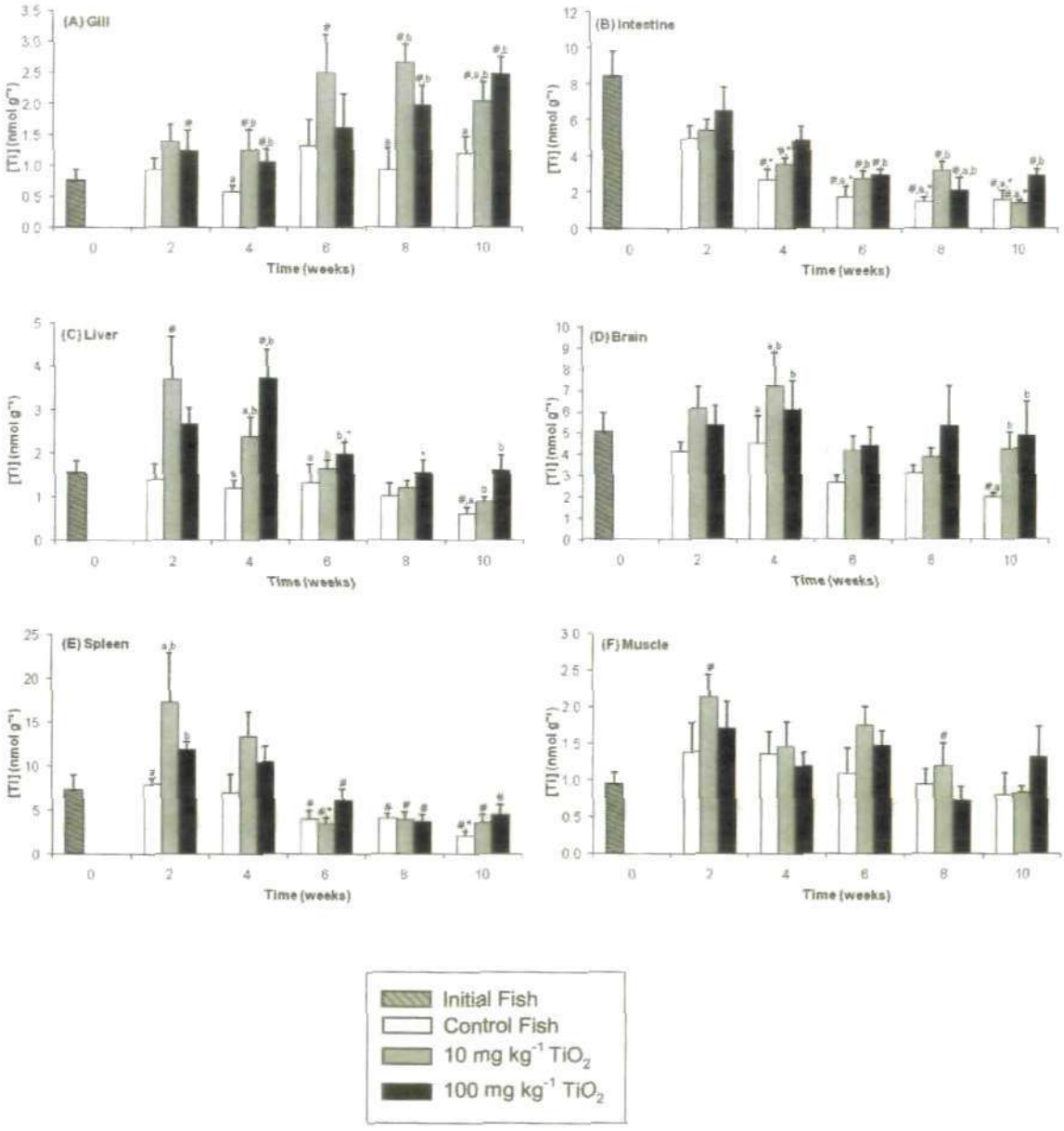


Figure 4.1. Titanium metal levels in the gill (A), intestine (B), liver (C), brain (D), spleen (E) and (F) muscle of trout after exposure to 0 (clear bars), 10 (grey bars) or 100 (black bars) mg kg^{-1} TiO_2 NP for 8 weeks (equivalent to 0, 0.125 and 1.252 mmol TiO_2 NPs kg^{-1} respectively), followed by a 2 week recovery period (week 10) with all fish fed normal food. The dashed line indicates the end of exposure and the return of all fish to normal food ("recovery phase"). Diagonal hatched bars are initial (day 0) fish. Data are mean ± S.E.M., nmol Ti g^{-1} dry weight tissue, $n = 6$ fish. Different letters within a time point indicate significant differences between tissues within each tissue (ANOVA or Kruskal-Wallis, $P < 0.05$). $^{\#}$ Significant time effect compared to initial fish (ANOVA or Kruskal-Wallis, $P < 0.05$). $^{+}$ Significant time effect within treatment compared to previous time point (ANOVA or Kruskal-Wallis, $P < 0.05$).

4.3.2 Growth and nutritional performance

Fish from all treatments gained body weight during the experiment (Fig. 4.2), with no statistically significant differences in mean final weights (ANOVA, $P > 0.05$), or differences in specific growth rates (Table 4.1), seen between tanks or treatments throughout. By week 10 there were slight differences in mean fish weight between treatments, where the treated fish weighed slightly more than the control fish, but this was not statistically significant (ANOVA, $P > 0.05$, Fig. 4.2). No differences were seen in time taken to commence, or in time spent, feeding (data not shown). There was no food refusal by fish from any treatments and no regurgitation of feed occurred, so mean daily ration size and FCR were similar for all treatments (Table 4.1). The slightly higher cumulative feed intake (Fig. 4.2) in the highest TiO_2 treatment reflects the elevated mean weight of these fish compared to control at the end of the experiment (Table 4.1). Condition factor and hepatosomatic index presented no significant differences over time or between treatments (Table 4.1). However, the spleen index increased 2 fold in treated fish compared to controls at week 8 (ANOVA, $P < 0.05$, Table 4.1), but recovered by week 10 when all fish were returned to the control diet. Carcass proximate composition was unaffected by TiO_2 exposure, except for a statistically significant decrease in lipid in the carcasses of fish fed the 100 mg kg^{-1} TiO_2 diet at week 8 compared to the controls or the 10 mg kg^{-1} TiO_2 treatment (ANOVA, $P < 0.05$, Table 4.1).

4.3.3 Haematology and blood plasma analysis

Dietary exposure to TiO_2 NPs did not cause any major haematological disturbances, with values remaining within the normal range for trout (Table 4.2). No statistically significant treatment-dependent changes were presented apart from a transient increase in

white blood cells at week 2 in fish exposed to 10 mg kg^{-1} TiO_2 NPs (ANOVA, $P < 0.05$). Also, haemoglobin, and subsequently MEH, showed transient changes during the experiment, with levels in both parameters decreasing in TiO_2 NP treated fish compared to controls at week 6 (ANOVA, $P < 0.05$; Table 4.2). Some statistically significant changes in haematological parameters were seen in fish after 8-10 weeks compared to initial fish (Table 4.2), but these were not TiO_2 treatment related and were attributed to growth effects over time. Analysis of osmotic pressure and plasma glucose showed no treatment or time-dependent statistically significant differences, apart from a transitory decrease in plasma glucose at week 2 in the control and 10 mg kg^{-1} TiO_2 NP treatments (ANOVA, $P < 0.05$, data not shown). Values remained between 252-342 mOsm kg^{-1} and 2.99-4.88 mmol l^{-1} for osmotic pressure and plasma glucose respectively. Plasma Na^+ did not differ significantly between treatments, however plasma K^+ showed small, but statistically significant increases at week 8 in the TiO_2 treatments compared to controls (Table 4.2). This effect on K^+ was no longer present by week 10 (Table 4.2).

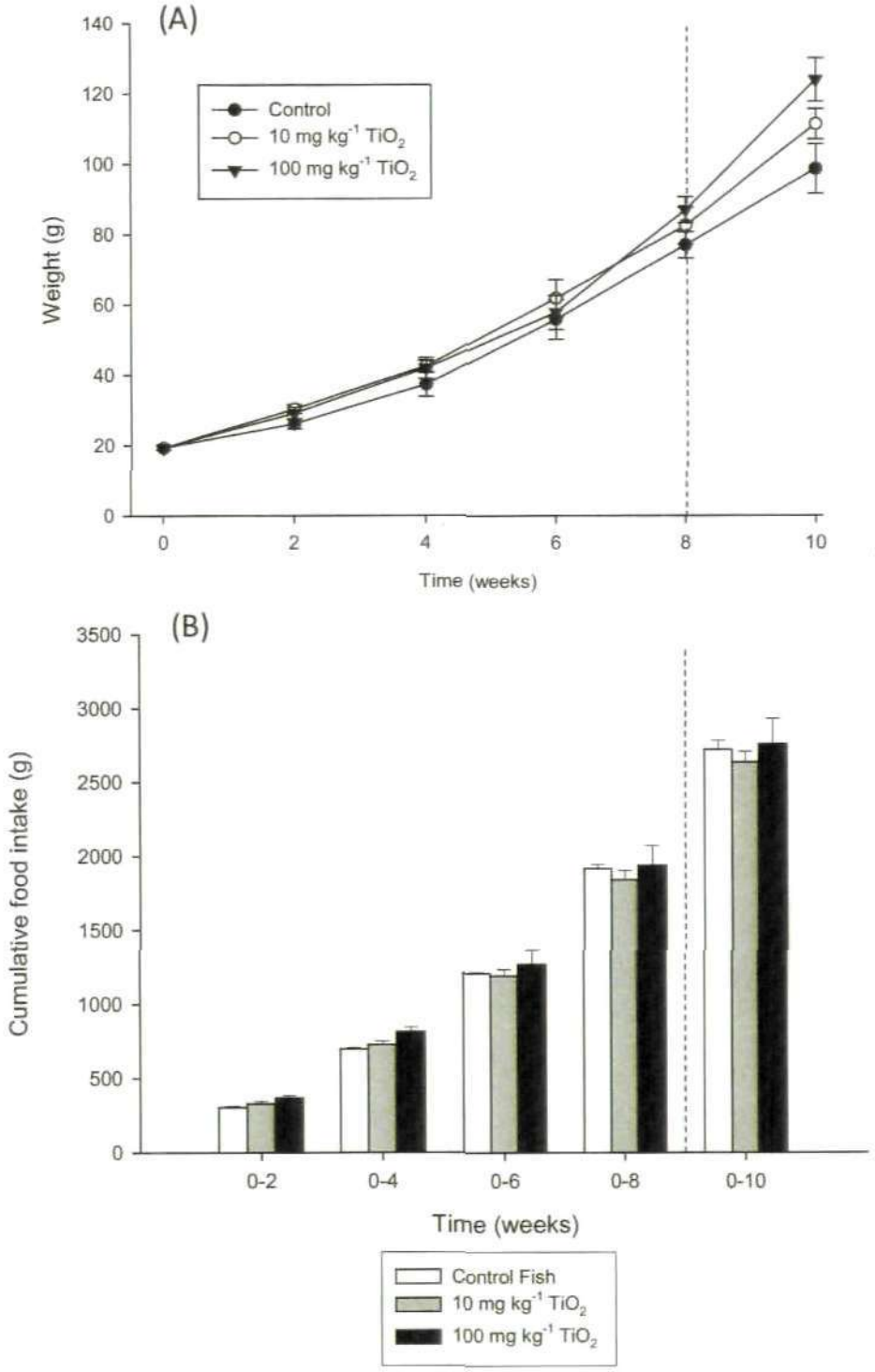


Figure 4.2. Body weight (A) and cumulative food intake (B) in rainbow trout fed 0, 10 or 100 mg kg^{-1} TiO_2 NPs for 8 weeks, followed by a recovery period with all fish fed normal food (no TiO_2 NP) for a further two weeks. In panel (A) data are mean ± S.E.M., n = 18 fish per treatment at each time point. In panel (B) data are means of triplicate tanks for each treatment. The dashed line indicates the end of exposure and the return of all fish to normal food ("recovery phase").

Table 4.1. Growth and nutritional performance of rainbow trout exposed to 0 (control with no TiO_2), 10 or 100 mg kg^{-1} TiO_2 NPs for 8 weeks, followed by 2 weeks recovery.

Parameter	Experimental Phase	Time (weeks)	Treatment		
			Control	10 mg kg^{-1} TiO_2	100 mg kg^{-1} TiO_2
Final mean weight (g)	Exposure	8	77.0	82.5	87.0
	Recovery	10	98.5	111.3	119.5
Mean weight gain (%)	Exposure	0-8	298.88	327.50	350.71
	Recovery	8-10	28.02	33.37	30.81
	Entire experiment	0-10	410.74	476.97	519.59
SGR (% day^{-1})	Exposure	0-8	2.43	2.55	2.64
	Recovery	8-10	1.76	2.06	1.92
	Entire experiment	0-10	2.30	2.47	2.57
FCR	Exposure	0-8	1.07	1.05	0.93
	Recovery	8-10	1.84	1.59	1.54
	Entire experiment	0-10	1.22	1.16	1.05
FCE	Exposure	0-8	0.97	0.97	1.10
	Recovery	8-10	0.54	0.63	0.65
	Entire experiment	0-10	0.90	0.91	1.02
Mean ration size (% bw day^{-1})	Exposure	0-8	2.60	2.68	2.46
	Recovery	8-10	3.24	3.27	2.96
	Entire experiment	0-10	2.81	2.86	2.71
Condition factor (%)	Initial fish	0	0.94 ± 0.02		
	End of exposure	8	1.16 ± 0.02$^{\#}$	1.16 ± 0.02$^{\#}$	1.18 ± 0.02$^{\#}$
	Recovery	10	1.23 ± 0.03$^{\#}$	1.24 ± 0.02$^{\#}$	1.23 ± 0.02$^{\#}$
Hepatosomatic index (%)	Initial fish	0	1.35 ± 0.16		
	End of exposure	8	1.61 ± 0.14	1.49 ± 0.14	1.41 ± 0.06
	Recovery	10	1.49 ± 0.08	1.54 ± 0.09	1.48 ± 0.12
Spleen index (%)	Initial fish	0	0.19 ± 0.04		
	End of exposure	8	0.08 ± 0.01$^{a,\#}$	0.16 ± 0.03^{b}	0.16 ± 0.03^{b}
	Recovery	10	0.10 ± 0.01$^{\#}$	0.13 ± 0.04	0.10 ± 0.01$^{\#}$

Table 4.1. continued.

Parameter	Experimental Phase	Time (weeks)	Treatment		
			Control	10 mg kg^{-1} TiO_2	100 mg kg^{-1} TiO_2
Proximate carcass composition*					
Moisture (%)	End of exposure	8	71.44 ± 1.11	70.36 ± 0.86#	71.30 ± 0.48
Lipid (%)	End of exposure	8	24.72 ± 1.38[a]	23.61 ± 1.30[a]	20.42 ± 1.23[b,#]
Protein (%)	End of exposure	8	53.83 ± 1.05	52.59 ± 0.69#	54.54 ± 0.66
Ash (%)	End of exposure	8	7.65 ± 0.43#	7.08 ± 0.26#	7.09 ± 0.13#

Data are mean ± S.E.M. Initial mean weight at Time 0 was 21.6 ± 0.15 g (n = 360 fish). Different letters within time point indicate significant differences between treatments (ANOVA or Kruskal-Wallis, $P < 0.05$). # Significantly different from initial fish (stock fish at time zero, ANOVA or Kruskal-Wallis, $P < 0.05$). * Values are for fish at the end of the experimental phase (week 8) expressed as a percentage of dry matter. Proximate carcass composition of initial fish for moisture, lipid, protein and ash, respectively, were (%, means ± S.E.M. n = 5 fish): 73.6, 24.96, 55.88, and 9.03.

4.3.4 Tissue electrolytes, trace metals and moisture content

Fish tissues (gill, intestine, liver, spleen, muscle, whole brain) were analysed for major tissue electrolytes (Na^+, K^+, Ca^{2+}) and trace elements (Cu, Zn, Mn). There were no major electrolyte disturbances with only some minor temporal changes seen throughout the duration of the experiment, which were not TiO_2 treatment related. There were no time or treatment effects on tissue K^+ or Ca^{2+} (data not shown; ANOVA or Kruskal-Wallis, $P > 0.05$). Tissue Na^+ did exhibit some transient, though statistically significant differences, in the gill, liver and spleen (ANOVA or Kruskal-Wallis, $P < 0.05$). However, no clear treatment-dependent trends were observed with these data remaining within normal levels for rainbow trout (e.g., control and highest TiO_2 diet respectively for gill, liver and spleen, mean ± S.E.M., $n = 6$, in μmol g^{-1} dry weight tissue: 206.4 ± 38.0 to 361.2 ± 117.6; 93.2 ± 4.0 to 152.1 ± 47.8; 66.4 ± 5.9 to 141.1 ± 67.1). Exposure to dietary TiO_2 NPs caused some statistically significant decreases in Cu levels in the intestine, brain and spleen of both TiO_2 treatments at some time points (Kruskal-Wallis, $P < 0.05$, Fig. 4.3). Notably, Cu depletion in spleen (Fig. 4.3) was coincident with the Ti peak in the tissue (Fig. 4.1). Some treatment-dependent and transient elevations in tissue Zn were noted (Fig. 4.4). Zinc in spleen of the 10 mg kg^{-1} TiO_2 NP treatment at week 4 were elevated to almost 2-fold that of control and the highest TiO_2 treatment (Kruskal-Wallis, $P < 0.05$; Fig. 4.4). Also in week 4, the brain tissue of fish from both TiO_2 treatments showed statistically significant increases in Zn compared to control (Kruskal-Wallis, $P < 0.05$, Fig. 4.4). Tissue Mn was unaffected by exposure to dietary TiO_2 NPs (data not shown), apart from a transient increase in the spleen. Fish from the highest TiO_2 treatment displayed a statistically significant increase in spleen Mn at week 6 compared to the control fish (Kruskal-Wallis, $P = 0.00659$, data mean ± S.E.M., $n = 6$, in

Table 4.2. Haematological parameters and plasma ions in rainbow trout fed 0 (control with no TiO_2), 10 or 100 mg kg^{-1} TiO_2 NPs for 8 weeks, followed by 2 week recovery.

Parameter	Time (weeks)	Treatment		
		Control	10 mg kg^{-1} TiO_2	100 mg kg^{-1} TiO_2
Haemoglobin (g dl^{-1})	0	5.68 ± 0.40		
	8	5.54 ± 0.32	5.51 ± 0.80	5.40 ± 0.51
	10	6.27 ± 0.33	5.93 ± 0.54	7.16 ± 0.36#
Haematocrit (%)	0	30.00 ± 2.23		
	8	34.83 ± 2.28	33.25 ± 4.15	32.67 ± 3.24
	10	30.25 ± 1.59	29.67 ± 2.28	34.58 ± 1.59
Red blood cell count	0	0.58 ± 0.07		
(*cells* $\times 10^6$ mm^3)	8	*0.58 ± 0.04*	*0.56 ± 0.02*	*0.53 ± 0.08*
	10	0.45 ± 0.04	0.43 ± 0.05#	0.46 ± 0.03
White blood cell count	0	13.26 ± 1.93		
(cells $\times 10^3$ mm^3)	8	29.46 ± 1.83#	30.83 ± 4.70#	26.75 ± 3.91#
	10	14.05 ± 1.48	17.10 ± 0.55	18.50 ± 3.10
Mean erythrocyte haemoglobin content	0	4.13 ± 0.49		
(MEH, µg $cell^{-1}$)	8	3.90 ± 0.25	4.44 ± 0.46	9.41 ± 5.64
	10	5.80 ± 0.48#	5.66 ± 0.54#	6.38 ± 0.32#
Mean erythrocyte volume	0	533.88 ± 35.38		
(MEV, nm^3)	8	608.01 ± 27.00	662.97 ± 66.69	656.11 ± 49.96
	10	694.22 ± 36.40#	700.34 ± 36.86#	769.71 ± 34.38#
Plasma Na^+ (mmol l^{-1})	0	128.17 ± 7.98		
	8	166.33 ± 1.69#	165.17 ± 5.29#	158.67 ± 2.64#
	10	158.33 ± 8.81#	168.67 ± 5.14#	169.33 ± 1.38#
Plasma K^+ (mmol l^{-1})	0	3.51 ± 0.12		
	8	4.26 ± 0.14a,#	4.96 ± 0.08b,#	4.88 ± 0.24b,#
	10	4.26 ± 0.33#	4.76 ± 0.20#	4.61 ± 0.18#

Data are mean ± S.E.M., $n = 6$ fish/treatment. Different letters within time point indicate significant differences between treatments (ANOVA or Kruskal-Wallis, $P < 0.05$). # Significantly different from initial fish (stock fish at time zero, ANOVA, $P < 0.05$).

μmol g^{-1} dry weight tissue: control, 0.016 ± 0.002; 100 mg kg^{-1} TiO_2 NP treatment, 0.028 ± 0.010), though this effect did not persist throughout the experiment. Tissue moisture was not affected by dietary exposure to either 10 or 100 mg kg^{-1} TiO_2 NPs (data not shown) apart from a transient decrease in spleen moisture content in the highest TiO_2 treatment at week 2 compared to control and low TiO_2 NP treatment (Kruskal-Wallis, $P < 0.0001$, data mean ± S.E.M., $n = 6$, as %: control, 71.8 ± 2.9; 10 mg kg^{-1} TiO_2 NP, 65.5 ± 5.2; 100 mg kg^{-1} TiO_2 NP, 58.4 ± 3.2).

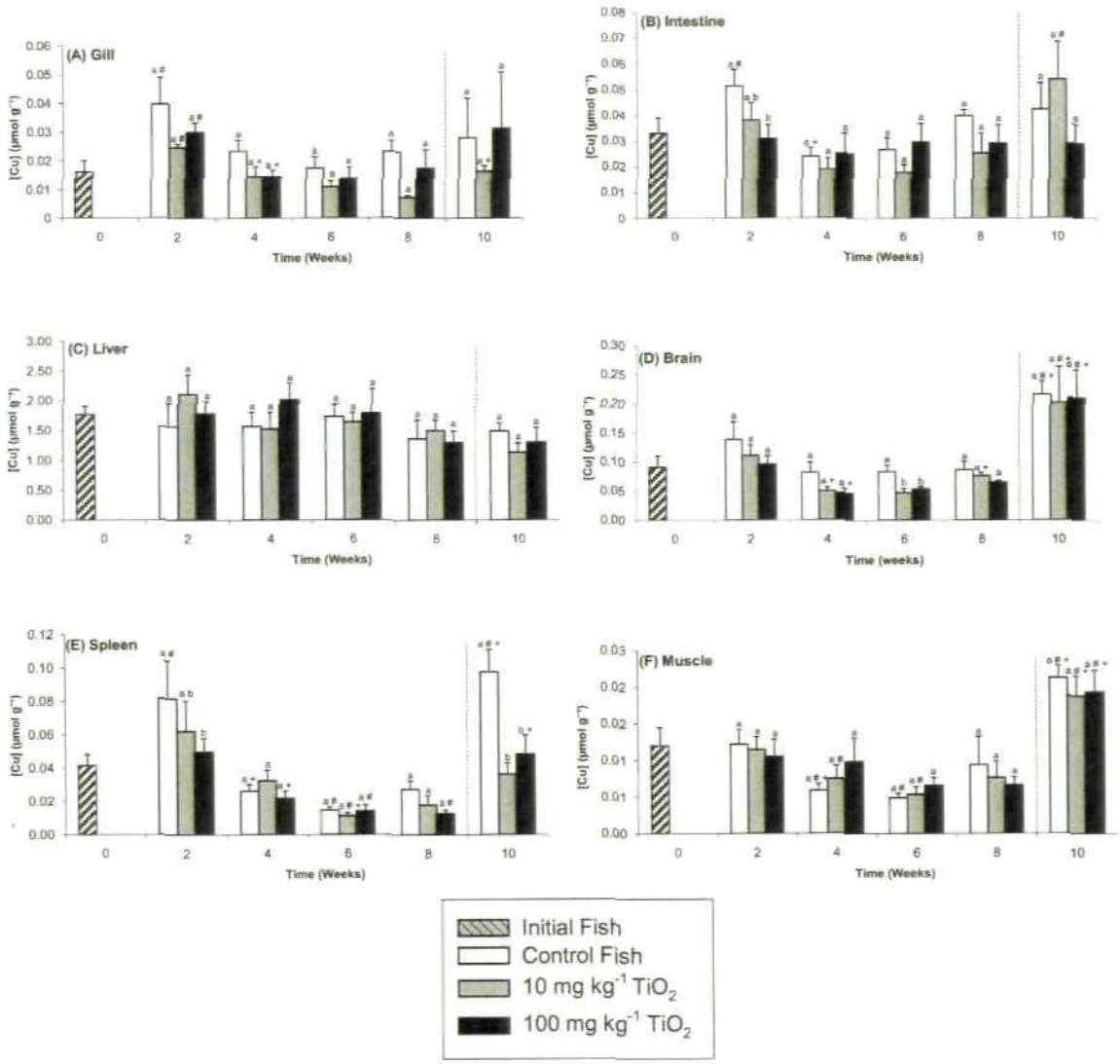


Figure 4.3. Copper levels in the gill (A), intestine (B), liver (C), brain (D), spleen (E) and (F) muscle of trout after exposure to 0 (clear bars), 10 (grey bars) or 100 (black bars) mg kg^{-1} TiO_2 NP for 8 weeks, followed by a 2 week recovery period (week 10) with all fish fed normal food. The dashed line indicates the end of exposure and the return of all fish to normal food ("recovery phase"). Diagonal hatched bars are initial (day 0) fish. Data are mean ± S.E.M., µmol Cu g^{-1} dry weight tissue, $n = 6$ fish. Different letters within a time point indicate significant differences between tissues within each tissue (ANOVA or Kruskal-Wallis, $P < 0.05$). $^{\#}$ Significant time effect compared to initial fish (ANOVA or Kruskal-Wallis, $P < 0.05$). $^{+}$ Significant time effect within treatment compared to previous time point (ANOVA or Kruskal-Wallis, $P < 0.05$).

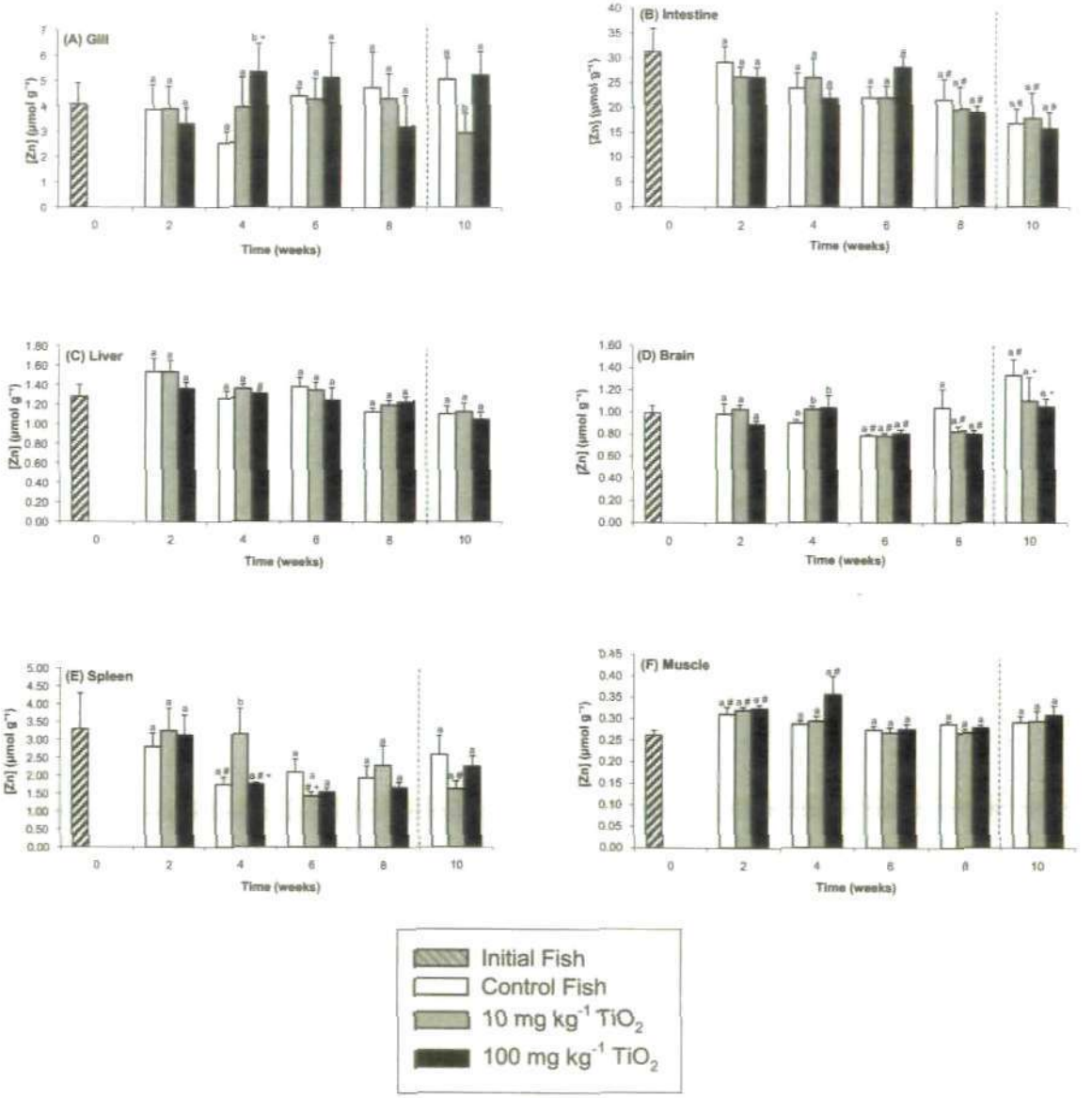


Figure 4.4. Zinc levels in the gill (A), intestine (B), liver (C), brain (D), spleen (E) and (F) muscle of trout after exposure to 0 (clear bars), 10 (grey bars) or 100 (black bars) mg kg^{-1} TiO_2 NP for 8 weeks, followed by a 2 week recovery period (week 10) with all fish fed normal food. The dashed line indicates the end of exposure and the return of all fish to normal food ("recovery phase"). Diagonal hatched bars are initial (day 0) fish. Data are mean ± S.E.M., µmol Zn g^{-1} dry weight tissue, $n = 6$ fish. Different letters within a time point indicate significant differences between treatments within each tissue (ANOVA or Kruskal-Wallis, $P < 0.05$). $^{\#}$ Significant time effect compared to initial fish (ANOVA or Kruskal-Wallis, $P < 0.05$). $^{+}$ Significant time effect within treatment compared to previous time point (ANOVA or Kruskal-Wallis, $P < 0.05$).

4.3.5 Na^+/K^+-ATPase

Na^+K^+-ATPase activity in the gill and intestine were unaffected by TiO_2 exposure, but the brain showed inhibition of Na^+/K^+-ATPase at the end of the exposure phase that did not recover by the end of the experiment (about a 2 fold decrease in enzyme activity at each time point, ANOVA, $P < 0.05$, Fig. 4.5). There was no apparent dose-effect within the TiO_2 treatments on the brain Na^+/K^+-ATPase inhibition with the 10 mg kg^{-1} diet causing the same level of inhibition as the 100 mg kg^{-1} diet.

4.3.6 TBARS and total glutathione

Fish exposed to TiO_2 NPs generally showed a decrease in TBARS compared to controls at the end of the experiment (Fig. 4.6). Significant differences were seen in the gills and intestine of TiO_2 NP exposed fish at week 8 with maximum decreases of 49 and 50 % in branchial and intestinal TBARS respectively in the 100 mg kg^{-1} TiO_2 NP treatment compared to control. However, TBARS in the liver and brain were unaffected by exposure to TiO_2 NPs (ANOVA, $P > 0.05$), although the latter was significantly lower than initial fish in all treatments, including control fish (ANOVA, $P < 0.05$). No notable changes in TBARS resulted from a recovery period where all fish were fed control diet (Fig. 4.6). However, TBARS in the gill of both TiO_2 NP treatments and in the intestine of the 100 mg TiO_2 NP kg^{-1} treatment only, remained significantly lower than control.

Only the gill showed statistically significant changes in total glutathione (GSH), with levels in the intestine, liver and brain remaining stable throughout with no treatment-dependent effects (ANOVA, $P > 0.05$). Values ranged between 0.49-2.13; 1.68-3.73 and 0.68-1.92 μmol g^{-1} wet weight tissue for intestine, liver and brain respectively. Following

exposure to 100 mg kg^{-1} TiO_2 NPs for 8 weeks the gills displayed significantly decreased levels of GSH compared to all other treatments and initial fish (ANOVA, $P < 0.05$). Glutathione levels in the gills at week 8 were (mean ± S.E.M., $n = 6$): 1.28 ± 0.13, 1.46 ± 0.13, 1.24 ± 0.12 and 0.92 ± 0.08 μmol g^{-1} wet weight tissue for initial fish, control, 10 and 100 mg kg^{-1} TiO_2 NP treatments respectively. This treatment-effect was lost after fish were returned to the control diet, and by week 10 no statistical differences were observed between treatments (ANOVA, $P > 0.05$; mean ± S.E.M., $n = 6$): 1.30 ± 0.12, 1.24 ± 0.16, and 1.20 ± 0.10 μmol g^{-1} wet weight tissue for control, 10 and 100 mg kg^{-1} TiO_2 NP treatments respectively.

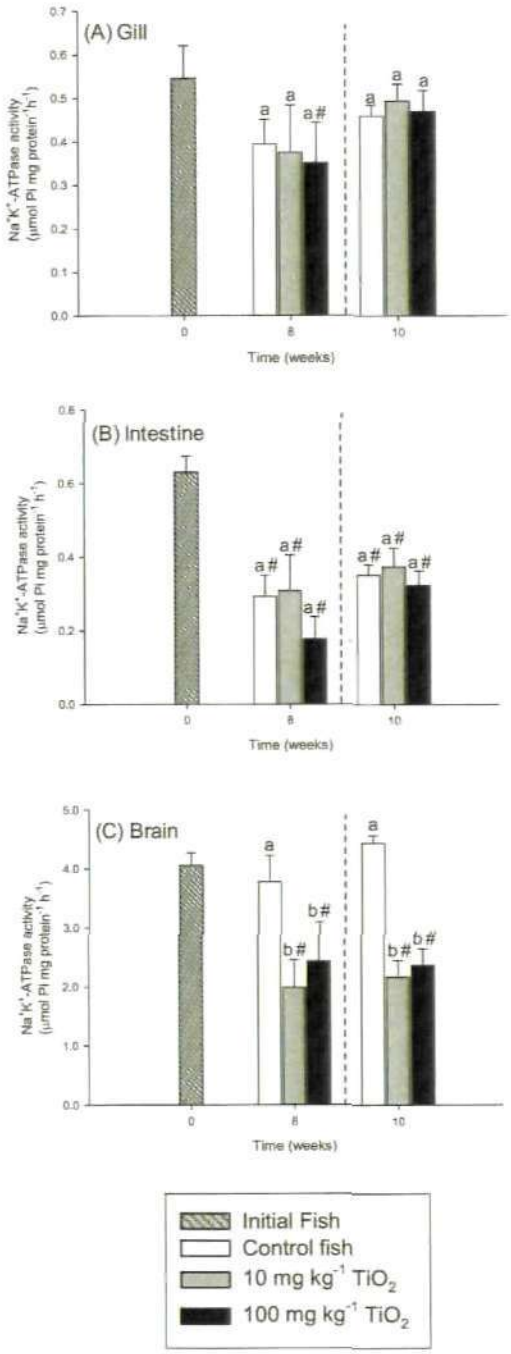


Figure 4.5. Na^+/K^+-ATPase activity in crude homogenates of the gill (A), intestine (B), and brain (C) of rainbow trout fed 0 (clear bars), 10 (grey bars) or 100 (black bars) mg kg^{-1} TiO_2 NPs for 8 weeks, followed by 2 week recovery (week 10). The dashed line indicates the end of exposure and the return of all fish to normal food ("recovery phase"). Data are mean ± S.E.M., $n = 6$ fish. Different letters within a time point indicate significant differences between tissues within each tissue (ANOVA or Kruskal-Wallis, $P < 0.05$). # Significant time effect compared to initial fish (ANOVA or Kruskal-Wallis, $P < 0.05$). Diagonal hatch bar are the initial fish at time zero collected immediately prior to starting the experimental diets.

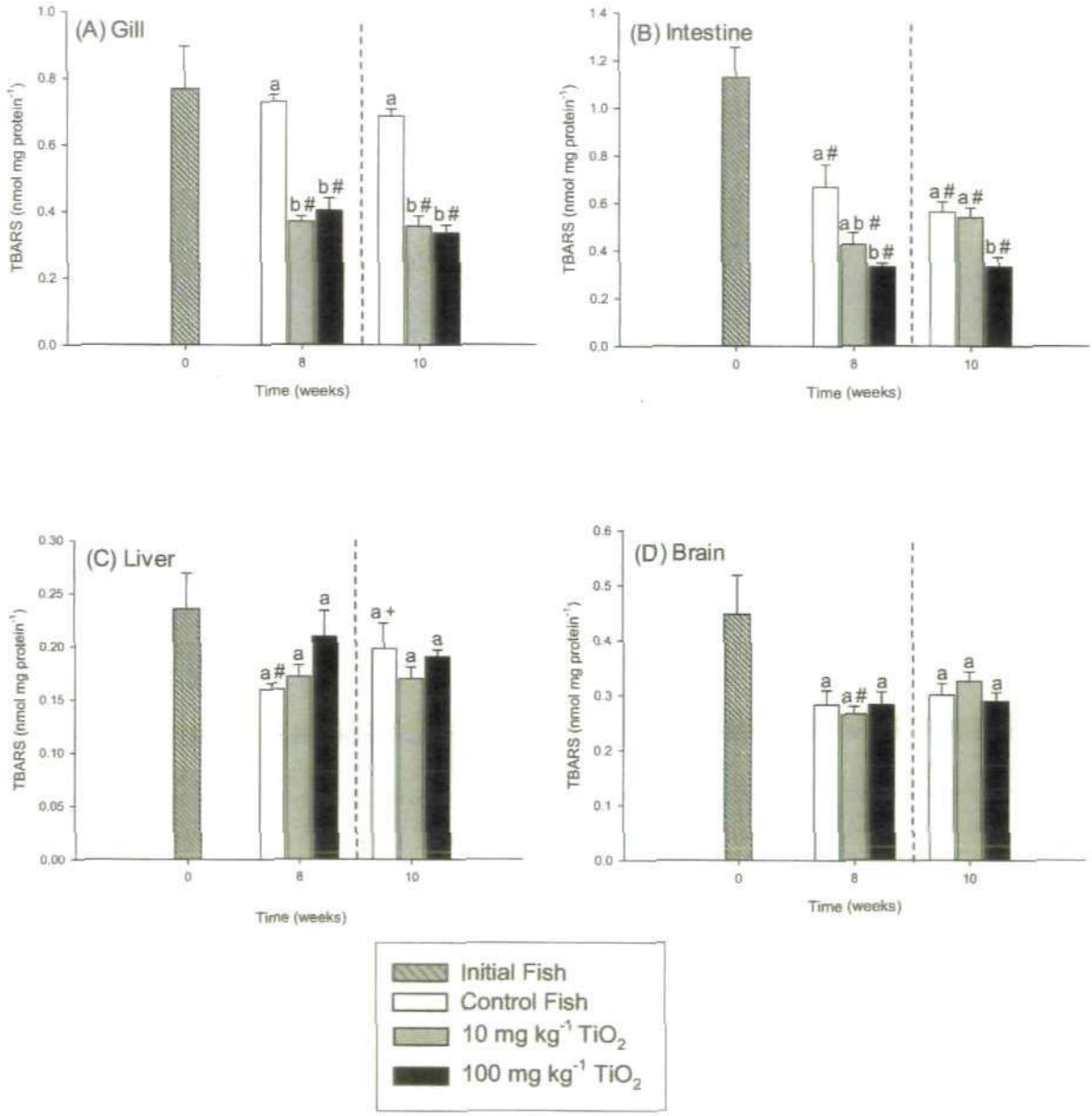


Figure 4.6. Thiobarbituric acid reactive substances (TBARS) in the gill (A), intestine (B), liver (C) and brain (D) of rainbow trout fed 0, 10 or 100 mg kg^{-1} TiO_2 NPs for 8 weeks, followed by 2 week recovery period (week 10). The dashed line indicates the end of exposure and the return of all fish to normal food ("recovery phase"). Data are mean ± S.E.M., $n = 6$ fish. Different letters within a time point indicate significant differences between tissues within each tissue (ANOVA or Kruskal-Wallis, $P < 0.05$). $^{\#}$ Significant time effect compared to initial fish (ANOVA or Kruskal-Wallis, $P < 0.05$). $^{+}$ Significant time effect within treatment compared to previous time point (ANOVA or Kruskal-Wallis, $P < 0.05$). Diagonal hatch bar are the initial fish at time zero collected immediately prior to starting the experimental diets.

4.4. Discussion

This study is one of the first reports of dietary exposure to NPs in fish, and shows that juvenile rainbow trout will eat diets containing TiO_2 NPs, and can accumulate the Ti in the gut tissue and other internal organs including the gill, liver, brain and spleen (albeit at low levels). Despite this Ti accumulation, the fish had relatively normal growth rates, suggesting that nutritional performance was not compromised at the dietary TiO_2 NP concentrations and exposure times used here. However the fish showed a number of transient physiological and biochemical disturbances during the exposure, and some organ pathologies (the latter not reported here, see Ramsden et al., 2007) which suggest the fish did suffer some sublethal effects from dietary TiO_2 NP exposure. The intention here, like previous studies on dietary metal toxicity (e.g., Cu, Handy et al., 1999; Shaw and Handy, 2006; Hoyle et al., 2007), was to simply show whether or not TiO_2 NPs as a potential dietary contaminant could cause sublethal effects compared to a control diet of normal food. This appears to be the case.

In the current experiment a commercially available NP ("Aeroxide" P25 TiO_2 NPs) was chosen because of the practical value to hazard assessment of using a material that is actually in use, rather than some other material purposefully manufactured for experiments. The P25 TiO_2 material has some complex physico-chemistry, and it was not the intention here (or in the experimental design) to investigate which aspect of the many physico-chemical properties of NPs (review, Handy et al., 2008a) is the cause of any adverse effects, but just to report what toxic effects are observed compared to an unexposed control. When the details of the chemistry are considered, caution is recommended regarding the use of commercially available bulk TiO_2 (ordinary TiO_2 powder, not nano) for comparisons of particle size effects in ecotoxicity tests (see discussion in Federici et al., 2007, and Crane et al., 2008 on NP reference materials for ecotoxicology).

There are very considerable differences in crystal structure, the proportions of different crystal structures, stochiometry, zeta potential, surface area, aggregation kinetics, chemical reactivity, the presence of different impurities associated with manufacturing processes, and many other physico-chemical properties between the P25 TiO_2 particles we have used and ordinary TiO_2 powders (e.g., Yan et al., 2005; Štengl et al., 2007; Warheit et al., 2007a; Behnajady et al., 2008; Uzunova-Bujnova et al., 2008); particle size is only one of many parameters that are different and there is therefore no logic for using commercially available bulk TiO_2 powders as a "particle size control" in the current TiO_2 NP experiment. Of course, one solution for a particle size control is to deliberately engineer a TiO_2 bulk powder where all of these physico-chemical properties are exactly identical to a nano version of the same powder, except for those associated with particle size. Such a material does not exist, and such an experiment would not be as relevant to the environment as using a real nanomaterial that is being used in many products. In any event, there remains an unresolved argument as to whether particle size, surface area or something else should be used as a metric for toxicity studies (e.g., Wittmaack, 2006). Particle size has been reported to have no effect on the toxicity of some TiO_2 NPs in rodents (Warheit et al., 2006), but instead, even small changes in crystal structure within the nano-size can produce dramatic differences in the reactivity of TiO_2 (Behnajady et al., 2008). It is therefore far from certain which physico-chemical parameters will be critical in aquatic toxicology, and the experimental approach here uses a classic nutritional toxicology study design. It is therefore possible to at least bench mark this work against identical study designs conducted with other dietary metals using similar sizes and species of salmonid fish (e.g., Handy et al., 1999; Berntssen et al., 2003; Campbell et al., 2005).

There is also a special ethical consideration for dietary TiO_2 experiments, which is different from aqueous studies. Ordinary TiO_2 powders have been used for many years as an

inert marker in fish nutrition studies (e.g., Lied et al., 1992, Weatherup and McCracken, 1998, Mamun et al., 2007), and such studies show the material to be safe to use (not toxic). The typical TiO_2 inclusion in fish diets is around 1 % of dry matter (i.e., 10 g kg^{-1} food), orders of magnitude higher than the TiO_2 NP inclusion in the current experiment. It could be argued that it is ethically unsound to repeat experiments with bulk TiO_2 powders (and therefore use more fish), when the safety of the bulk material is already established in fish nutrition. The present experiments were subject to rigorous ethical scrutiny, and for the above ethical reasons, and the many differences in chemistry, a separate bulk TiO_2 experiment as a very questionable "control experiment" for the oral route was considered unethical. It can also be argued that this is not necessary as ordinary fish foods inevitably contain some TiO_2 as background in the ingredients used for making fish diets, and would therefore already be accounted for in the unexposed controls, and the tissue levels of Ti reported in these control animals (e.g., Fig. 4.1).

4.4.1 Dietary TiO_2 NP exposure and titanium accumulation in the tissues

The exposure can be regarded as sublethal, and the background 2 % mortality (9 out of 400 fish died, no treatment-effect) was typical of the spontaneous losses of juvenile trout held in recirculating aquaria for nutrition trials (e.g., Handy et al., 1999). The dietary exposure was verified by the measured Ti in the feed, the fact that fish ate the food (Table 4.1), and that measurable increases in Ti metal were found in the tissues of the fish (Fig. 4.1) in the absence of any changes in background Ti levels in the water. In addition, the fish showed no signs of the gill pathology (see Ramsden et al., 2009) that has been associated with waterborne exposure to TiO_2 NPs (Federici et al., 2007), and this further supports the notion of an exclusive dietary exposure.

There are almost no reports of background Ti levels in juvenile rainbow trout. In this study values were around a few nmol g^{-1} dw, depending on the tissue examined (Fig. 4.1), and are broadly within the wide range reported for fish and shellfish (nmol - μmol g^{-1} levels, Bustamante and Miramand, 2005; about 0.2 nmol g^{-1} in Atlantic salmon, *Salmo salar*, Dubé et al., 2005). However, the values (Fig. 4.1) are at least 10 fold lower than those previously reported for trout (Federici et al., 2007), and are attributed to the differences in the supply of stock trout. In the latter study, the fish were obtained from a fish farm where the natural Ti levels in the environment and the background levels of Ti in the commercial food at the farm were higher than those used here. Nonetheless, in this study the trout showed measurable Ti accumulation in several tissues including the gill, gut, liver, brain and spleen during dietary TiO_2 exposure (Fig. 4.1). The levels of accumulation remained in the nmol g^{-1} range, despite the large mg levels in the food, suggesting that only a small fraction of the dietary dose was absorbed. This is consistent with other sublethal dietary metal toxicity studies where only a few % of the metal dose is absorbed (reviews, Clearwater et al., 2002; Handy et al., 2005). For Ti, this is perhaps no surprise given the absence of measurable TiO_2 NP accumulation over 14 days in our previous (shorter) aqueous study (Federici et al., 2007), and the use of bulk TiO_2 powder (> 100 nm particle size) as an inert digestibility marker in fish nutrition studies (e.g., Lied et al., 1992, Weatherup and McCracken, 1998, Mamun et al., 2007), where only about 1 % or much less of the ingested dose is accumulated (Vandenberg and De La Noüe, 2001; Richter et al., 2003). The behaviour of the NPs during the preparation of the diet might also suggest low bioavailability. The NP solutions used to spray onto the food were initially dispersed (as reported in Federici et al., 2007), but the material (not surprisingly) quickly aggregated onto the surface of the food matrix. Once in the gut lumen, the colloid behaviour of NPs (Handy et al., 2008a) suggests the high fibre/organic content and ionic

strength of partly digested food would also promote NP aggregation, and therefore probably also reduce the uptake of the ingested TiO_2 NPs.

The pattern of metal accumulation in fish tissues is usually characteristic of the route of exposure, and dietary metals usually accumulate in the gut mucosa (i.e., the route of entry), and are then carried via the hepatic portal vein to the liver, and then perhaps to other internal organs (review, Handy et al., 2005; e.g., dietary Cu, Shaw and Handy, 2006; Hoyle et al., 2007). Dietary TiO_2 NP exposure also seems to fit this general pattern for dietary exposure.

Ti did not clear quickly from all of the tissues after exposure (Fig. 4.1), with the brain, liver, intestine and gill also showing elevated Ti concentrations in treated animals compared to controls at the end of the experiment. This is similar to the findings in mice which do not clear Ti from the tissues 2 weeks after a single oral exposure to TiO_2 NPs (Wang et al., 2007). Hepatic Ti levels peaked in the tissues of exposed fish at week 4 (after the spleen), and then started to decrease back towards control levels in both TiO_2 NP treatments (Fig. 4.1), suggesting the liver was able to excrete some of the Ti. The liver also seems to be an important target organ for TiO_2 NPs in rodents. Fabian et al., (2008) showed that the livers of rats after intravenous administration of TiO_2 NPs accumulated the highest proportion of Ti, followed by spleen, lung, and kidney.

3.4.2 Growth and nutritional performance

The fish from all treatments showed steady weight gain and cumulative food intake, with no adverse effects of either TiO_2 NP inclusion (Fig. 4.2). Fish fed the 100 mg kg^{-1} TiO_2 diet showed a marginally higher final weight than the controls after 8 weeks (not statistically significant) because the fish ate slightly more food, and this trend became more apparent in the post-exposure period (but not statistically significant, Fig. 4.2). There were no treatments

effects on mean ration size, SGR, FCR or FCE throughout the experiment (Table 4.1) and these values were comparable to previous reports on trout in dietary metal studies in juvenile trout from our laboratory (e.g., Handy et al., 1999). Indicators of nutrition health such as condition factor and HSI also did not show any treatment-dependent effects (Table 4.1). Taken together these observations indicate that dietary TiO_2 NPs do not adversely affect growth or nutritional performance in rainbow trout at the inclusion levels and exposure times used here. However there was a statistically significant loss of lipid from the carcass of 100 mg kg^{-1} TiO_2 NP exposed fish compared to controls (Table 4.1), suggesting that the exposed-fish may have utilised more lipid than controls (e.g., as part of a metabolic strategy to maintain growth, Handy et al., 1999), or as a result of some lipid peroxidation (see below). However, the latter seems unlikely since the bulk of the carcass analysis will reflect the skeletal muscle which did not accumulate Ti (Fig. 4.1).

4.4.3 Haematology and ionoregulation

Dietary TiO_2 NP exposure had no effect on haematology, or plasma ions apart from a small but statistically significant rise in plasma K^+ in both TiO_2 treatments compared to controls at 8 weeks (Table 4.2). Haematological parameters remained within the expected range for trout, and showed expected small time-dependent change in some parameters associated with normal growth (Table 4.2). The haematology data (Table 4.2) were broadly similar to previous reports using rainbow trout (e.g., haemoglobin, 4-7 g dl^{-1}; haematocrit, 21-29 %; red cell counts, 0.4-0.7 x 10^6 cells mm^3; white cell counts, 6-15 x 10^3 cells mm^3; Federici et al., 2007). Bulk tissue electrolytes (Na^+, K^+, Ca^{2+}) and tissue moisture content also did not exhibit any treatment-related effects. The normal plasma ions, plasma osmolarity, and major electrolytes in the tissues, was supported by a lack of Na^+/K^+-ATPase inhibition in the

gill and intestine (Fig. 4.5); which suggests these tissues retained their ability for active ion uptake from the water and food respectively. Taken together these findings indicate that TiO_2 NPs are not potent ionoregulatory toxicants via the dietary route, and this is consistent with the findings for waterborne exposure to the same NPs (Federici et al., 2007).

4.4.4 Effects on the brain

In this study, Ti accumulated in the brain of trout (Fig. 4.1), and this has also been reported in the brain of mice following gut gavage (Wang et al., 2007). We noted some incidence of necrotic cell bodies in the brains of exposed fish with vacuolation, and Wang et al. (2007) also found vacuolation in the hippocampus of mouse brains. Notably in this study (Fig. 4.1), and in Wang et al. (2007), the Ti accumulation in the brain persisted 2 weeks after the exposure, suggesting that the brain does not clear Ti or does so very slowly. In terms of brain as a target organ, Ti is similar to dietary Hg exposure in fish where the brain tissue also accumulates metal during exposure (Berntssen et al., 2003).

Brain tissue showed transient, but statistically significant depletions of Cu at week 6 in fish from both treatments compared to controls (Fig. 4.3). Federici et al. (2007) also noted transient depletion of tissue Cu during TiO_2 NP exposure, especially in the brain. The cause of the Cu depletion remains uncertain, but effects of TiO_2 NPs on Cu transporters in the brain cannot be excluded (e.g., inhibition of Cu-ATPases) given that the closely related Na^+/K^+-ATPase is also inhibited (Fig. 4.5). Notably, the Na^+/K^+-ATPase activity in the brain showed large decreases (around 50 % inhibition, Fig. 4.5), and this effect was much greater than that observed with aqueous exposures to NPs in trout (TiO_2, Federici et al., 2007; carbon nanotubes, Smith et al., 2007).

Zinc levels in the brain also showed some small, but statistically significant, transient increases (Fig. 4.4). This was also noted in the brain after 14 days of waterborne exposure to TiO_2 NPs (Federici et al., 2007). Elevations of brain zinc levels are implicated in many processes in the brain including neuro-endocrine functions (e.g., Su et al., 1997), long term potentiation and memory formation (Takeda et al., 2008), and the regulation of proteins involved in controlling neuroactivity in the CNS (e.g., c-Fos, Matsuoka et al., 1998). These complex neurological processes were not measured during this experiment, but it is clear that Ti exposure may cause some neurological disturbances via interference with Zn homeostasis. The mechanism of this Ti effect on tissue Zn (and Cu) remains unclear, but the fact that two different routes of exposure (via the water or the food) can produce similar trace element disturbances in the brain requires further investigation.

It remains unclear whether these changes in brain chemistry caused changes in behaviour in the present experiment. In this study TiO_2 did not interfere with feeding behaviour, but feeding motivation is a strong driver, and metal-contaminated fish are known to fight for possession of food (Campbell et al., 2005) even when neuro-endocrine functions of the brain may be disrupted (Handy, 2003). Disturbances to Cu homeostasis in trout (Handy, 2003; Campbell et al., 2005), and brain lesions leading to decreased brain Na^+/K^+-ATPase activity (e.g., in rodents, Lima et al., 2008) are implicated in alteration of more complex behaviours. We also observed large reductions in Na^+/K^+-ATPase in trout brain homogenates during TiO_2 NP exposure (Fig. 4.5). Lima et al. (2008) demonstrated that loss of Na^+/K^+-ATPase activity in the cortex of the rat brain was associated with poor performance in the Barnes maze test, and a consequent delay in escape from the maze. Unfortunately, such complex behaviours such as social hierarchy formation and schooling behaviours of the trout were not measured here.

4.4.5 Functions of the spleen

The spleen showed a rapid rise in Ti in week 2, prior to Ti increasing in the other internal organs (Fig. 4.1), and spleens from exposed animals also increased in size during the exposure (Table 4.1) with some pathology at the end of the experiment (Fig. 4.8). One of the main functions of the spleen is to filter damaged cells and foreign material from the blood, and given the apparently normal haematology (Table 4.2), this seems to have been successful. The spleens of fish from the 100 mg kg^{-1} TiO_2 NP treated showed a statistically significant increase in red pulp by the end of the exposure period (week 8) consistent with the role of the spleen in filtering damaged red blood cells, and the presence of granular deposits associated with the activity of fixed macrophages in the spleen from all treatments suggested normal phagocytic functions. In the recovery phase (by week 10), there was a statistically significant reduction in the red pulp and concomitant increase in the white pulp in spleens for the highest TiO_2 diet indicating that the spleen had recovered from this activity and returned to the control proportions of red and white pulp (see Ramsden et al., 2007). The absence of changes in the red and white pulp proportions at the lower 10 mg kg^{-1} TiO_2 NP treatment, suggested that the spleen was working harder than normal to filter the blood at the 100 mg kg^{-1} TiO_2 NP concentration. These observations taken together indicate an important protective role of the spleen during Ti exposure. The spleen normally responds to damage to the blood cells and so it is assumed that it is the blood cells triggering this response. The fixed macrophage activity, while present in all treatments, was not higher in the NP treated animals (Ramsden et al., 2007), suggesting that particulate matter was not being phagocytised in the spleen, or at least not visibly at the level of the light microscope. Wang et al. (2007) also noted increased Ti levels in the spleen of mice following a single dose (gut gavage 5 g kg^{-1} of either 25 or 80 nm diameter TiO_2 NPs), but did report spleen function. Detailed studies of splenic morphology in

fish are rare (e.g., Sticklebacks, Handy et al., 2002a), but changes in the proportions of red and white pulp and the presence of foci of necrosis associated with the management of damaged red cells are typical during oral exposures to chemicals in rodents (e.g., pesticides, Handy et al., 2002b), and overall very similar responses are observed here for TiO_2 NP exposure (Ramsden et al., 2007).

In this study, Ti levels in the spleens of treated animals returned to control levels by week 4, and this was coincident with Ti elevations in some of the other internal organs (Fig. 4.1), suggesting redistribution of Ti away from the spleen to other tissues such as the liver and gill. This also implies the spleen has a finite ability to protect the internal organs from Ti in the circulation. The changes in Ti levels in the spleen also imply that the TiO_2 NPs or Ti metal entering the blood within the first 2 weeks of exposure becomes associated with (on or within) the blood cells, and is therefore trapped in the spleen. The precise aetiology of events in the spleen requires a more detailed investigation of the haemopoietic system, but the redistribution of measured Ti away from the spleen by week 4 suggests that either the spleen was working at capacity and no longer able to protect the other internal organs from exposure due to pathology (not a likely explanation as the spleen seemed to be working within its normal capacity, Fig. 4.8), or that the Ti was in a form that the spleen could not trap (e.g., not in blood cells or large aggregates that could be phagocytised by fixed macrophages in the spleen, but in solution). The experimental design was not aimed at testing particle size effects on splenic function, and the ability of fish spleen to filter any kinds of manufactured particulate matter (nano or otherwise) remains undocumented. Methods for counting particulate matter in the spleen need to be developed, but would be very problematic for NPs, because of the background particulate matter generated during normal macrophage activity in the spleen.

There were also some transient changes in Cu and Zn in the spleen (Figs. 4.3 and 4.4). The spleen showed a statistically significant depletion of Cu at week 2 in fish from both treatments compared to controls (Fig. 4.3). Copper depletion has long been implicated in altered cell surface markers on splenocytes and therefore modulation of spleen functions (Flynn, 1984). Zinc levels in the spleen also showed some small, but statistically significant, transient increases (Fig. 4.4), and a role for Zn in immunity is also well known (Rink and Gabriel, 2000). Fish do not have distinct lymph nodes (unlike mammals) and the spleen is therefore considered a more important organ in the haemopoietic system of fish. The effect of NPs on the function of the spleen and immunity in fish clearly requires further investigation.

4.4.6 Oxidative stress and histology

Several *in vitro* studies have demonstrated the potential for TiO_2 NPs to generate reactive oxygen species (ROS) or other products associated with oxidative stress in fish cell lines (Reeves et al., 2008; Vevers et al., 2008). Oxidative stress is also reported *in vivo* in trout during waterborne exposures to TiO_2 where the gills, intestine and brain showed increases in thiobarbituric acid reactive substances (Federici et al., 2007). However, TBARS did not increase during dietary TiO_2 NPs exposure, but instead showed some small (but statistically significant) decreases in the gill and intestine (Fig. 4.6). This phenomenon has also been observed during waterborne SWCNT exposure in trout, and in the absence of changes in the total glutathione pool, suggested that the fish were probably up regulating other anti-oxidant defences to cause a fall in background level of TBARS (Smith et al., 2007).

Histological observations in the liver (Ramsden et al., 2009) also support the notion that the changes in TBARS and glutathione in the current study were subtle background

effects that were not likely to compromise organ function. Biologically important levels of lipid peroxidation are often associated with vacuolation and extensive lipidosis in the tissues (Handy, 2003; Shaw and Handy, 2006; Hoyle et al., 2007).

4.4.7 Hazard assessment implications

This study demonstrates that fish can accumulate Ti from a dietary TiO_2 NP exposure, and that a number of subtle physiological and biochemical disturbances, including organ pathologies can occur. This information is collected against an historic use of ordinary TiO_2 powder (bulk TiO_2) in many fish nutrition studies and animal feeds over many years where no such toxic effects have been observed, partly because the fish do not absorb the bulk TiO_2 powder from the gut lumen (e.g., digestibility studies, Vandenberg and De La Noüe, 2001; Richter et al., 2003). This at least suggests some circumstantial evidence that there may be a different hazard from the commercially available nano TiO_2 product used in this study and ordinary TiO_2 powders that have been used in nutritional research and foods.

Perhaps a more important question for risk assessors examining exposure via the food chain in ecosystems, is whether or not the hazard presented by TiO_2 NPs is more or less than existing dietary metal toxicants of considerable concern such as Cd, Hg, Zn or Cu (review, Handy et al., 2005). If this experiment is compared against dietary metal concentrations in other studies on salmonids where growth rate was maintained, but subtle biochemical disturbances and pathologies were observed over 8 weeks or more (e.g., Cu, 500 mg kg^{-1}, Handy et al., 1999; Zn, 590-1520 mg kg^{-1}, Clearwater et al., 2002; Cd, 250 mg kg^{-1}, Lundebye et al., 1999; Inorganic Hg, 100 mg kg^{-1}, Berntssen et al., 2003); then TiO_2 NPs might be considered more toxic than dietary Cu and Zn, and at least as toxic as Hg and Cd. The human health hazard from eating contaminated fish is also a concern, and the risk from

accidental ingestion of TiO_2 NP contaminated trout may be limited, because the Ti does not appear to accumulate in the edible muscle at the concentrations and time scales used here.

4.4.8 Addendum

The research presented in the current chapter was conducted in 2008, and the subsequent paper published in 2009 (two years prior to the submission of the thesis). Notably, this chapter and the associated research paper are almost identical (histology and minor edits aside). As such, it is prudent to revisit the literature since this publication in order to assess the current state of knowledge regarding the toxicity of TiO_2 NPs to fish.

Zebrafish chronically (6 months) exposed to relatively low levels of TiO_2 NPs (1-7 mg l^{-1}), showed that Ti was accumulated and distributed in the gills, liver, heart and brain (ng g^{-1} levels), with some histological effects in the gills (Chen et al., 2011a). However, similarly to the study by Federici et al. (2007), Johnston et al. (2010) also observed a lack of uptake of TiO_2 NPs in rainbow trout following waterborne exposure (500 or 5000 μg l^{-1} for 14 days), and only a small elevation in gut Ti following dietary exposure (30 or 300 g kg^{-1} for 21 days; no increased Ti burden in the gill, liver, brain, skin, or blood of exposed fish). The amount of Ti found in the gut of dietary exposed trout after 21 days represented approximately 0.83 % of the total concentration of Ti metal in the feed, which is very similar to that seen in the study here where the Ti concentration in the gut after 14 days dietary exposure represented approximately 0.92 % of the total Ti metal concentration in the feed (assuming that Ti metal constitutes approximately 60 % of TiO_2). However, there is no definitive evidence as to whether this is from uptake of TiO_2 NPs, uptake of Ti released from the NPs, or surface bound metal or NPs (although attempts were made to rinse the guts in the current study to remove any surface bound NPs). The spleen experienced a transient increase in Ti levels and

some deleterious effects in the current study, though to date these have not been replicated in studies within the literature, though it is quite possible that no attempts have been made to do so. Likewise the increase in Ti concentration in the liver and brain of dietary exposed fish seen currently has not been observed in other studies (e.g., Scown et al., 2009; Johnston et al., 2010), though the data pool is currently relatively small. In the injection study by Scown et al. (2009), the kidney was the main storage compartment for Ti, however this may not be ecologically relevant due to the lack of evidence of significant TiO_2 NP uptake from the water and diet meaning that it is unlikely that TiO_2 NPs or Ti metal will be transported to the kidney following environmental exposure. Also, although there were some differences in Ti levels between treatments in the current study, it is notable that the levels are still very low (nmol g^{-1} levels). At these low levels the analytical instrumentation (i.e., ICP) is close to running at the detection limit and caution may be required in interpretation in such instances.

In the waterborne TiO_2 NP study by Federici et al. (2007) and the present dietary TiO_2 NP exposure there was some evidence of oxidative stress. Hao et al. (2009) observed statistically significant decreases in superoxide dismutase, catalase, and peroxidase activities and a significant increase in lipid peroxidation levels in the liver, gill and brain of juvenile carp (*Cyprinus carpio*) following exposure to 100 or 200 mg l^{-1} TiO_2 NPs. The liver was the organ most affected; however, attributing a positive correlation between the effects seen and the NPs is difficult as tissue Ti levels were either not assessed or not described. Scown et al. (2009) intravenously injected rainbow trout with 100 µg TiO_2 and found Ti concentrated in the kidney for up to 21 days, but observed no deleterious effects to kidney function and no evidence of lipid peroxidation (TBARS) in kidney, liver or blood plasma. No evidence of oxidative stress was observed in zebrafish larvae exposed to TiO2 NPs (Chen et al., 2011b), Some *in vitro* studies have shown oxidative stress to be one mechanism of toxicity following exposure to TiO_2 NPs (e.g., Reeves et al., 2008), however, Thomas et al. (2011) saw no

increase in reactive oxygen species in rainbow trout hepatocytes exposed to two different sizes of TiO_2 NPs. Significant changes in innate immune function at the levels of gene expression and cellular function have also been seen following zebrafish exposure to waterborne TiO_2 NPs. Palaniappan and Pramod, (2010) exposed zebrafish to nanosized and bulk TiO_2 and saw that the structural conformation of proteins in gill tissue was significantly influenced by NP exposure when compared to the bulk form.

Further empirical evidence is clearly required before a more definitive judgement can be made as to the level of bioavailability and toxic effect of TiO_2 NPs in fish, particularly in view of some of the irregularities between different studies in the literature where fish were exposed to TiO_2 NPs via the water or diet. For example, fathead minnow (*Pimephales promelas*), had a 48 h LC_{50} value of > 500 mg l^{-1} (Hall et al., 2009), whilst rainbow trout displayed a 96 h LC_{50} of > 100 mg l^{-1} (Warheit et al., 2007b). Zhu et al. (2008) observed no toxic effects to zebrafish (*Danio rerio*) embryos and larvae exposed to 1-500 mg l^{-1} TiO_2 NPs, whilst Paterson et al. (2011) observed some effects to the development of TiO_2 NP (0-14 mg l^{-1}) exposed Japanese medaka (*Oryzias latipes*), particularly post-hatching. Chen et al. (2001b) saw no effects to hatchability, survival, and no signs of malformation of zebrafish embryos and larvae exposed to 0.1-10 mg l^{-1} TiO_2 NPs, whilst effects to reproductive success were seen in zebrafish chronically exposed to TiO_2 NPs (Wang et al., 2011). Contrary to trout exposed to dietary TiO_2 NPs here, fathead minnow exposed to waterborne TiO_2 NPs (542 mg l^{-1} for 7 days) in the study by Hall et al. (2009) experienced a 25 % inhibition of growth. Inhibition of growth from a waterborne TiO_2 NP exposure route was also seen in zebrafish exposed to 1-7 mg l^{-1} for 6 months (Chen et al., 2011a). Some dietary studies have shown no uptake of TiO_2 NPs (e.g., Johnston et al., 2010), whilst others have shown that there was uptake (e.g., Zhu et al., 2010), although the mode of exposure may have some bearing upon

this (NP incorporation into pelleted, manmade feed in the former compared to NPs biologically incorporated into prey items in the latter).

It is clear that two years following the study contained within this chapter, that there is still no general consensus on the nanotoxicity of TiO_2 and it remains prudent to conduct more research, but perhaps with a more collaborative effort to coordinate and standardise toxicity tests (experimental design etc) to allow a rational comparison.

Chapter 5. The toxicological effects of waterborne copper nanoparticles versus copper sulphate on rainbow trout, *(Oncorhynchus mykiss)*.

Abstract

Emerging data suggests that some types of nanoparticles (NPs) are toxic to fish, and given the well known toxicity of dissolved metals, there are also concerns about metal-NPs. One central question for risk assessment is whether or not nanometals represent a greater hazard than traditional dissolved forms of metals. In this study, fish (mean ± S.E.M., $n = 210$; 37.4 ± 1.9 g) were exposed in triplicate to either control, 20 or 100 μg l^{-1} of either soluble Cu (as $CuSO_4.5H_2O$) or Cu-NPs (99.9+ % purity; 87 ± 27 nm mean particle size) in a semi-static aqueous exposure regime. Fish were sampled at days 0, 4, and 10 for tissue trace elements, haematology, and biochemistry. By day 4, fish from the 100 μg l^{-1} $CuSO_4$ treatment experienced 85 % mortality (treatment subsequently terminated) compared to 14 % in the 100 μg l^{-1} Cu-NP exposed fish. Mortality at day 10 was 4, 17, 10, and 19 % in the control, low $CuSO_4$, and low and high Cu-NP treatments respectively. Copper accumulation was seen in the gills of fish from both Cu treatments, and was statistically significant in both $CuSO_4$ treatments at day 4 and all Cu treatments at day 10 compared to controls. No statistically significant Cu uptake was seen in the spleen, brain or muscle of fish from any treatment, although an elevation in intestinal Cu was seen in the high Cu-NP treatment throughout. Although there were some statistically significant effects observed in some blood parameters (i.e., haemoglobin, haematocrit, and red blood cells), there were no obvious treatment-related trends and Cu-NPs were not haemolytic. However, a parallel decreases in Na^+/K^+-ATPase activity (almost 6-fold in all Cu treatments compared to controls) and plasma and carcass ions suggests that Cu-NPs are an ionoregulatory toxicant to rainbow trout. Significant decreases in Na^+/K^+-ATPase activity were also seen in the brains of all Cu exposed fish compared to controls and in the intestine of fish exposed to high Cu-NPs, along with changes to levels of thiobarbituric acid reactive substances (TBARS, as an indicator of lipid peroxidation), also observed in the gills. The brain and intestine as target organs of waterborne NP exposure has been noted previously and requires further study. In conclusion, Cu-NPs were not as acutely toxic as $CuSO_4$, but did present a sublethal risk to rainbow trout that was similar to the metal salt.

5.1. Introduction

The effects of dissolved trace metals (i.e., metal ions in aqueous solution) on fish have been extensively studied (e.g., Cu: Bury and Handy, 2010; Zn: Hogstrand and Wood, 1996: Cd: Sprague, 1987). This has led to a consensus view that aqueous metal toxicity and/or bioavailability is often a function of the free metal ion concentration, or the complexes that the metal ion will form in aqueous solution (Paquin et al., 2002). However, recent advances in materials science have produced nanoparticulate forms of metals or metal oxides, including Cu nanoparticles (Cu-NPs). The ecotoxicology and chemistry of nanomaterials has recently been reviewed (Ju-Nam and Lead, 2008; Handy et al., 2008a; Klaine et al., 2008; Kahru and Dubourguier, 2010; Shaw and Handy, 2011). Nanoparticles form dispersions or emulsions, rather than *aqueous* solutions in water (Handy et al., 2008a) and there are concerns that the toxicity of metal NPs may be different to the traditional dissolved forms of the same metal. Or alternatively, that metal NPs may gradually dissolve to release metal ions by dissolution from the surface of the particle, causing latent free ion toxicity. However, little is known about the bioavailability of metal NPs and their subsequent accumulation and body systems effects compared to the traditional dissolved form (review, Handy et al., 2008b).

Following waterborne exposure, the traditional form of dissolved copper from metal salts tends to accumulate in the gills first, reflecting the route of exposure, and then the internal organs (e.g., Stagg and Shuttleworth, 1982; Grosell et al., 1997; McGeer et al., 2000). The mechanisms of acute toxicity in the gill are well established and can be explained by the direct effect of Cu on the structural integrity of the gill epithelium and loss of branchial ionoregulatory functions (Taylor et al., 1996; Campbell et al., 1999; Wood, 2001; Grosell et al., 2002; Handy, 2003) which is largely a function of the amount of free Cu ions

in the water (review; Meyer et al., 2007). Consequently, there is a direct correlation between Cu toxicity and abiotic factors such as increased water hardness and reduced pH which can provide other ions (Ca^{2+}, H^{+}) to compete with dissolved Cu (Di Toro et al., 2001; Handy and Eddy, 2004). The presence of other ligands in the water which can also complex Cu ions (e.g., natural organic matter; Hollis et al., 1997; Schwartz et al., 2004).

Research on dissolved Cu toxicity has therefore focused on metal speciation in the water, and the details of effects on gill functions including inhibition of branchial Na^{+}/K^{+}-ATPase (e.g., Li et al., 1998), as well as respiratory acid-base disturbances (e.g., Wilson and Taylor, 1993; Taylor et al., 1996). In acute Cu toxicity, the loss of branchial ionoregulatory control, efflux of electrolytes from the blood over the gill epithelium, and subsequent cardiovascular collapse leads to death (review; Handy, 2003). Sublethal concentrations of Cu (low $\mu g\ l^{-1}$ levels) will result in Cu accumulation in the internal organs, with the liver being the central compartment in Cu metabolism (Grosell et al., 1998; 2001). Numerous sublethal effects following waterborne Cu exposure have been reported including changes to haematology (e.g., Cerqueira and Fernandes, 2002), vacuole formation and necrosis in the liver (Figueiredo-Fernandes et al., 2007), and oxidative stress (e.g., Sanchez et al., 2005).

Currently only a handful of studies have assessed the effects of Cu-NPs on fish, and details of the mechanisms of toxicity are currently unclear. It remains to be seen if the factors that affect dissolved Cu toxicity (e.g., metal speciation and water chemistry) will also apply to Cu-NPs. Cu ions may be released from the NP surface so that the fish are exposed to dissolved Cu as well as the nano-form, but the toxicity of free Cu compared to Cu-NPs has not been quantified in the same experiment with fish. Studies with Cu-NPs have demonstrated quite low acute toxicity of the nano form. The 48 h LC_{50} of Cu-NPs to

zebrafish (*Danio rerio*) was 1.5 mg l^{-1} compared to 250 µg l^{-1} in fish exposed to dissolved Cu (Griffitt et al., 2007). Some of this difference in toxicity may have been due to the loss of Cu-NPs due to particle aggregation in the suspension, but nonetheless, toxicity associated with Cu-NPs was observed. Similar to traditional dissolved Cu, the gills were the organ primarily affected by exposure with concentration-dependent damage to the lamellae; characterised by proliferation of epithelial cells, as well as oedema of primary and secondary gill filaments. An inhibition of branchial Na^+/K^+-ATPase activity was also observed (Griffitt et al., 2007). Subsequent work by Griffitt and colleagues also showed that Cu-NPs were also toxic to juvenile zebrafish (48 h LC_{50}, Griffitt et al., 2008), and that Cu accumulation was seen in the gills of zebrafish exposed to Cu-NPs for up to 48 h (Griffitt et al., 2009). Cu-NPs were also seen to be acutely toxic to zebrafish embryos in a 96 h exposure, with 100 % mortality in animals exposed to 1 and 0.5 mg l^{-1} Cu-NPs at 8 and 24 hours post-fertilisation (hpf) respectively (Bai et al., 2010). Concentration-dependent decreases in hatching success were also observed along with a statistically significant concentration-dependent decrease in larvae length.

To the authors knowledge the effects of waterborne Cu-NPs on rainbow trout have not been investigated and therefore the aim of the current study was to compare the effects of Cu-NPs to equal concentrations of soluble Cu (as $CuSO_4$) following aqueous exposure to juvenile rainbow trout (*Oncorhynchus mykiss*). Two concentrations of $CuSO_4$ and Cu-NPs were selected for the exposure. A high Cu concentration (100 µg l^{-1}) was chosen as an approximation of the 96 h LC_{50} for waterborne Cu in salmonids (e.g., Shaw and Brown, 1974; Spear and Pierce, 1979) so that the toxicity of Cu-NPs could be compared to a concentration of soluble Cu that would be overtly toxic to trout, whilst the lower concentration of 20 µg l^{-1} was selected in order to asses for any sublethal affects at more

environmentally realistic levels. Analogous to previous work on the effects of waterborne NPs on fish (e.g., Federici et al., 2007, Smith et al., 2007), a body systems physiological approach was employed which assessed haematology and plasma ions, disturbances associated with osmoregulation (trace element profiles of the internal organs, Na^+/K^+-ATPase activity) and oxidative stress (thiobarbituric acid reactive substances (TBARS), tissue glutathione content).

5.2. Materials and methods

5.2.1 Experimental design

Juvenile rainbow trout (n = 300) were obtained from Hatchlands Trout Farm, Rattery, Devon, UK, and held for 4 weeks in a stock aquaria with flowing, aerated, dechlorinated Plymouth tap water (see below). Stock animals were fed to satiation on a commercial trout food. Fish weighing 29.4 ± 1.0 g (mean ± S.E.M., n = 210) were then transferred into fifteen aerated 20 l experimental glass aquaria (14 fish/tank; identical water conditions) and acclimated for 48 h prior to experiments. Three tanks per treatment were randomly allocated and fish exposed in triplicate to one of the following treatments for 10 days: control (no added Cu), 20 or 100 μg l^{-1} Cu (as $CuSO_4.5H_2O$) or copper nanoparticles (Cu-NPs), equating to molar concentrations of 0.315 and 1.574 μmol Cu l^{-1} respectively. Note, when concentrations of $CuSO_4$ are mentioned here on, it is referring to the actual Cu concentration (i.e., 100 μg l^{-1} $CuSO_4$ is actually 100 μg l^{-1} Cu), with the whole compound name used in order to make distinction between the two Cu forms and thus treatments easier. A semi-static exposure regime (80% water change every 12 h with re-dosing after each change) was

employed following a pilot study where water changes after 12 h were shown to adequately maintain water quality.

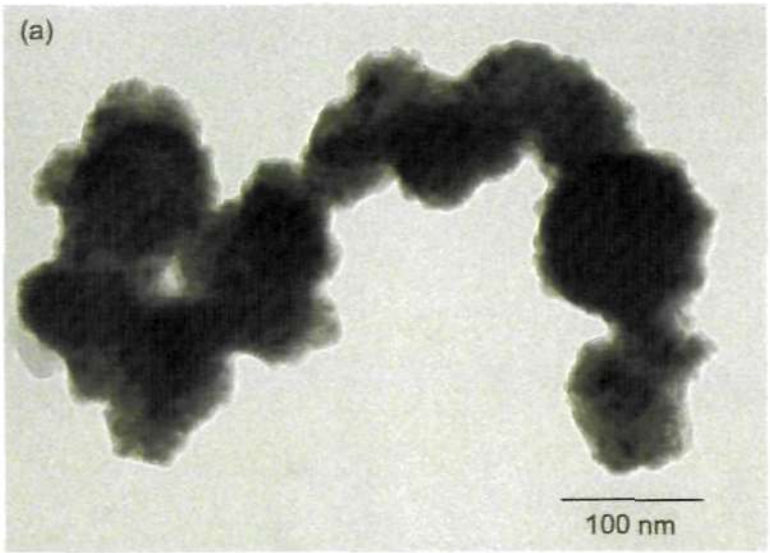
Fish were not fed 24 h prior to, or during the experiment in order to minimise the risk of the Cu-NPs absorbinzyg to food or faecal material, and to help maintain water quality. Water samples were taken before and after each water change for pH, temperature, saturated oxygen (HACH HQ40d multi reader), total ammonia (HACH LANGE GMBH LCK kit 304 read on a HACH LANGE GmbH DR 2800 spectrophotometer) and water hardness (Ca and Mg measured by inductively coupled plasma optical emission spectroscopy (ICP-OES)). As there were no significant differences between any tanks in water quality, data were pooled and were (means ± S.E.M., n = 240-528 samples); pH, 6.98 ± 0.004; temperature, 16.0 ± 0.01 °C; oxygen saturation, 90.9 ± 0.2 %; total ammonia, 0.85 ± 0.05 mg l^{-1}, total hardness (mg l^{-1} $CaCO_3$), 52.02 ± 1.2. Photoperiod was 12 h light: 12 h dark. The electrolyte composition of the dechlorinated Plymouth tap water used was 9.89, 1.56, and 18.05 mg l^{-1} Na^+, K^+ and Ca^{2+} respectively (0.43, 0.04, and 0.45 mmol l^{-1} respectively). Background Cu levels in the water were 6.04 ± 0.27 µg Cu l^{-1} (0.095 ± 0.004 µmol Cu l^{-1}).

Fish were randomly sampled on day 0 (initial fish from the stock), day 4, and day 10 for haematology, plasma analysis, tissue electrolytes, and tissue biochemistry.

5.2.2 Copper nanoparticle stock solution and dosing

The Cu-NPs used were purchased from Sigma Aldrich and had (from manufacturer's information) an average particle size < 50 nm and 99.9 % purity. Subsequent image analysis by transmission electron microscopy (TEM, JEOL 1200EX II) revealed a mean particle of 87 ± 27 nm (mean ± S.D., n = 50, Fig. 5.1a), with mean aggregates of 216 ± 122 nm with a mode of 48 nm (NanoSight LM_{10}, Fig. 5.1b). Notably, analysis of Cu-NP aggregates in the

experimental tanks (NanoSight LM_{10}) following dosing was not possible due to the low concentrations and interference from other particulates in the water. Inductively couple plasma optical emission spectroscopy (ICP-OES, Varian 725 ES) analysis revealed no metal impurities (data not shown). A fresh 50 ml stock solution of 0.5 g l^{-1} Cu-NPs was made at 6 pm daily by dispersing the NPs in ultrapure (Milli-Q) water without solvents and stirring (magnetic stirrer IKA Werke RET basic C, at 300 rpm) for 4 h in a low-density polyethylene (LDPE) plastic. This stock was then used to dose the fish at 10 pm following the water change and again at 10 am the following morning (the stock was stirred for a further hour prior to the morning dosing event). A 10 ml subsample was then taken from the stock for analysis of total Cu by ICP-OES and the remaining stock discarded.



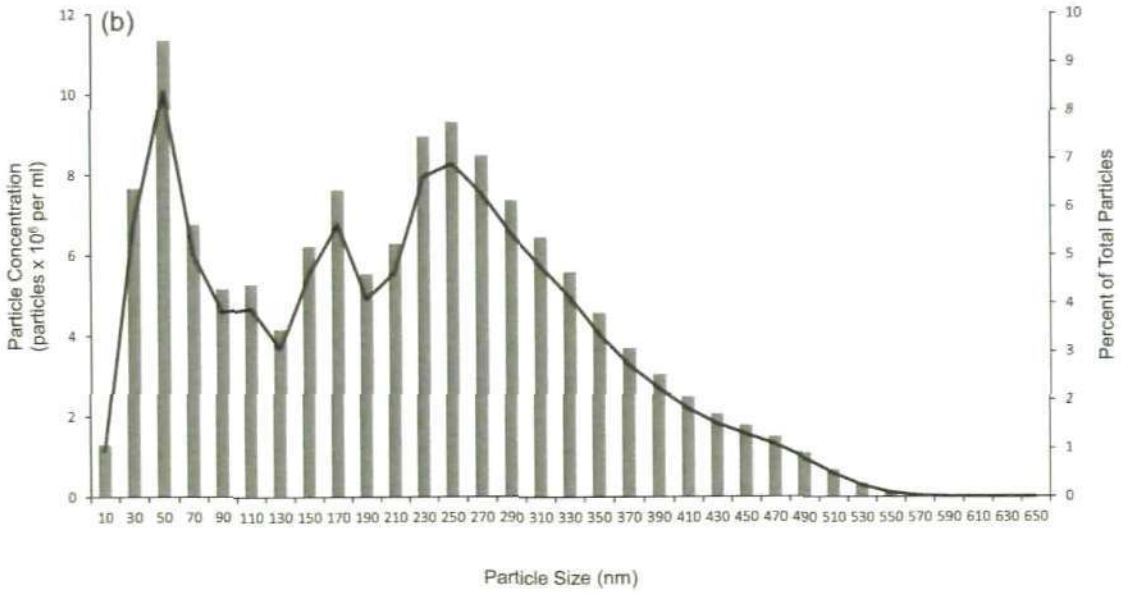


Figure. 5.1. Particle size and particle size distribution and aggregation of 1 g l^{-1} Cu-NP stock. (a) Electron micrograph showing aggregated Cu-NPs with a mean particle size of 87 ± 27 nm (mean ± S.D., $n = 50$), and (b) NanoSight LM_{10} graph showing the concentration of particles at each size (vertical bars) and the percent of total particles at each size (line) with a calculated mean aggregate size of 216 ± 122 nm and a mode of 48 nm.

5.2.3 Haematology and plasma analysis

Haematology and plasma analysis were performed as described in Chapter 2. Briefly, two fish were randomly collected from each tank (six fish/treatment and initial fish) at days 0, 4, and 10 and anaesthetised with buffered MS222. Whole blood was collected via the caudal vein into heparinised syringes, and the fish were then weighed and the total length recorded. Immediately following blood collection the haematocrit value (HCT) and haemoglobin concentration were determined. Plasma analysis was carried out for Na^+, K^+, Cl^- and glucose.

5.2.4 Tissue ion analysis

Following blood sampling, fish were terminally anaesthetised with MS222 and dissected for tissue ion analysis. Gill, liver, intestine, spleen, whole brain, and skinned muscle from the flank were harvested, rinsed in deionised water and processed for Cu, Zn, Mn, Ca, Na and K by inductively coupled plasma optical emission spectrometry (ICP-OES, Varian 725 ES) according to Chapter 2. In the absence of certified fish reference tissues for Cu-NPs, spike recovery tests were performed with both $CuSO_4$ and Cu-NPs using rainbow trout gill and liver. Recovery was good in all tissues measured with recovery values from nominal concentrations all over 90 % (data not shown).

5.2.5 Biochemistry

Biochemistry was performed exactly as described in Chapter 2. Briefly an additional two fish were randomly collected from each tank (6 fish/treatment, and initial fish) at days 0, 4, and 10 for biochemistry. Gill, liver, intestine, and whole brain were removed and

immediately snap frozen in liquid nitrogen, then stored at -80 °C until required for analysis. Tissues (approximately 0.2-0.3 g or the whole brain) were homogenised and stored in 0.5 ml aliquots at -80 °C until required. Homogenates were analysed (in triplicate) for Na^+/K^+-ATPase activity, and thiobarbituric acid reactive substances (TBARS) and total glutathione (GSH).

5.2.6 Statistical analysis

All data were analysed by StatGraphics Plus version 5.1 as described in Chapter 2. No tank effects were observed throughout the experiment, so data were pooled by treatment for statistical analysis. Briefly, parametric data were analysed for treatment, time, and treatment x time interactions by multifactor ANOVA (see Chapter 2 for description of pre-requisite tests and observations to allow the appropriate use of ANOVA and how non-parametric data were analysed). When a statistically significant effect was observed by this model a one-way ANOVA (or equivalent non-parametric test) was employed to assess for simple effects. Where a statistical significance is indicated within the data (i.e., $P < 0.05$, ANOVA) this refers to the simple effect observed by one-way ANOVA following the multifactor ANOVA model showing there was an overall effect.

5.3. Results

5.3.1. Waterborne exposure to dissolved $CuSO_4$ and Cu-NPs

Waterborne copper exposure was confirmed by ICP-OES analysis of water samples taken 10 minutes following the dosing of the experimental tanks. Copper levels were (mean ± SEM., $n = 12$ water samples per treatment) 3.01 ± 0.02, 22.3 ± 1.7, 102.3 ± 5.7, 19.7 ± 2.8,

and 100.8 ± 6.9 μg l^{-1} (for the control, 20 and 100 μg l^{-1} $CuSO_4$ and Cu-NP treatments respectively), representing recoveries of 111.5, 102.3, 98.5, and 100.8 % of the nominal concentrations respectively. Mean recoveries taken over the 12 h exposure period between water changes (samples taken at 10 min, 1, 2, 6, and 12 h post dosing) were (data; mean %, *n* = 12 samples per treatment) 94.7, 95.2, 88.3, and 84.6 % for the control, 20 and 100 μg l^{-1} $CuSO_4$ and Cu-NP treatments respectively. Analysis of Cu-NP stocks used to dose the fish showed that values never deviated by more than 2 % of the nominal concentration (mean values ranged between 98.9-101.2 % of nominal concentrations). Aqueous exposure to both $CuSO_4$ and Cu-NPs caused mortality in rainbow trout, though the former was far more acutely toxic (Fig. 5.2). Exposure to 100 μg l^{-1} $CuSO_4$ caused 80 % mortality in 96 h and this treatment was therefore terminated at this point. However, the same concentration of Cu-NPs only caused an approximate 20 % mortality after 10 days exposure. No signs of stress, aggression or unusual swimming behaviour were noted in any tank prior to the fish becoming moribund. Whilst the control fish did experience some mortality (5 % after 10 days, Fig. 5.2), this is not unusual for juvenile rainbow trout and within acceptable levels.

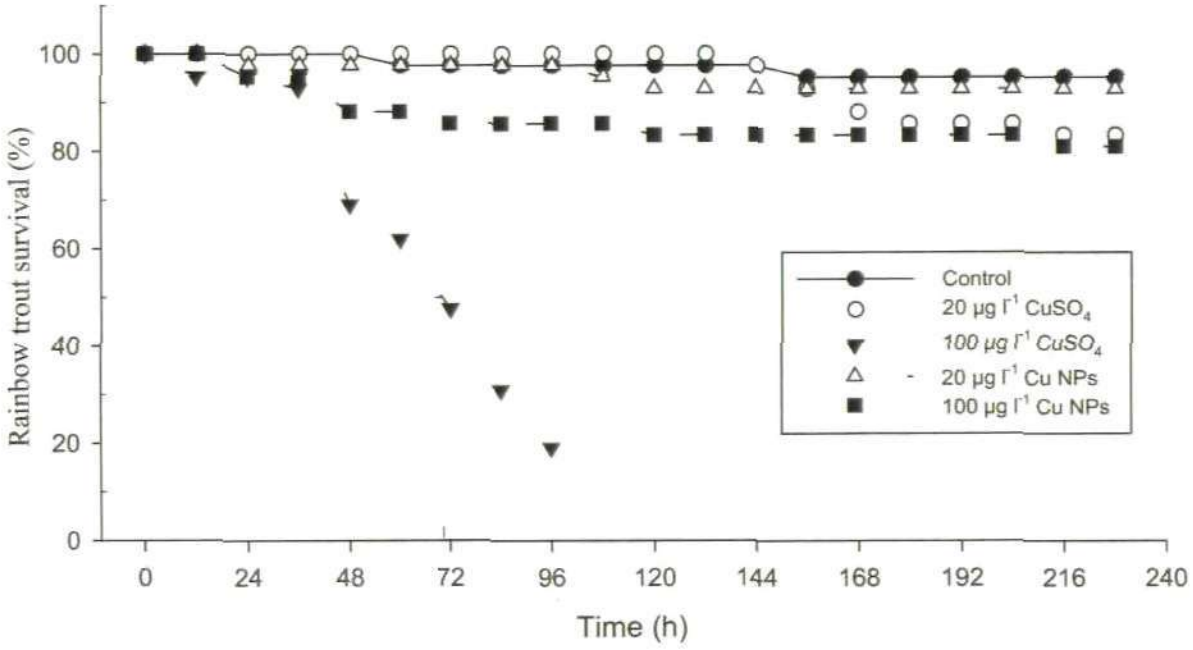


Figure. 5.2. The effects of $CuSO_4$ and Cu-NPs on survival in rainbow trout. Data are mean of triplicate treatments (errors bars are not shown for clarity). Note the termination of the 100 µg l^{-1} $CuSO_4$ treatment after 4 days due to high mortality.

Copper accumulation was seen in the gills of fish from both Cu treatments, statistically significant increases in the tissue Cu levels in gills from the $CuSO_4$ treatments at day 4, and all Cu treatments at day 10 compared to controls (Fig. 5.3). The elevated mortality rates observed in fish from the high $CuSO_4$ treatment (Fig. 5.2) was associated with an almost 9-fold increase in branchial Cu levels compared to control at day 4, Fig. 5.3). Notably, throughout the exposure branchial Cu burden was more pronounced in the $CuSO_4$ treatments compared to Cu-NPs, whilst the livers of fish exposed to $CuSO_4$ also had elevated Cu levels compared to Cu-NP exposed animals, though this was only statistically significant in the low $CuSO_4$ treatment at day 10. Contrary to the aqueous exposure route, intestinal Cu levels were significantly elevated in the high Cu-NP treatment at day 4 (not observed in the $CuSO_4$ treatments), which persisted until the end of the exposure (day 10). No statistically significant ($P > 0.05$, ANOVA) Cu uptake was seen in the spleen, brain or muscle of fish from any treatment (Fig. 5.3).

5.3.2 Haematology and blood plasma analysis

Although there were some statistically significant effects observed in some blood parameters (Table 1), there were no obvious treatment-related trends. Fish exposed to both $CuSO_4$ and Cu-NPs showed decreases in haemoglobin levels after 10 days, although this trend was also seen in the control fish compared to initial (T0) fish though to a lesser extent. Similarly, haematocrit and red blood cell counts decreased throughout the experiment

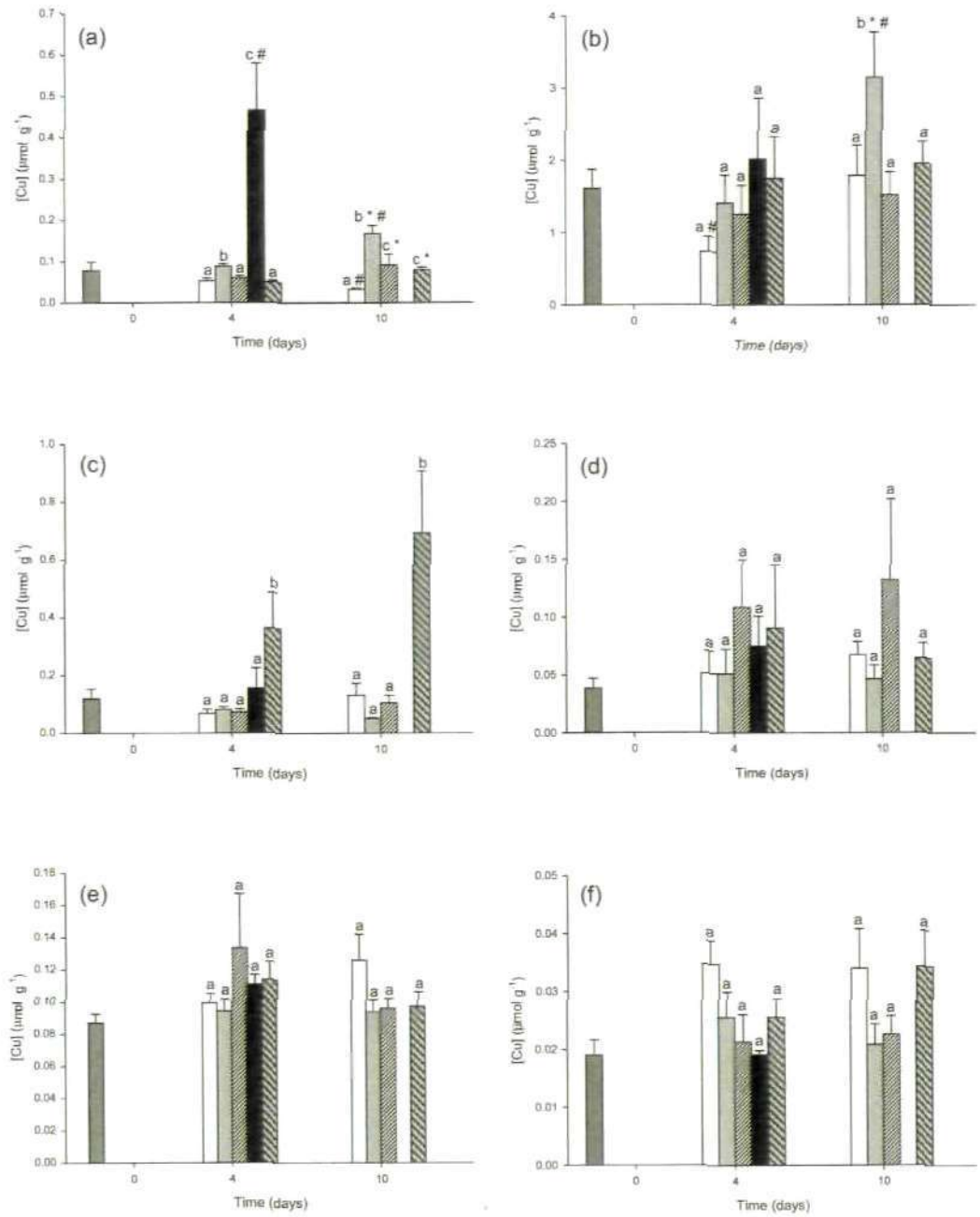


Figure. 5.3. Copper levels in (a) gills; (b) liver; (c) intestine; (d) spleen; (e) brain; (f) muscle of trout after 4 and 10 days of exposure. Data are mean ± S.E.M., dry weight tissue, $n = 6$ fish/treatment. Data were analysed by multifactor ANOVA and when an overall significant effect ($P < 0.05$) was indicated, data were then analysed by one-way ANOVA (or non-parametric equivalent) for simple effects. Different letter denotes a statistically significant (simple) difference between treatments at each time point (ANOVA or Kruskal–Wallis, $P < 0.05$); # indicates significantly different than initial fish (ANOVA, $P < 0.05$), * indicates significantly different from same treatment at the previous time point (ANOVA, $P < 0.05$). Note that initial (time zero fish) had been recently fed and as such may have dietary derived Cu remaining in their system at this time point.

compared to initial fish and control (though in the latter, the day 10 control fish also displayed reduced red blood cell count similar to that of the Cu treated groups indicating no treatment effects). Haematocrit values were seen to decrease in both Cu-NP treatments by day 10. These were statistically significant in the high Cu-NP treatment compared to day 4 values for that treatment (time effect), whilst day 10 haematocrit values in fish from the low Cu-NP treatment were significantly lower compared to fish exposed to low $CuSO_4$ at that time point ($P < 0.05$, ANOVA).

Some treatment related changes were seen in blood plasma analysis which were potentially treatment related (Table 1). By day 4 fish exposed to low Cu-NPs showed a significant decrease in plasma Na^+ compared to all other treatments and initial fish (ANOVA, $P < 0.05$), though this did not persist at day 10, at which time fish exposed to high Cu-NPs also experienced reduced plasma Na^+ levels (significantly reduced compared to the previous time point, and the low Cu-NPs at day 10, ANOVA, $P < 0.05$). A time-dependent decrease in plasma K^+ was seen in fish exposed to low $CuSO_4$ and high Cu-NPs, which was significant in the high Cu-NP treatment compared to control fish at day 10 as well as the previous time point within that treatment and initial fish (ANOVA, $P < 0.05$, Table 1). Plasma glucose levels were also measured and showed depletion in fish exposed to both Cu-NP treatments by day 10; statistically different compared to initial fish at both NP concentrations and to control fish in the high Cu-NP treatment only ($P < 0.05$; Table 1).

Table 5.1. Haematological parameters and plasma ion, Cl^- and glucose concentrations in rainbow trout exposed to control (no added Cu), 20 or 100 μg l^{-1} $CuSO_4$ or Cu-NPs for up to 14 days.

Parameter	Time (days)	Treatment				
		Control	20 μg l^{-1} Cu (as $CuSO_4$)	20 μg l^{-1} Cu-NPs	100 μg l^{-1} Cu (as $CuSO_4$)	100 μg l^{-1} Cu-NPs
Haemoglobin	0	5.25 ± 0.51				
(g dl^{-1})	4	4.58 ± 0.53	3.74 ± 0.56	$3.68 \pm 0.37^{\#}$	$3.95 \pm 0.415^{\#}$	4.39 ± 0.32
	10	4.70 ± 0.17	$3.80 \pm 0.61^{+\#}$	$3.64 \pm 0.25^{+\#}$	-	$3.84 \pm 0.22^{+\#}$
Haematocrit	0	26.8 ± 1.1				
(%)	4	$22.8 \pm 0.8^{\#}$	$22.1 \pm 1.0^{\#}$	$21.4 \pm 2.1^{\#}$	$20.7 \pm 0.9^{\#}$	$23.4 \pm 0.2^{\Delta\#}$
	10	$22.3 \pm 1.0^{\#}$	23.7 ± 1.3	$18.1 \pm 1.7^{+\Delta\#}$	-	$19.4 \pm 1.2^{\#*}$
Red blood cell count	0	0.79 ± 0.09				
(cells x10^6 mm^3)	4	0.76 ± 0.06	$0.52 \pm 0.08^{+}$	$0.66 \pm 0.04^{\Delta}$	$0.54 \pm 0.05^{+\#}$	$0.62 \pm 0.03^{\Delta}$
	10	$0.59 \pm 0.03^{\#}$	$0.53 \pm 0.07^{\#}$	0.55 ± 0.04	-	$0.50 \pm 0.04^{\#}$
Plasma Na^+ (mmol l^{-1})	0	129.17 ± 3.95				
	4	138.33 ± 6.19	141.83 ± 4.64	$97.83 \pm 6.70^{+\Delta\#}$	142.00 ± 3.61	136.67 ± 2.93^{D}
	10	125.67 ± 4.01	115.5 ± 11.23*	131.00 ± 6.54*	-	$102.83 \pm 4.35^{*\#D}$
Plasma K^+ (mmol l^{-1})	0	3.27 ± 0.08				
	4	3.35 ± 0.12	3.09 ± 0.08	$2.37 \pm 0.09^{+\Delta\#}$	3.03 ± 0.09	3.34 ± 0.25^{D}
	10	3.22 ± 0.20	2.72 ± 0.24	$2.37 \pm 0.17^{+\#}$	-	$2.50 \pm 0.16^{+*\#}$
Plasma Cl^- (mmol l^{-1})	0	163.67 ± 14.55				
	4	$148.33 \pm 12.83^{\#}$	$153.73 \pm 8.09^{\#}$	$130.38 \pm 7.84^{\#}$	$143.00 \pm 4.36^{\#}$	$139.45 \pm 3.28^{\#}$
	10	$137.17 \pm 6.89^{\#}$	$146.19 \pm 3.53^{\#}$	$136.00 \pm 4.58^{\#}$	-	$139.55 \pm 4.71^{\#}$
Plasma glucose (mmol l^{-1})	0	3.87 ± 0.35				
	4	3.65 ± 0.54	3.04 ± 0.47	2.83 ± 0.12	3.10 ± 0.26	3.42 ± 0.23^{D}
	10	3.27 ± 0.36	3.27 ± 0.25	$2.66 \pm 0.30^{\#}$	-	$2.06 \pm 0.11^{+\#}$

Data are mean ± S.E.M. ($n = 6$ fish/treatment). Data were analysed by multifactor ANOVA and when an overall significant effect ($P < 0.05$) was indicated, data were then analysed by one-way ANOVA (or non-parametric equivalent) for simple effects. $^{+}$, significant difference from control within rows (ANOVA or Kruskal-Wallis, $P < 0.05$). $^{\Delta}$, indicates that the Cu-NPs displayed a significant difference from the $CuSO_4$ treatment at

each concentration (NP effect, ANOVA, $P < 0.05$). *, significant difference between day 7 and day 14 within treatment (time-effect, ANOVA, $P < 0.05$). ^{D}, significantly different from the previous $CuSO_4$ or Cu-NP concentration within row (dose-effect within time point, ANOVA, $P < 0.05$). $^{\#}$, significantly different from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). Note that there is no data for the 100 $\mu g\ l^{-1}$ $CuSO_4$ treatment at day 10 due to the cessation of this treatment at day 4 following high mortality.

5.3.3 Tissue ion concentrations and water content

During the exposure there were some statistically significant differences to ion levels in some tissues, most notably Na^+ levels in the gills, liver, intestine, and muscle. However, only the gills displayed any obvious trends and these were both treatment and time related (ANOVA, $P < 0.05$, Fig. 5.4). Branchial Na^+ levels decreased in all Cu treatments (apart from the 20 μg l^{-1} $CuSO_4$ treatment) at day 4 and this persisted at day 10. Although liver, intestine and muscle tissue Na^+ levels also displayed some statistically significant differences (ANOVA, $P < 0.05$), these data did not show any notable time or concentration-dependent trends (Fig. 5.4). However, a transient decrease (day 4 only) in hepatic K^+ was observed in fish exposed to both of the high Cu treatments along with reduced Ca^{2+} in the liver at day 10 in both of the Cu-NP treatments (ANOVA, $P < 0.05$; Ca^{2+} day 10 values were (mean ± SEM, $n = 6$ samples per treatment) 19.5 ± 2.8, 21.4 ± 3.6, 12.8 ± 4.7, and 5.3 ± 0.4 μmol Ca^{2+} g^{-1} for the control, low $CuSO_4$, low and high Cu-NP treatments respectively). No significant changes in tissue Zn and Mn levels were seen in any treatment (ANOVA, $P > 0.05$) with values remaining in the expected range for rainbow trout. In the present study Mn and Zn values ranged between 0.02 – 0.16 and 1.4 – 7.1 μmol g^{-1} dry weight tissue respectively depending upon tissue (i.e., gill, liver, intestine, spleen, muscle or brain). Tissue moisture content did not differ significantly between treatments or over time with values remaining between 58-89 % depending upon the tissue.

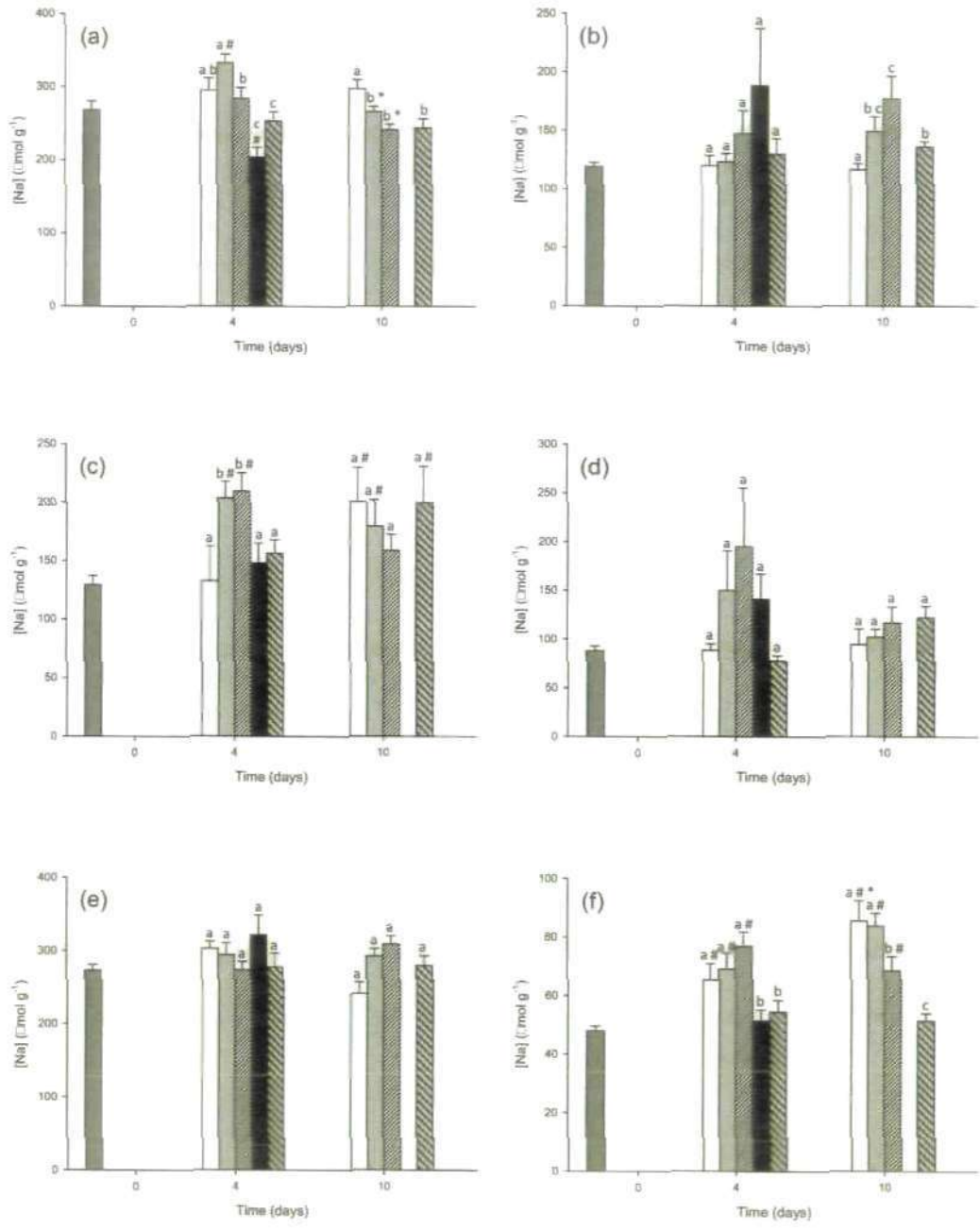


Figure. 5.4. Sodium levels in (a) gills; (b) liver; (c) intestine; (d) spleen; (e) brain; (f) muscle of trout after 4 and 10 days of exposure. Data are mean ± S.E.M., dry weight tissue, $n = 6$ fish/treatment. Data were analysed by multifactor ANOVA and when an overall significant effect ($P < 0.05$) was indicated, data were then analysed by one-way ANOVA (or non-parametric equivalent) for simple effects. Different letter denotes a statistically significant (simple) difference between treatments at each time point (ANOVA or Kruskal–Wallis, $P < 0.05$); # indicates significantly different than initial fish (ANOVA, $P < 0.05$), * indicates significantly different from same treatment at the previous time point (ANOVA, $P < 0.05$). Note that initial (time zero fish) had been recently fed and as such may have dietary derived Na^+ remaining in their system at this time point.

5.3.4 Na^+/K^+-ATPase

Exposure to both $CuSO_4$ and Cu-NPs caused decreases in Na^+/K^+-ATPase activity. The gills of fish exposed to 20 $\mu g\ l^{-1}$ $CuSO_4$ and both Cu-NP treatments all experienced significant reductions with an almost 6-fold difference compared to control at day 10 (Kruskal-Wallis, $P < 0.05$, Fig. 5.5) with little difference seen between fish exposed to $CuSO_4$ or Cu-NPs. Brain and intestinal Na^+/K^+-ATPase activity were also affected, with all Cu exposed fish showing significantly reduced levels in the brain compared to control fish and in the intestine of fish from the high Cu-NP treatment compared to all other treatments (ANOVA, $P < 0.05$; Fig. 5.5).

5.3.5 Total glutathione content and TBARS

Biochemical analysis was carried out on day 10 and showed that total glutathione (GSH) content of the liver, intestine, and brain were not affected by waterborne exposure to $CuSO_4$ or Cu-NPs and remained stable (ANOVA, $P > 0.05$). Day 10 GSH values ranged between 2.3-3.4 (liver), 0.7-1.3 (intestine), and 0.6-1.1 (brain) $\mu mol\ g^{-1}$ wet weight (ww) tissue. A small, but insignificant increase in branchial GSH was noted at day 10, with values of 1.8 ± 0.5, 2.8 ± 0.8, 2.0 ± 0.7, and 2.6 ± 1.0 $\mu mol\ g^{-1}$ ww tissue in the control, low $CuSO_4$, and low and high Cu-NP treatments respectively (ANOVA, $P < 0.05$). However, significant differences in levels thiobarbituric acid reactive substance were seen between some treatments (ANOVA, $P < 0.05$, Fig. 5.6). The brains of fish exposed to $CuSO_4$ and both Cu-NP treatments showed reduced levels of TBARS compared to control fish, whilst fish exposed to $CuSO_4$ only showed reduced branchial levels (ANOVA, $P < 0.05$, Fig. 5.6). A non-significant trend of increasing TBARS was also seen in the gills of fish exposed to Cu-

NPs (ANOVA, $P > 0.05$). Fish exposed to low $CuSO_4$ and low Cu-NPs did not experience any changes in intestinal TBARS, but fish exposed to high Cu-NPs showed a significant increase, approximately 1.7-fold compared to control fish (ANOVA, $P < 0.05$, Fig. 5.6).

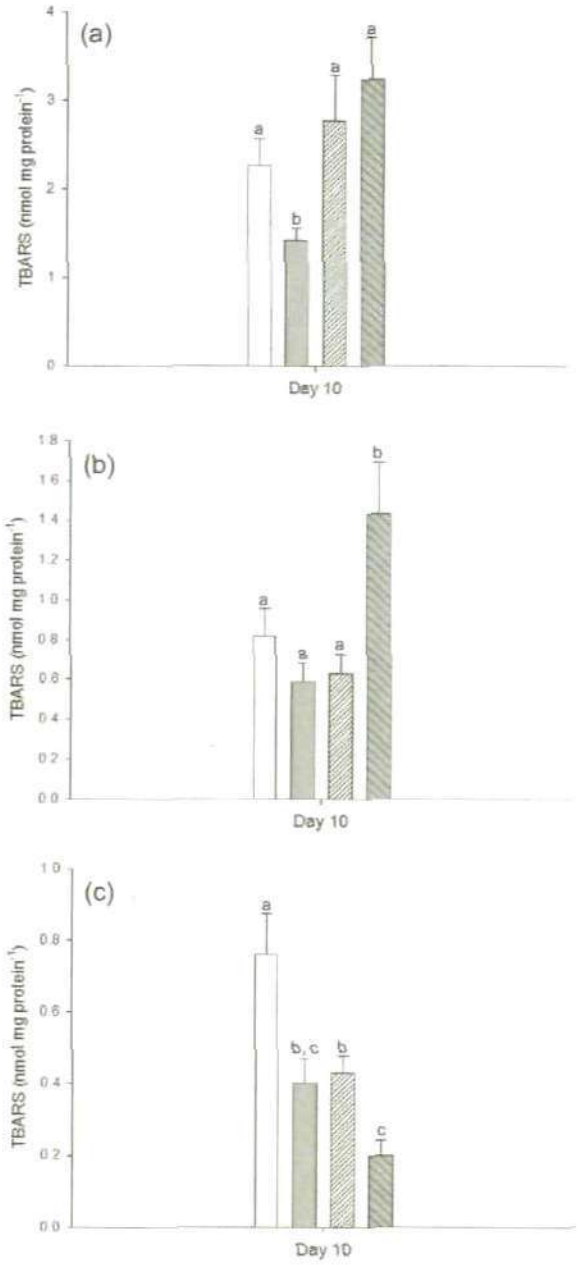


Figure. 5.6. Thiobarbituric acid reactive substances (TBARS) in crude homogenates from (a) gills; (b) intestine; (c) brain of trout after 10 days of exposure to 0 (clear bar), 20 μg l^{-1} $CuSO_4$ (grey bar), 20 μg l^{-1} Cu-NPs (white bar with diagonal lines), or 100 μg l^{-1} Cu-NPs (grey bar with diagonal lines). Data are mean ± S.E.M., $n = 6$ fish/treatment. Data were analysed by multifactor ANOVA and when an overall significant effect ($P < 0.05$) was indicated, data were then analysed by one-way ANOVA (or non-parametric equivalent) for simple effects. Different letters indicate significant (simple) differences between treatments within tissues (ANOVA or Kruskal-Wallis, $P < 0.05$). Note that there was no 100 μg l^{-1} $CuSO_4$ treatment at this time point and he different *y*-axis scales on each graph.

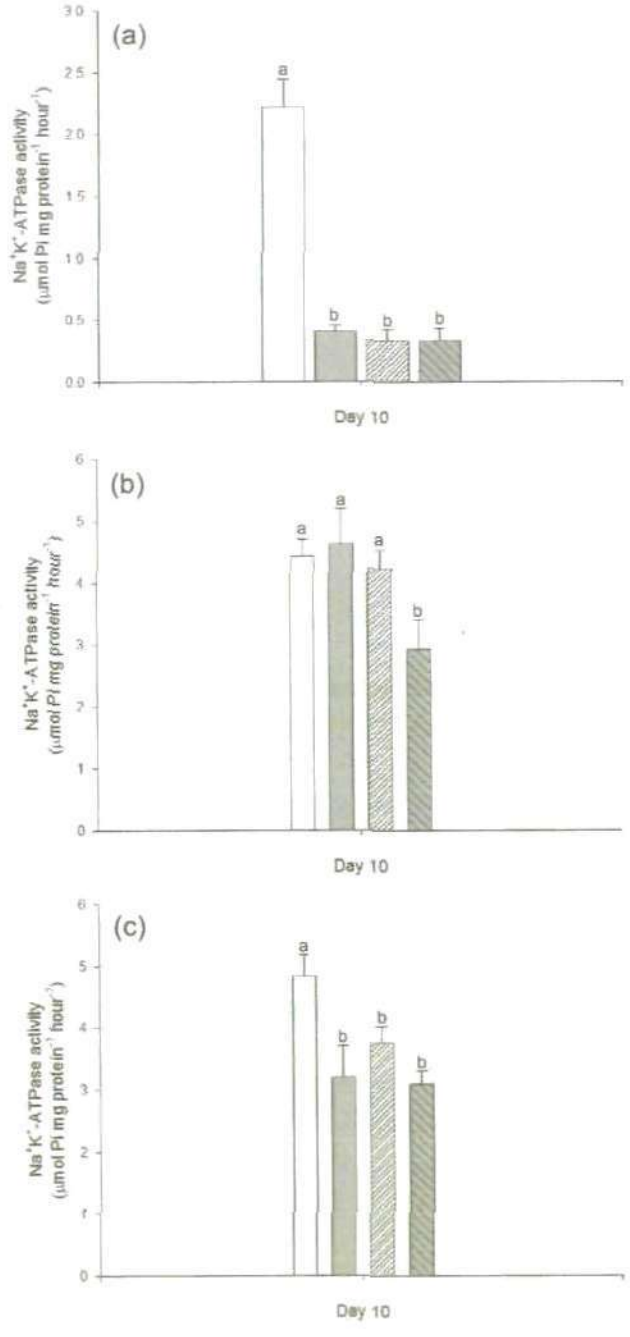


Figure. 5.5. Na^+/K^+-ATPase activity in crude homogenates from (a) gills; (b) intestine; (c) brain of trout after 10 days of exposure to 0 (clear bar), 20 μg l^{-1} $CuSO_4$ (grey bar), 20 μg l^{-1} Cu-NPs (white bar with diagonal lines), or 100 μg l^{-1} Cu-NPs (grey bar with diagonal lines). Data are mean ± S.E.M., $n = 6$ fish/treatment. Data were analysed by multifactor ANOVA and when an overall significant effect ($P < 0.05$) was indicated, data were then analysed by one-way ANOVA (or non-parametric equivalent) for simple effects. Different letters indicate statistically significant (simple) differences between treatments within tissues (ANOVA or Kruskal-Wallis, $P < 0.05$). Note that there was no 100 μg l^{-1} $CuSO_4$ treatment at this time point. Note the different *y*-axis scale for the gill data (panel A).

5.4. Discussion

Few, if any, studies have so far investigated the effects of waterborne Cu-NPs on trout and here we present a comparison with equal mass Cu (as $CuSO_4$) using a whole body systems approach. Exposure to Cu-NPs caused significant changes to plasma and tissue Na^+ levels along with reduced Na^+/K^+-ATPase activity in the gills, intestine, and brain, indicating that Cu-NPs, like dissolved Cu, are an ionoregulatory toxicant to trout. It is possible that oxidative stress was a factor in the observed toxicity with levels of TBARS (as an indicator of lipid peroxides in the tissue) significantly different to that of controls in some treatments, though total glutathione pools were not affected.

5.4.1 Particle characterisation, exposure, and metal ion dissolution

Primary particle size characterisation of the Cu-NPs used here showed a disparity with the manufacturer's information (measured mean particle size of 87 ± 27 nm compared to < 50 nm primary particle size claimed by the manufacturers). This is a phenomenon that has previously been noted by several researchers and emphasises the need for characterisation to take place prior to toxicity testing (Scown et al., 2010), particularly as particle size dependent toxicity (different sizes of the same NP) has been observed (e.g., Ag-NPs; Bar-Ilan et al., 2009). Although it is unlikely that the fish were predominantly exposed to single particles here (with a mean aggregate size of 216 ± 122 nm seen in 1 g l^{-1} Cu-NP stock solution in pure water), NanoSight analysis did show that particles less than 100 nm in size were present in the stock before addition to the experimental tanks. Aggregation of Cu-NPs may well differ in tank water, which has significantly increased ionic strength compared to pure water and also contained fish and thus organic matter (e.g., mucous secretions). Although attempts were

made to assess Cu-NP aggregation and distribution in tank water this was not possible due to the interference of other particles present (e.g., natural colloids), whilst the concentrations of Cu-NPs used in the exposures were too low to successfully assess in pure water using NanoSight. Exposure to $CuSO_4$ and Cu-NPs was confirmed by ICP-OES analysis of water samples taken mid-water column 10 minutes, 1, 2, 6, and 12 hours following its addition to the experimental tanks. These analyses showed that concentrations of total Cu did not drop below 84 %, throughout in any treatment, with the 12 h period between water changes and subsequent redosing adequate to maintain exposure. This is despite that at stock concentrations at least (i.e., 1 g l^{-1}) it was evident by observation that there was NP aggregation and some sedimentation. Confirmation of waterborne exposure was also demonstrated by elevation of branchial Cu, with the gills being the primary target organ for this route of exposure. Indicative of waterborne exposure, fish in the $CuSO_4$ exposed groups had significantly elevated branchial Cu after 4 days of exposure compared to all other treatments, whilst all Cu exposed fish ($CuSO_4$ and Cu-NPs) had Cu levels that were significantly higher than controls at day 10 (Fig. 5.3).

Separate experiments using the bulk equilibrium reverse dialysis bag technique showed that over 12 h (i.e., the time between water changes and subsequent redosing) there was an approximate 6.1 % dissolution of Cu ions from the Cu-NPs (Boden, 2011, personal communication). In the current experiment this rate of loss would equate to approximately 1.22 and 6.1 μg l^{-1} free Cu in the 20 and 100 μg l^{-1} Cu-NP treatments 12 h after dosing respectively. Therefore, it can be concluded that the toxicity seen in the current experiment was not a product of free ion activity.

5.4.2 Acute toxicity

Although the aim of this study was to investigate the sublethal affects of Cu-NPs some mortality did occur (Fig. 5.2). Fish from both the $CuSO_4$ and Cu-NPs treatments died during the exposure, though the former was far more acutely toxic with the high $CuSO_4$ treatment terminated after 4 days due to high mortality. These fish experienced 50 % mortality after approximately72 h exposure to 100 $\mu g\ l^{-1}$ $CuSO_4$, with over 80 % dead by 96 h. All other treatments including control fish experienced less than 20 % mortality over 10 days. At the start of the experiment it was accepted that the high $CuSO_4$ concentration used here could potentially cause mortality. For example, Cu 24-96 h LC_{50} values in trout typically range from 91-210 $\mu g\ l^{-1}$ (e.g., Shaw and Brown, 1974; Taylor et al., 2000; De Boeck et al., 2004) depending upon water quality (review; Meyer et al., 2007), although more extreme values can be found within the literature (e.g., Howarth and Sprague, 1978). Therefore a high concentration of 100 $\mu g\ l^{-1}$ was chosen here in order to expose fish to a concentration of Cu-NPs that although could prove lethal to trout exposed to an equal concentration of soluble Cu and would certainly elicit sublethal affects. Subsequently at the concentrations used here, despite some mortality, Cu-NPs can be considered sublethal toxicants to trout. That the Cu-NP concentrations used here were not acutely toxic to rainbow trout is unsurprising with two 48 h Cu-NP LC_{50} values of 1.5 and 0.94 $mg\ l^{-1}$ previously established in adult zebrafish (Griffitt et al., 2007; 2008), though sensitivity may well differ from rainbow trout.

5.4.3 Copper accumulation during waterborne exposure

The accumulation of Cu in the gills seen here after exposure to Cu-NPs (Fig. 5.3) is in agreement with Griffitt et al. (2007) who also saw branchial Cu levels rise in zebrafish after

exposure to 100 μg l^{-1} Cu-NPs. Though in the latter study this may have been as a result of dissolution of Cu ions from the NPs. Following waterborne exposure, fish will likely experience an elevation in hepatic Cu levels, with the liver being an important organ in Cu homeostasis (Grosell et al., 1998a; b), though other internal organs can also show increases in Cu levels (Kamunde and Wood, 2004). Unlike fish exposed to $CuSO_4$, there was no accumulation of Cu in the livers of Cu-NP exposed fish (Fig. 5.3). This presents an interesting quandary in that there was clearly Cu accumulation (though maybe not actual uptake) at the gills, with measures taken to remove surface bound particles from the analysis, but this Cu was not translocated to other internal organs. It is possible that Cu-NPs became associated with mucous within the gill microenvironment (similar to other NPs, e.g., CNT; Smith et al., 2007) and this was not easily dislodged by rinsing with deionised water. Another explanation could be that Cu ions dissolved from the Cu-NPs at the gill and were taken up, though if this was of appreciable concentrations then one would expect liver levels to rise accordingly. Clearly, more research is necessary to understand the processes by which Cu-NPs associate and/or accumulate within the gills (or at least the gill microenvironment) of exposed fish. Of the other tissues analysed for Cu, only the gut of fish exposed to high Cu-NPs displayed significantly elevated levels, with no changes compared to control, or between $CuSO_4$ and Cu-NP exposed fish in the spleen, brain or muscle (Fig. 5.3). This elevation of Cu in the gut occurred even though the fish were not fed during the exposure and the gut tissue was cleared of surface bound Cu-NPs by washing both the interior and exterior in deionised water during dissection (the risk of oral exposure is discussed further below).

5.4.4 Haematology and ionoregulatory disturbances

Blood chemistry and haematology is often used to assess the health of animals and waterborne Cu exposure has been shown to affect haematological parameters in fish. For example, changes (often seen as decreases) have been observed in Hb, RBC, and HCT levels (e.g., Khangarot and Tripathi, 1991; James et al., 1998; Singh et al., 2008), and can be an indication of ionoregulatory or respiratory disturbances (Carvalho and Fernandes, 2006). However, no major disturbances to haematology resulted from exposure to $CuSO_4$ or Cu-NPs in the current study, with no clear concentration or Cu-form trends evident. These results suggest that Cu-NPs are not haemolytic to rainbow trout, although some statistically significant changes were seen in some of the blood parameters at some time points (Table 1). However, changes seen over time compared to initial fish may be associated with the animals not being fed during the experiment (e.g., Rios et al., 2005) or just background 'noise'. During the exposure, plasma glucose levels remained below 5 mmol l^{-1} in all treatments indicating that the fish were not overly stressed. However, plasma glucose levels were significantly reduced in both Cu-NP treatments at day 10 (compared to control and $CuSO_4$ exposed fish, Table 1). This may have been related to the lack of feeding during the experiment, though as it was only seen in the Cu-NP exposed fish could suggest a depletion of glucose stores following increased stress levels in those treatments at an earlier time not assessed.

The lack of overt haemolytic toxicity is in agreement with the limited effects seen from previous studies where fish were exposed to different forms of NPs via the water or diet (e.g., single-walled carbon nanotubes (SWCNT), Smith et al., 2007; Fraser et al., 2011, and TiO_2 NPs, Federici et al., 2007; Ramsden et al., 2009). However, significant reductions in Na^+/K^+-ATPase activity and effects on tissue electrolytes in the current study show that Cu-

NPs are an ionoregulatory toxicant to rainbow trout. The gills play an important role in osmoregulation and the ionoregulatory toxicity of Cu is well established (e.g., Laurén and McDonald, 1987a,b; Li et al., 1998), with sodium homeostasis being a key target (Grosell et al., 2002). Subsequently the effects on the Na pump and tissue and plasma Na^+ levels here suggests this is also true for Cu-NPs. Griffitt et al. (2007) also reported decreased branchial Na^+/K^+-ATPase activity, in zebrafish exposed to Cu-NPs, though a far greater inhibition (approximately 5-fold or greater) was seen in fish exposed to $CuSO_4$. Contrary to the work by Griffitt and workers no differences were seen here between fish exposed to $CuSO_4$ or Cu-NPs with the significant decreases in branchial and brain Na^+/K^+-ATPase activity compared to controls similar between both treatments (Fig. 5.5). Of particular note here are effects to the brains of fish, a recurrent theme in NP toxicity. For example, as well as affecting the sodium pump, 100 $\mu g\ l^{-1}$ Cu-NPs also caused a reduction in brain Na^+ levels at day 4 (also seen in the high $CuSO_4$ treatment at this time point), which was persistent at day 10. By day 10 the low $CuSO_4$ treatment also displayed a significant decrease in brain Na^+ levels, although notably this was not seen in the low Cu-NP treatment.

5.4.5 Oxidative stress

Oxidative stress is a known mechanism of Cu toxicity to fish. For example, in previous studies fish exposed to Cu have shown changes to antioxidants levels (Eyckmans et al., 2011), hepatic catalase gene expression (Craig et al., 2007), and branchial and intestinal TBARS (Hoyle et al., 2007). Some recent studies have also shown that NPs can cause oxidative stress, though without appreciable metal accumulation in the affected organs (e.g., TiO_2 NPs, Federici et al., 2007). Oxidative stress was also implicated in a study exposing Japanese medaka (*Oryzias latipes*) to Fe-NPs (Li et al., 2009). In the current study, fish

exposed to 20 μg l^{-1} $CuSO_4$ for 10 days had significantly decreased TBARS in the gills compared to all other treatments, whilst a non-significant trend of increasing TBARS was seen in the gills of fish exposed to both concentrations of Cu-NPs (Fig. 5.6). Total glutathione pools in this organ were not significantly different between treatments, though values in the Cu treated fish were slightly higher than controls. However, these data cannot sufficiently explain the changes to levels of TBARS and it is possible that other antioxidants, not measured here, may have been induced.

Following the trend of Cu accumulation and subsequent ionoregulatory inhibition seen in the gut of trout exposed to the high Cu-NP treatment, fish from that treatment also experienced a significant increase (approximately 2-fold compared to all other treatments) in TBARS at day 10, although levels remained in the range previously reported for control fish in similar conditions (e.g., 1-3 nmol mg $protein^{-1}$, Federici et al., 2007; Smith et al., 2007). However, it is clear that Cu-NPs were present in the gut of fish from the high treatment and it is thus likely that the elevated TBARS seen in this organ was not 'background noise', but an artefact of oxidative stress.

5.4.6 Risk of oral exposure to NPs from waterborne sources

The phenomenon of NPs being present in the gut of fish following exposure via the water has been noted in previous studies. Rainbow trout exposed to waterborne SWCNT had visible aggregates of the NPs in the gut lumen of exposed fish along with pathology to the mucosa (Smith et al., 2007), whilst TiO_2 NP exposed fish displayed luminal fluid of a milky-white appearance, presumed to be ingested TiO_2, also with concomitant gut pathologies (Federici et al., 2007). This is perhaps not surprising seeing as stressors such as pollutants have been seen to increase the drinking rate of fish (e.g., Best et al., 2003), which in normal

(unstressed) conditions can be a few ml kg^{-1} h^{-1} (Eddy, 1982). The elevated intestinal Cu (Fig. 5.3) and changes to Na+/K+-ATPase activity (Fig. 5.4) and levels of TBARS (Fig. 5.5) of fish exposed to the high Cu-NP treatment seen here support the notion of the gut being a target organ from waterborne exposure. Although there is the possibility of NPs being a delivery vehicle for metal ions (presenting ions to the gut mucosa with subsequent toxicity, Handy et al., 2008), the gut lumen is a high ionic strength environment that may promote particle aggregation, making dissolution less likely. Therefore the effects seen here may be NP driven and thus from the evidence beginning to emerge there are clearly reasons to be concerned regarding the risk of oral exposure of fish to NPs.

5.4.7 Toxicity of Cu-NPs compared to $CuSO_4$

From the results seen here it is clear that Cu-NPs present a similar sublethal risk to trout as $CuSO_4$ with generic stress responses, though they are less of an acute risk. For example, both forms of Cu had no effect on haematology, but both affected ionoregulation, with similar inhibition of the sodium pump and subsequent imbalances to Na^+ levels (Fig. 5.5 and Table 5.1). This was in spite of the almost 2-fold greater Cu accumulation in the gills of fish exposed to 20 $\mu g\ l^{-1}$ $CuSO_4$ than either Cu-NP treatment after 10 days (Fig. 5.3). In terms of uptake, Cu-NPs did not exhibit the trends frequently associated with dissolved Cu exposure (e.g., elevated hepatic Cu), but fish did, somewhat unusually, experience a significant increase in intestinal Cu (over 3-fold in the high Cu-NP exposed fish at day 10 compared to other treatments). These data suggest that the mechanisms of toxicity may differ between $CuSO_4$ and Cu-NPs. It is unlikely that dissolved Cu from the NPs played a significant role in toxicity and the concern that NPs could be potent surface acting toxicants has been suggested (Handy et al., 2008b) with Cu-NPs potentially falling into this category.

The phenomenon of indirect systemic toxicological effects without a positive correlation to internal metal accumulation has previously been seen with aluminium exposure (e.g., Handy and Eddy, 1989, 1991), and with exposure to a metal-NP (TiO_2 NPs; Federici et al., 2007). The brain of Cu-NP exposed fish in the current study showed reduced sodium pump activity and changes to levels of TBARS without Cu accumulation in this organ, although this also occurred in fish exposed to $CuSO_4$. However, the content of Cu in the brain appears to be a neglected aspect of Cu accumulation studies (Handy, 2003), and similarly, data on deleterious effects in the brain are lacking in the literature, rendering a comparison difficult. Federici et al. (2007) discuss the possibility that from the site of injury (at the gills) there could be a diffusion of highly mobile hydroxyl radicals or a cascade of inflammatory responses that are rapidly mediated around the body by specific signalling pathways, and similar responses cannot be disregarded here. Overall it appears that Cu-NPs elicit toxicological effects akin to dissolved Cu and at similar levels of severity, but that there are potential differences in the mechanism of toxicity as indicated by the accumulation data, although there is some ambiguity regarding the brain.

5.4.8 Conclusions

Although less acutely toxic than soluble Cu, Cu-NPs present a similar sublethal risk to rainbow trout. The effects observed here displayed the usual traits of traditional Cu toxicity, along with concerns regarding oxidative stress. There were no overt toxicological effects on haematology, though other blood constituents were not assessed (e.g., plasma proteins, lipids, and enzymes). These results are in agreement with other studies on the toxicity of waterborne NPs to fish where similar effects were seen without high levels of accumulation. As in traditional trace metal toxicology, the gills were the target organ here, but analogous to other

NP studies, the brain also appears to be a target organ following waterborne NP exposure, as does the gut, and as such both require further investigation. Although it is possible that ions dissolving from the metal-NPs in the water column and being taken up by the gills of the experimental fish could affect toxicity, the limited rate of dissolution seen over 12 h in separate experiments for the Cu-NPs used here suggests that this is relatively negligible. Further studies may be required to assess the possibility of the Cu-NPs releasing Cu ions within the gill microenvironment (thus acting as a delivery vehicle), though with current analytical techniques routinely employed in ecotoxicology, differentiation in this environment could be difficult.

Chapter 6. The acute toxicity of waterborne copper nanoparticles to early life stage zebrafish, (*Danio rerio*) and the influence of some abiotic factors.

Abstract

Embryonic and larval zebrafish (*Danio rerio*) were exposed in a series of experiments to either control, Cu as ($CuSO_4$) or Cu-NPs (99.9+ % purity; 87 ± 27 nm mean particle size). Acute static toxicity bioassays established 48 and 96 h-LC_{50} values in embryos (24 hpf) and larvae (< 4 hph) respectively. This was followed by experiments investigating the effects of abiotic factors on acute toxicity in static bioassays (Ca^{2+}, a Cu chelator (EDTA), humic acid and pH). Cu concentrations in water samples were determined by ICP-OES and were ± 10 % of nominal concentrations at all times. Dose-dependent mortality was seen in embryos and larvae exposed to both treatments and based on Cu concentrations, $CuSO_4$ was more acutely toxic to larvae than Cu-NPs. However, little difference was seen in the acute toxicity of $CuSO_4$ or Cu-NPs to embryos. Larvae were generally more sensitive to Cu-NPs than embryos (mean 96 h median lethal concentration (LC_{50}) values of 427.1 and 580.1 μg l^{-1} respectively). Larvae exposed to $CuSO_4$ in the presence of Ca^{2+} (0-80 mg l^{-1}) experienced reduced mortality, whilst Ca did not reduce Cu-NP toxicity, with a slight increase in acute Cu-NP toxicity with increasing Ca concentrations. A further Ca experiment was conducted with larvae exposed to either 100 μg l^{-1} $CuSO_4$ or 500 μg l^{-1} Cu-NPs in the presence of 0-2000 mg l^{-1} Ca^{2+}, with an overall trend of increased survival with increasing Ca^{2+}. Larvae exposed to 100 μg l^{-1} $CuSO_4$ or 1000 μg l^{-1} Cu-NPs with increasing EDTA concentrations (0-800 nM) showed a dose dependent decrease in acute toxicity in $CuSO_4$ exposed larvae, but not Cu-NPs. Humic acid (10 mg l^{-1}) was seen to elevate the LC_{50} values for both $CuSO_4$ and Cu-NPs (i.e., reducing acute toxicity), whilst altered pH had a similar, though more exaggerated, effect on Cu-NPs as did $CuSO_4$. These results show that both $CuSO_4$ and Cu-NPs are acutely toxic to early life stage fish and whilst some effects may be due to dissolved Cu ions there are also effects that seem to be attributable to the NPs themselves. Aggregation appears to play a key role in the observed effects, but further experiments are required to clarify this.

6.1 Introduction

Advancements in materials science and technology have led to increased production and use of engineered nanoparticles (NPs), with a predicted one trillion dollar industry by 2015 (Maynard, 2006; Meng et al., 2009b; Xia et al., 2009). The production and use of metallic or metal oxide nanomaterials and nanoparticles (nanometals) is a growing area of nanotechnology and there are concerns that these may be toxic to aquatic wildlife. One of these is nanosized copper which has uses in antimicrobial coatings, fuel cells, water electrolysis, air and water purification and biomedical imaging contrast agents (Griffitt et al., 2008). With the extensive research into the uptake and toxicity of trace metals in the aquatic environment several paradigms have emerged regarding the affects (often protective) that certain abiotic factors have on metal availability and toxicity (e.g., pH, alkalinity, water hardness, temperature, competing cations, dissolved organic matter, and suspended solids, review; Meyer et al., 2007). However, it remains to be seen if similar protective effects occur with nanometals. Furthermore, successful reproduction and subsequent uptake of young fish into wild stocks is critical for their ongoing survival and as a consequence it is essential to carry out early life stage (ELS) toxicity tests with NPs.

Experiments investigating the effects on fish at various life stages have highlighted differences in sensitivity to trace metals, with ELS fish often more sensitive than their older counterparts (e.g., Holcombe et al., 1979; Van der Merwe et al., 1993). Larval fish have often proven more sensitive to Cu than embryos (e.g., McKim et al., 1978; Scudder et al., 1988), most likely due to the protective nature of the chorion surrounding the embryos. Therefore it is prudent to carry out toxicity bioassays with the most sensitive life stage of the test organism (Hynes, 1960). Copper NPs were seen to be less toxic to adult zebrafish (*Danio*

rerio) than soluble Cu (Griffitt et al., 2007), though it remains to be seen if this is the case for ELS fish. However, to date few studies have assessed the effects of NPs to ELS fish. Griffitt et al. (2008) discovered that zebrafish larvae were slightly more sensitive to Cu-NPs than adults (48 h LC_{50} values of 0.71 and 0.94 mg l^{-1} respectively). Somewhat unusually in that study adult fish exposed to soluble Cu (as $CuCl_2$) were more sensitive than larvae (48 h LC_{50} values of 0.13 and 1.78 mg l^{-1} respectively), with larvae over 2.5-fold more sensitive to Cu-NPs than soluble Cu. Retarded hatching success in zebrafish embryos has also been seen following exposure to Cu-NPs, along with mortality in embryos exposed to concentration over 0.1 mg l^{-1} (Bai et al., 2010). Further studies have investigated the effects of other nanometals on fish. Asharani et al. (2008) exposed zebrafish embryos to Ag-NPs and saw concentration-dependent increases in mortality and delays in hatching. Zebrafish embryos experienced a concentration-dependent increase in mortality when exposed to Ag-NPs (Lee et al., 2007), whilst Yeo and Kang, (2008) saw significantly decreased hatching rates in zebrafish embryos exposed to Ag-NPs. Zinc-NPs imparted similar acute toxicity as bulk ZnO in zebrafish embryos (Zhu et al., 2008), along with decreased survival and reduced hatching rates. Ispas et al. (2009) found little difference in lethal toxicity between soluble Ni salts and Ni-NPs to zebrafish embryos, with the latter slightly less toxic, although dentric clusters (consisting of aggregated 60 nm particles with particle size distribution of 540 nm) of Ni-NPs were most acutely toxic of all. In a study exposing adult zebrafish for up to 13 weeks to TiO_2 NPs, Wang et al. (2011), observed impairment to reproduction and a significant reduction in the survival of embryos whose parents had been exposed to the NPs for 8 weeks or more.

The influence of abiotic factors on Cu toxicity in fish has long been recognised (e.g., water hardness; Shaw and Brown, 1974), with risk assessment often difficult due to the large and complex effects of water characteristics on metal toxicity (Erickson et al., 2008). Our

understanding of these complex interactions following many years of research has led to exposure prediction models such as the biotic ligand model (BLM, technical review; Di Toro, 2001) and it remains to be seen if these can be applied to Cu-NPs. Copper accumulation and subsequent toxic action depend on bioavailability (Campbell, 1995), which can mostly be explained by Cu speciation with the two often interlinked. For example, Cu speciation can be affected by pH, with the more toxic and bioavailable cupric species present at low (acidic) pH levels (e.g., Howarth and Sprague, 1978). Bioavailability can also be affected by the presence of divalent cations such as Ca^{2+}, which can out compete Cu for binding sites on the gills (Matsuo et al., 2005), with the propensity for reduced Cu toxicity with increasing water hardness not surprising (e.g., Perschbacher and Wurts, 1999). Elevated Na^{+}, as well as Ca^{2+}, was also protective to zebrafish exposed to low levels of soluble Cu (Craig et al., 2007). The complexation of Cu by various inorganic and organic ligands, such as natural organic matter (NOM), also serves to reduce toxicity in fish, presumably due to reduced bioavailability (Erickson et al., 2008). However, few studies to date have assessed the effects of abiotic factors on waterborne NP fate and behaviour or on toxicity to fish, rendering comparison with dissolved metals difficult. The size of NP aggregates and the rate at which they were formed has been observed to increase with increasing particle concentration (e.g., nanoscale zerovalent iron particles, Phenrat et al., 2007), but some abiotic factors may also influence this. Handy et al. (2008a) suggest that NPs with a metal ion functional surface should behave in the same way as dissolved metals, but factors such as elevated hardness, changes to pH, and ionic strength may alter NP aggregation, though with currently unknown consequences. Data regarding any effects upon toxicity is currently lacking and for future environmental studies, nanospecific reactions between dissolved organic matter and NPs need to be considered (Aiken et al., 2011), along with other abiotic factors such as pH, ionic strength, water hardness etc.

To the authors knowledge the effects of abiotic factors on the acute toxicity of waterborne Cu-NPs on zebrafish embryos and larvae has not been investigated, so the aim of the current study was firstly to assess the acute toxicity of Cu-NPs to zebrafish (*Danio rerio*) embryos and larvae (range finding experiments). Our knowledge of the effects of abiotic factors on the ecotoxicity of NPs is poor and requires work (Handy et al., 2008a), with NP interactions with natural water components (e.g., environmental colloids and natural organic matter) under a variety of physicochemical conditions crucial to our understanding of the fate and behaviour of NPs in aquatic systems (Christian et al., 2008). With this in mind the second phase of the current study aimed to investigate the influences of certain abiotic factors on the acute toxicity of Cu-NPs to larvae compared to a Cu salt. Early life stage fish are considered a fairly rapid, low-cost and comparable to screen pollutants (Woltering, 1984) and zebrafish were chosen due to their importance as a research model (Lawrence, 2007).

6.2 Methodology

6.2.1 Experimental animals

Breeding stocks of adult zebrafish were maintained at the University of Plymouth in 20 l aerated, flow-through glass aquaria containing dechlorinated Plymouth tap water (see below). Fish were held 28 °C with a photoperiod of 14 h light: 10 h dark (as was used under experimental conditions). Parent fish (> 3 months old), were fed *ad libitum* three times daily (twice on flake and once on *Artemia nauplii*). Male and female fish were kept in separate tanks until the night before breeding, at which point they were placed in an equal ratio of sexes into breeding aquaria. Following spawning at first light the eggs were collected and

placed into 400 ml beakers containing dechlorinated Plymouth tap water at 28 °C. Dead embryos and debris were periodically removed and viable embryos removed into beakers containing clean water. The profile of the water used in for routine fish maintenance and also for experiments was (mean data) 11.43, 1.40, and 17.47 mg l^{-1} (0.50, 0.04, and 0.44 mmol l^{-1}) for Na^{+}, K^{+} and Ca^{2+} respectively. Total hardness was 37.7 ± 1.0 mg $CaCO_3$ l^{-1}, whilst background Cu levels in the water were 5.72 ± 0.31 µg Cu l^{-1} (0.090 ± 0.005 µmol Cu l^{-1}; data mean ± SEM). A circumneutral pH and saturated oxygen above 85 % were maintained throughout, and total ammonia did not rise above 0.03 mg l^{-1} in experimental beakers. Temperature was maintained by ambient heating in a dedicated tropical aquarium.

Criteria for confirming mortality were as suggested by OECD, (1992). For embryos this is defined as a marked loss of translucency and change in colouration, caused by coagulation and/or precipitation of protein, leading to a white opaque appearance (particularly in the early stages), and an absence of body movement and/or absence of heart-beat in later stages. For larvae, one or a combination of the following were used to confirm death; immobility, absence of respiratory movement, absence of heart-beat, an opaque colouration of the central nervous system or a lack of reaction to mechanical stimulus. For the most part dead larvae would appear opaque and assume a curved position, however if there was some doubt (e.g., when there was no natural movement) mechanical stimulus would be used.

6.2.2 Copper nanoparticles

The Cu-NPs used here were from the same batch used in Chapter 5 and characterisation and stock solution data can be viewed there.

6.2.3 Confirmation of exposure

Exposure to $CuSO_4$ and Cu-NPs was confirmed throughout by inductively couple plasma optical emission spectrometry (ICP-OES) as described in Chapter 2. Note, as in Chapter 5 when concentrations of $CuSO_4$ are mentioned here on, it is referring to the actual Cu concentration in water (i.e., 100 $\mu g\ l^{-1}$ $CuSO_4$ is actually 100 $\mu g\ l^{-1}$ Cu), with the whole compound name used in order to make distinction between the two Cu forms and thus treatments easier. Analysed exposure concentrations of Cu, and Ca when required, never deviated more than ± 10 % of the nominal concentration and exact values are only reported where necessary due to the large volume of experiments conducted here. Total organic carbon (TOC) was assessed in experiments using humic acid. Briefly, acidified water samples were kept in the dark at 4 °C until required and then processed using a Shimadzu TOC 500A total organic carbon analyser. Automatic sparging was conducted as a pre-treatment within the instrument in order to remove inorganic carbon prior to analysis of TOC.

6.2.3 Acute toxicity bioassays

Embryos or larvae (n = 15-30) were randomly added to 400 ml Pyrex beakers (pre-washed in 3 % HNO_3) containing dechlorinated Plymouth tap water. Acute static non-renewal tests were conducted according to standard methods with mortality as the endpoint (LC_{50}). Embryos were exposed at the blastula stage (~2-4 h post-fertilisation (hpf)) to serial dilutions of either Cu (as $CuSO_4.5H_2O$) or Cu-NPs for 48 h. Larvae were similarly exposed, but for a period of 96 h. The duration of the exposures was chosen to approximately reflect the time before hatching in embryos (i.e., 48 h) and the time before first feed for larvae (i.e., 96 h), as well as enabling comparison to values in the literature. During this period the larvae

also do not require feeding as nutrition is delivered via the yolk-sac. Beakers were checked every 24 h and dead embryos or larvae were immediately removed. The concentration of Cu in acidified water samples (one drop of 6 M HNO_3) in ion clean tubes was determined for each treatment by ICP-OES every 24 h.

6.2.4 Acute toxicity bioassays with varying abiotic factors

For experiments investigating the influence of abiotic factors on acute toxicity, zebrafish larvae were exposed to Cu (as $CuSO_4$) or Cu-NPs in 96 h non-renewal tests a mortality endpoint as described above, but with the addition of various constituents to the water. Firstly, the affects of calcium (as $CaCl_2.2H_2O$, Sigma Aldrich) were assessed whereby larvae were exposed to serial dilutions of $CuSO_4$ or Cu-NPs in the presence of 0 (no added Ca), 40, or 80 mg l^{-1} Ca^{2+}. Following the results of this bioassay a further experiment was conducted whereby fish were exposed to 500 µg l^{-1} Cu-NPs in the presence of an extended concentration of Ca^{2+} (0-2000 mg l^{-1}). In all Ca experiments, as well as no added Cu controls, Ca controls were also established, whereby Ca was added at the concentrations used in Cu experiments to ensure there were no Ca affects. Finally, a lethal-time experiment was conducted where the time taken for 50 % of the test larvae to die whilst exposed to 350 µg l^{-1} Cu-NPs in the presence of 0, 40, or 80 mg l^{-1} Ca^{2+} was assessed.

In order to explore whether free Cu (dissolved from the Cu-NPs) was causing acute toxicity, larvae were exposed to $CuSO_4$ or Cu-NPs in the presence of a known Cu chelator; ethylenediaminetetraacetic acid (EDTA, ACS reagent quality, 99.4-100.6 %, Sigma Aldrich). A concentration of $CuSO_4$ at 100 µg l^{-1} and Cu-NPs at 1000 µg l^{-1} were chosen for the exposure as these had been seen to cause ≥ 50 % mortality in acute toxicity tests. Larvae were added to experimental beakers (n = 30 per beaker) and acclimated for 6 h. Then, following

the addition of either 100 μg l^{-1} $CuSO_4$ or 1000 μg l^{-1} Cu-NPs to the appropriate experimental beakers, 0-800 nmol l^{-1} EDTA was added from either a 1 or 100 μmol l^{-1} stock (in pure, Milli-Q, water) and survival assessed over 96 h. EDTA controls were employed whereby larvae were exposed in normal tank water with added EDTA at the concentrations used in the experiments.

Further experiments assessed the possible protective effects of humic acid (representative of natural organic matter) on the acute toxicity of $CuSO_4$ and Cu-NPs. A 100 mg l^{-1} stock solution of humic acid was prepared by adding the appropriate mass of Aldrich humic acid to ultrapure (Milli-Q) water and stirring for 6 hours (magnetic stirrer IKA Werke RET basic C, at 150 rpm). Then 40 ml of stock was added to a series of 500 ml beakers and dechlorinated Plymouth tap water added to achieve a final volume of 400 ml. Zebrafish larvae were added (n = 15 per beaker) and acclimated for 24 h. Larvae were then exposed to serial dilutions of $CuSO_4$ or Cu-NPs in the presence of 0 or 10 mg l^{-1} humic acid and survival assessed over 96 h. Controls exposing larvae to humic acid only were also utilised. The pH of test solutions containing humic acid remained between 6.7-6.9 throughout.

The effects of pH on acute toxicity of both Cu treatments to zebrafish larvae was then assessed with larvae exposed in triplicate to either 100 μg l^{-1} $CuSO_4$ or 500 μg l^{-1} Cu-NPs at either pH 5, 7, or 9. The adjustment of pH in the experimental beakers was carried out prior to the addition of larvae. Sulphuric acid (Sigma-Aldrich) was used to adjust to pH 5, whilst sodium hydroxide (Sigma-Aldrich) was used to adjust to pH 7 and 9. Larvae were allowed to acclimate for 24 h in experimental conditions (without added Cu) prior to commencement of the experiments, during which time no mortalities occurred. As pH did not drift over 96 h there was no need to readjust pH in any treatments.

6.2.5 Statistics

The medium lethal concentration values (i.e., to kill 50 % of the population) were calculated by the trimmed Spearman-Karber method with 95 % confidence intervals as described in Chapter 2. Where possible a sigmoidal curve was fitted to dose-response curves, and where this was not appropriate linear regression was used. Correlation coefficients were calculated by the Pearson Product Moment Correlation.

6.3 Results

6.3.1 Acute toxicology

Zebrafish embryos and larvae were exposed to either $CuSO_4$ or Cu-NPs in a series of 48 or 96 h acute toxicity tests (LC_{50}). No significant differences were seen between the acute toxicity of $CuSO_4$ and Cu-NPs following exposure to zebrafish embryos (Fig. 6.1) with similar LC_{50} values (Table 6.1). However, significant differences between treatments were seen in larval lethal toxicity bioassays with $CuSO_4$ proving more acutely toxic than Cu-NPs (*Fig. 6.1, Table 6.1*). *Notably, repetition of experiments under identical conditions revealed* that there was some statistically significant differences in some larvae Cu-NP LC_{50} values (Table 6.1), though Cu-NPs were not more acutely toxic than $CuSO_4$. For ethical reasons, following two identical experiments to determine the $CuSO_4$ LC_{50} for larvae (alongside that of Cu-NPs), a further two acute toxicity experiments with Cu-NPs (not including those exploring the influence of abiotic factors) did not require concomitant $CuSO_4$ treatments, though control fish with no added Cu were still utilised. Three of the four Cu-NP larval exposure tests revealed similar LC_{50} values, whilst the other produced and LC_{50} values over

2-fold higher (Table 6.1). Notably, no differences were seen in LC_{50} values from embryo toxicity tests, with the two separate embryo exposures conducted returning very similar results (LC_{50} values of 662.77 and 638.88 $\mu g\ l^{-1}$ respectively; note only one of these is presented in Fig. 6.1). Correlation coefficients and resulting *P* values showed a positive correlation between concentration and mortality in all acute toxicity tests with both $CuSO_4$ and Cu-NPs (Table 6.1).

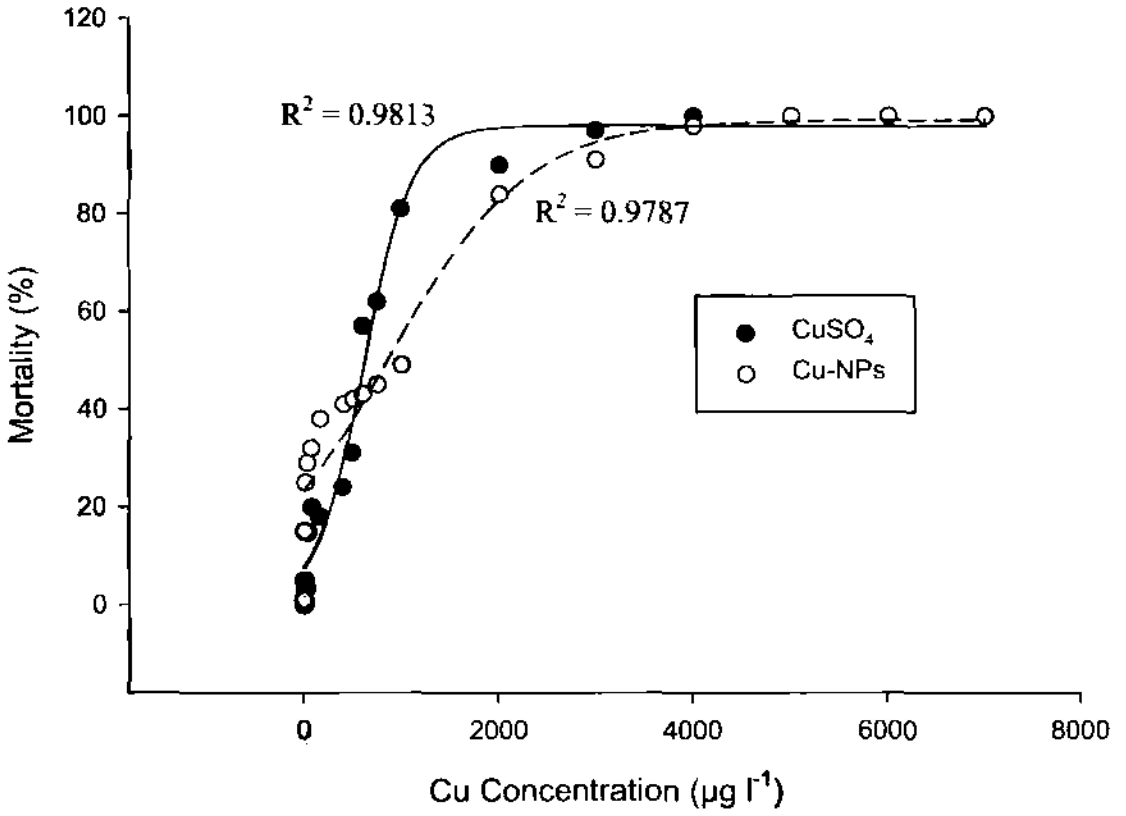


Fig. 6.1. Dose-response curves for zebrafish embryos exposed to either $CuSO_4$ or Cu-NPs for 48 h. LC_{50} and 95 % confidence level values can be found in Table 6.1. Data are raw values (no replication of each concentration). Sigmoidal curves are fitted to both data sets.

Table 6.1. Zebrafish embryo (48 h) and larvae (96 h) LC_{50} values and correlation coefficients following acute toxicity bioassays.

Life stage	Cu Form	LC_{50} ($\mu g\ l^{-1}$)	Lower confidence limit (%)	Upper confidence limit (%)	Correlation coefficient	Correlation coefficient (*P* value)	Figure (panel)
Embryonic	$CuSO_4$	526.72	433.63	639.78	0.832	0.0000351	6.1
Embryonic	$CuSO_4$	553.45	528.90	569.06	0.877	0.00054	-
Embryonic	Cu-NPs	582.56	527.53	639.78	0.901	0.000000786	6.1
Embryonic	Cu-NPs	577.63	554.32	603.66	0.876	0.0000641	-
Larval	$CuSO_4$	89.76[a]	82.50	97.66	0.632	0.0930	6.2 (a)
Larval	$CuSO_4$	100.60[a]	93.91	110.94	0.870	0.000492	-
Larval	Cu-NPs	783.61[b]	688.64	891.69	0.992	0.00000122	6.2 (a)
Larval	Cu-NPs	326.87[c]	298.40	358.05	0.792	0.0190	6.2 (b)
Larval	Cu-NPs	327.20[c]	297.99	359.27	0.980	0.000000658	6.2 (c)
Larval	Cu-NPs	270.85[c]	158.98	461.44	0.893	0.0167	6.2 (d)

Dashed line (-) = not displayed in a graph; Different letters donate significant differences between LC_{50} values for larvae (no significant differences seen in embryo values). Correlation coefficients are for toxicity data (i.e., not for the goodness of curve fit).

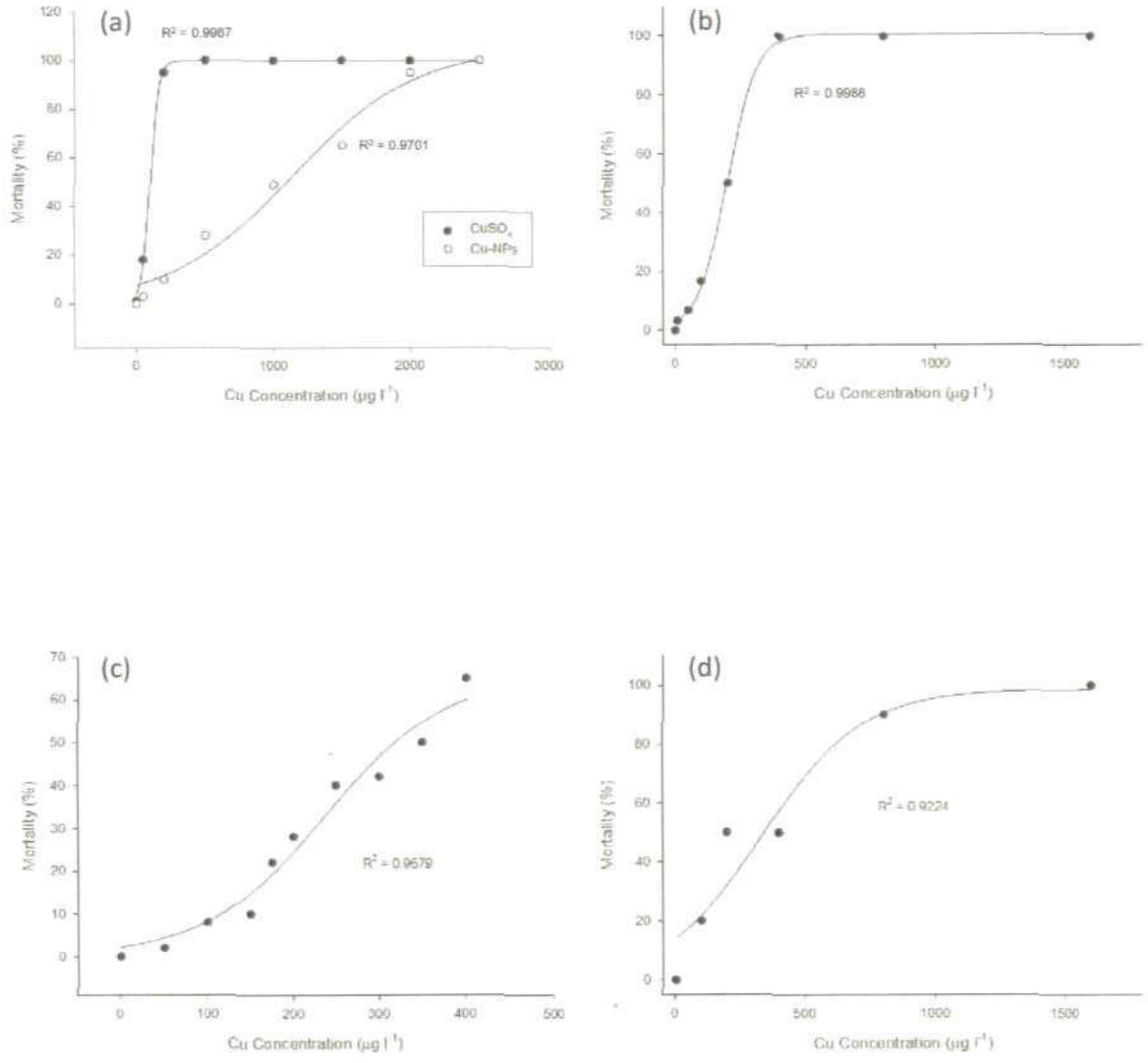


Fig. 6.2. Dose-response curves for zebrafish larvae exposed to either $CuSO_4$ or Cu-NPs for 96 h. Note only panel (a) has a dose-response for $CuSO_4$ compared to Cu-NPs, whilst panels (b, c, and d) display Cu-NP data only. LC_{50} and 95 % confidence level values can be found in Table 6.1. Data are raw values (no replication of each concentration). Sigmoidal curves are fitted throughout.

6.3.2. The influence of abiotic factors acute toxicology

6.3.2.1 Effects of Ca^{2+} on $CuSO_4$ and Cu-NP toxicity

During experiments investigating the effects of elevated aqueous Ca^{2+} on Cu toxicity, no mortalities occurred in Ca^{2+} control treatments (elevated Ca^{2+} at the levels used in experiments, but with no added Cu, data not shown). Background Ca^{2+} levels were (data mean ± SEM, $n = 6$ samples) 17.7 ± 0.18 mg l^{-1} and where a Ca^{2+} concentration is mentioned hereafter the actual value would be this naturally occurring background level. Larvae exposed to $CuSO_4$ in the presence of 40 or 80 mg l^{-1} Ca^{2+} experienced a Ca-dependent reduction in toxicity with LC_{50} values increasing with increasing Ca^{2+} (Fig. 6.3 and Table 6.2). This relationship was confirmed with positive correlation coefficients and P values less than 0.05. However, Cu-NPs displayed an inverse relationship, whereby larvae experienced increased mortality with increasing Ca^{2+} (Fig. 6.3). This is evident in the medium lethal concentration data obtained following exposure to a serial dilution of Cu-NPs in the presence of Ca^{2+} (Fig. 6.3b), with LC_{50} values of 309.04, 223.78, and 189.27 µg l^{-1} at Ca^{2+} levels of 0, 40, or 80 mg l^{-1} Ca^{2+} respectively (Table 6.2). Although the confidence intervals of the latter two LC_{50} values do have some crossover, the first two do not (Table 6.2), and it is likely there are statistically significant differences between the three LC_{50} values. In order to further investigate the effects of Ca, an experiment was conducted whereby larvae were exposed to either 100 µg l^{-1} $CuSO_4$ or 500 µg l^{-1} Cu-NPs (with no added Cu controls) in the presence of 0-2000 mg l^{-1} Ca^{2+}. Here, survival was plotted (Fig. 6.4), and although there is an overall trend of increased survival with increasing Ca^{2+} (positively correlated), the relationship is not significant ($P = 0.107$; Table 6.).

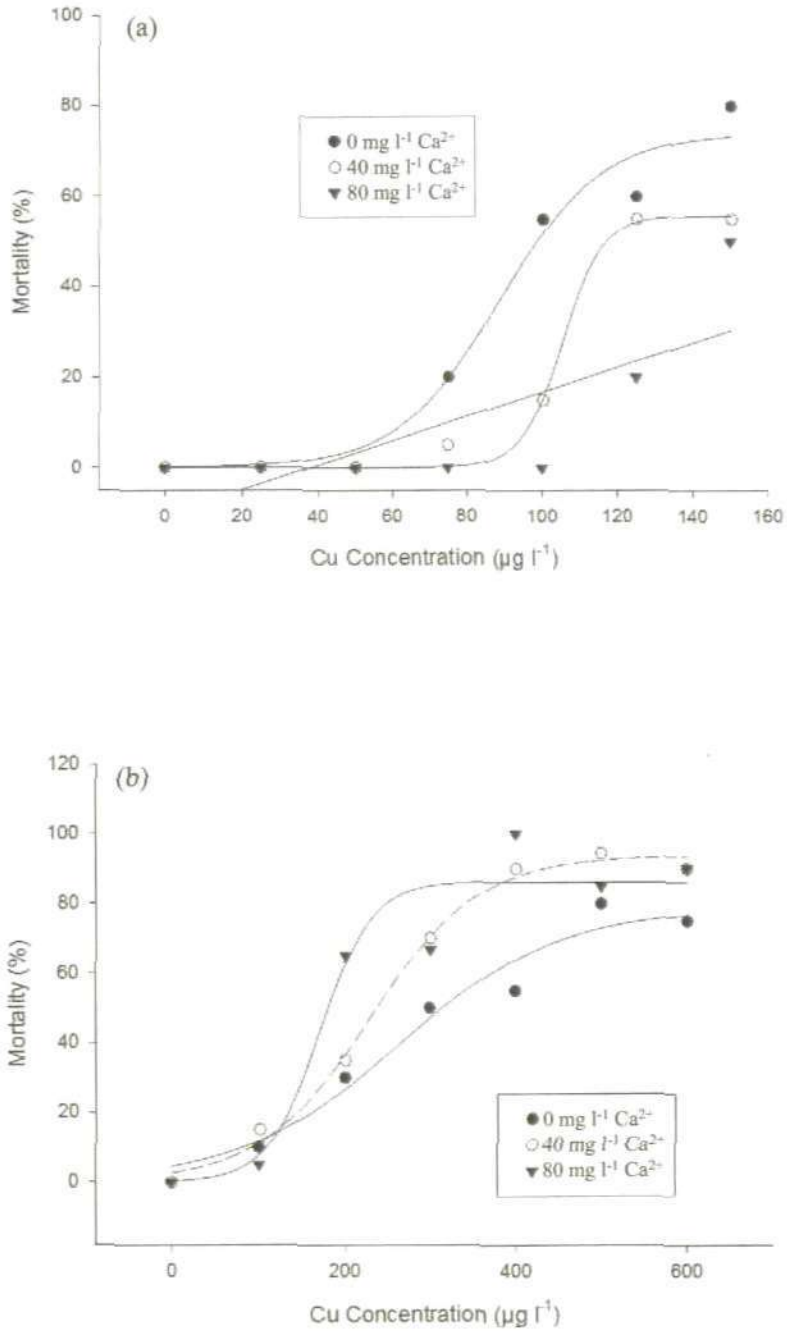


Fig. 6.3. The influence of Ca^{2+} at two different concentrations (and no added Ca control) on acute toxicity of serial dilutions of (a) $CuSO_4$ and (b) Cu-NPs to zebrafish larvae at 96 h. Data are raw values (no replication of each concentration). LC_{50} and 95 % confidence level values can be found in Table 6.2. Sigmoidal curves are fitted throughout both panels apart from the 80 mg Ca^{2+} l^{-1} treatment in panel (a) where mortality was too low, and so a linear curve has been fitted.

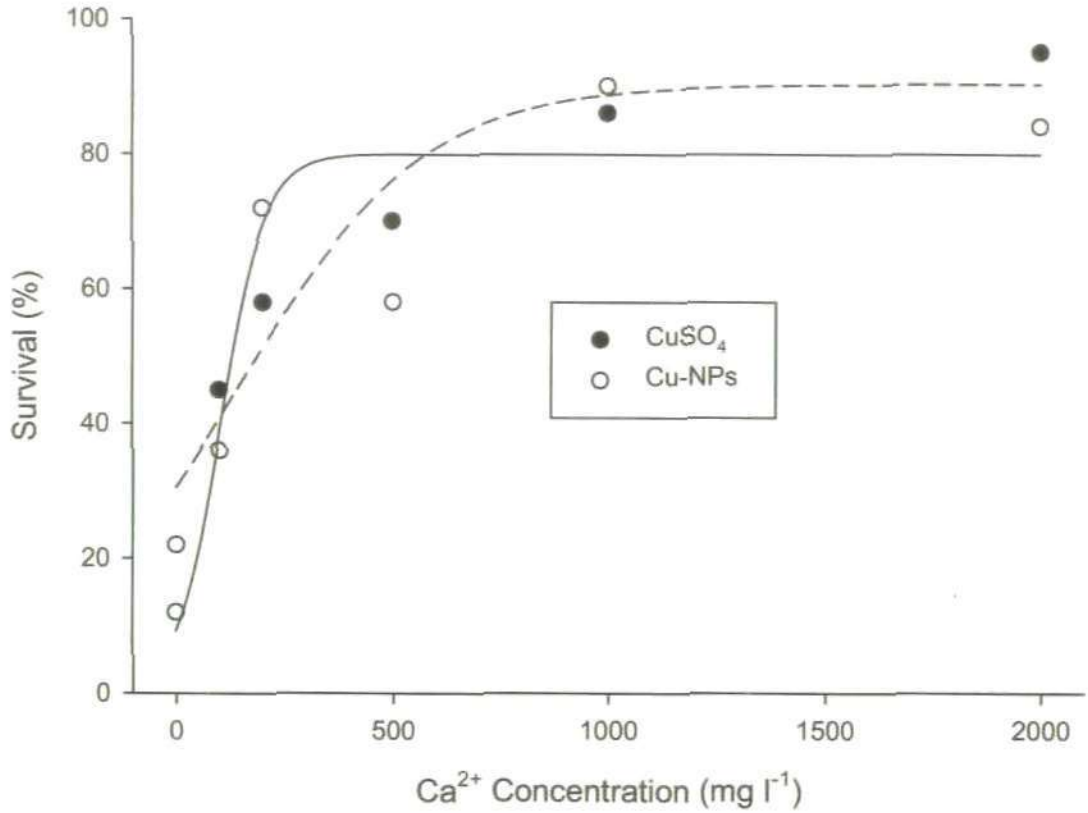


Fig. 6.4. The influence of 0-2000 mg l^{-1} Ca^{2+} on the acute toxicity of 100 µg l^{-1} $CuSO_4$ and 500 µg l^{-1} Cu-NPs to zebrafish larvae at 96 h. Dashed line curve = Cu-NPs, unbroken line curve = $CuSO_4$. Data are raw values (no replication of each concentration).

Table 6.2. The influence of 0-80 mg l^{-1} Ca^{2+} on acute toxicity of $CuSO_4$ or Cu-NPs to larvae

Cu form	Ca^{2+} (mg l^{-1})	LC_{50} (µg l^{-1})	Lower confidence limit (%)	Upper confidence limit (%)	Correlation coefficient	Correlation coefficient (*P* value)	Figure (panel)
$CuSO_4$	0	103.06	97.22	109.26	0.950	0.00107	6.3 (a)
$CuSO_4$	40	146.62	140.89	152.59	0.879	0.00912	6.3 (a)
$CuSO_4$	80	150	*	*	0.766	0.0449	6.3 (a)
Cu-NPs	0	309.04	278.03	343.50	0.977	0.000155	6.3 (b)
Cu-NPs	40	223.78	203.68	245.86	0.951	0.000986	6.3 (b)
Cu-NPs	80	189.27	175.37	204.28	0.887	0.00771	6.3 (b)

Correlation coefficients are for toxicity data (i.e., not for the goodness of curve fit). * Could not be calculated due to a lack of reliability as 50 % mortality was reached at the highest $CuSO_4$ concentration (150 µg l^{-1}) only in the presence of 80 mg l^{-1} treatment.

Table 6.3. The relationship between exposure to 100 or 500 µg l^{-1} $CuSO_4$ or Cu-NPs *respectively in the presence of 0-2000 mg* l^{-1} Ca^{2+}

Cu form	Cu (µg l^{-1})	Correlation coefficient	Correlation coefficient (*P* value)	Figure (panel)
$CuSO_4$	100	0.868	0.0250	6.4
Cu-NPs	500	0.720	0.107	6.4

Correlation coefficients are for toxicity data (i.e., not for the goodness of curve fit).

6.3.2.2 Effects of EDTA on $CuSO_4$ and Cu-NP toxicity

The effects of a Cu chelator were then assessed, with larvae exposed to 100 or 1000 μg l^{-1} $CuSO_4$ or Cu-NPs respectively, in water containing EDTA (0-800 nmol l^{-1}). Survival increased in both treatments with increasing EDTA (Fig. 6.5), though there was only a significant correlation in $CuSO_4$ exposed fish (Table 6.3), with larvae exposed to Cu-NPs and EDTA showing a positive correlation, but insignificant *P* value (i.e., correlation coefficient of 0.443, $P = 0.319$, Table 6.3).

6.3.2.3 Effects of humic acid on $CuSO_4$ and Cu-NP toxicity

Larvae exposed to both $CuSO_4$ and Cu-NPs in water containing 10 mg l^{-1} humic acid (Fig. 6.6) experienced elevated LC_{50} values (Table 6.3) compared to acute toxicity tests without the addition of natural organic matter (Table 6.1). Notably, this was far more pronounced in larvae exposed to Cu-NPs (LC_{50} of 1131.37 μg l^{-1}), with increases between 1.4-4.2-fold compared to larvae exposed to Cu-NPs without humic acid (Table 6.1). Analysis of water samples containing humic acid showed the TOC concentration to be 9.1 ± 0.3 mg C l^{-1} (mean ± SEM, $n = 3$).

6.3.2.4 Effects of pH on $CuSO_4$ and Cu-NP toxicity

Larvae exposed to 100 μg l^{-1} $CuSO_4$ at varying pH in triplicate showed increased survival with increasing pH (80 % mortality at pH 5, compared to 40 % mortality at pH 9, Fig. 6.7). A strong relationship between survival in these larvae and pH was observed with a correlation coefficient of 1 and *P* value of 0.0 (Table 6.4). Cu-NP exposed fish also

experienced increased survival with increasing pH (Fig. 6.7), though the correlation was not as strong as with the $CuSO_4$ treatments ($P = 0.0913$, Table 6.4). Notably mortality was higher at low pH in larvae exposed to Cu-NPs compared to $CuSO_4$, whilst more survived at high pH. No larvae died in control beakers with no added $CuSO_4$ or Cu-NPs at any pH assessed (i.e., pH 5, 7, or 9).

Table 6.4. The influence of EDTA, humic acid, or pH on the toxicity of $CuSO_4$ or Cu-NPs to larvae.

Cu form	Cu ($\mu g\ l^{-1}$)	Abiotic factor	LC_{50} ($\mu g\ l^{-1}$)	Lower confidence limit (%)	Upper confidence limit (%)	Correlation coefficient	Correlation coefficient (*P* value)	Figure (panel)
$CuSO_4$	100	EDTA	-	-	-	0.868	0.0250	6.5
Cu-NPs	1000	EDTA	-	-	-	0.720	0.107	6.5
$CuSO_4$	0-320	HA	142.54[a]	120.28	168.92	0.987	0.000267	6.6 (a)
$CuSO_4$	0-320	No HA	70.26[#]	56.56	87.27	0.890	0.0176	6.6 (a)
Cu-NPs	0-1600	HA	1131.37[b]	889.87	1438.42	0.978	0.000751	6.6 (b)
Cu-NPs	0-1600	No HA	300.42[#]	254.06	355.24	0.910	0.0117	6.6 (b)
Control	0	pH (5-9)	-	-	-	*	*	6.7
$CuSO_4$	100	pH (5-9)	-	-	-	1.000	0.0	6.7
Cu-NPs	500	pH (5-9)	-	-	-	0.990	0.0913	6.7

HA = humic acid. Dash (-) = not applicable as Cu dose-response not assessed. Different letters donates significant difference in LC_{50} values between $CuSO_4$ and Cu-NPs within that experiment. [#] = significant difference between $CuSO_4$ or Cu-NPs exposed larvae with no added HA compared to those exposed in the presence of 10 mg l^{-1} HA. * = data values were all equal and therefore correlation was not useful. Correlation coefficients are for toxicity data (i.e., not for the goodness of curve fit).

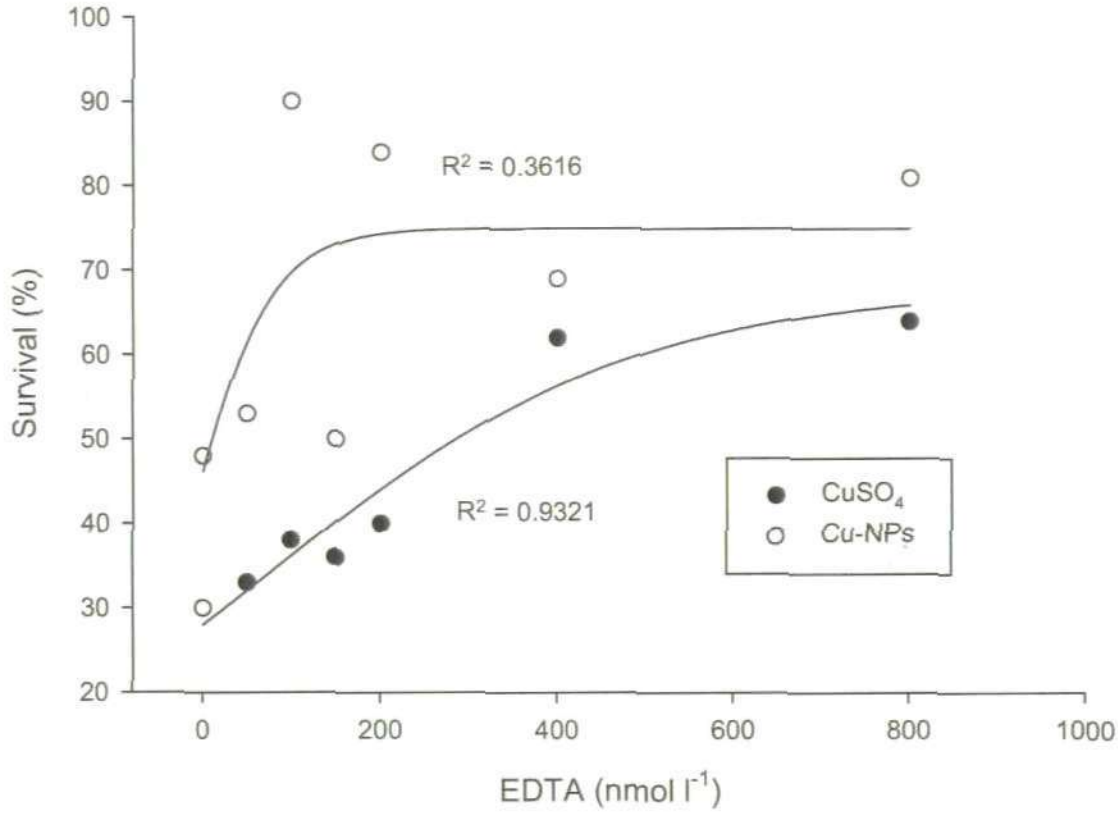
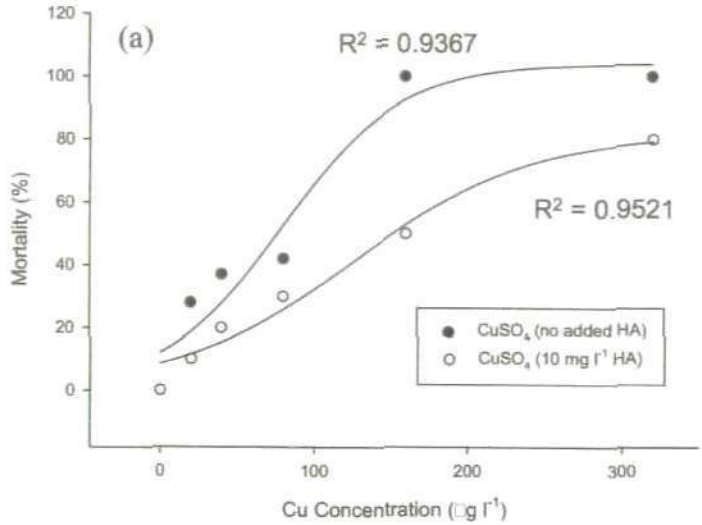
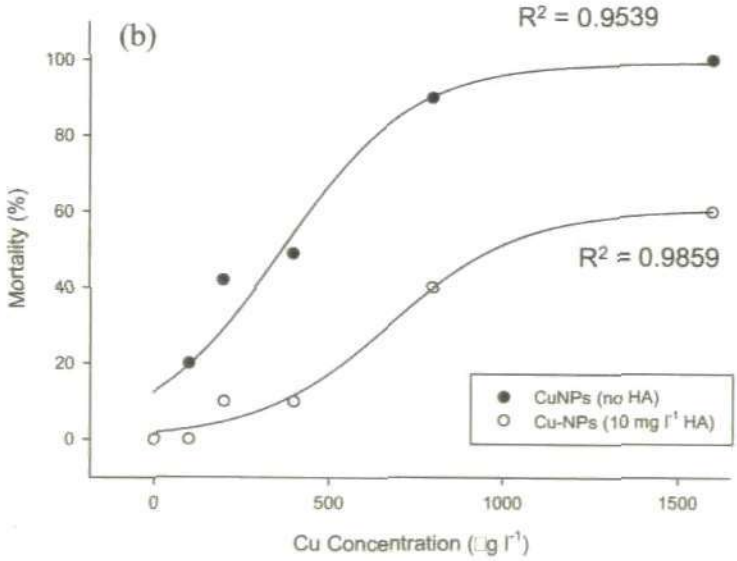


Fig. 6.5. The influence of 0-800 nmol l^{-1} EDTA on the acute toxicity of 100 µg l^{-1} $CuSO_4$ and 1000 µg l^{-1} Cu-NPs to zebrafish larvae at 96 h. Data are raw values (no replication of each concentration). Sigmoidal curves are fitted to both data sets.


(a)
R² = 0.9367
R² = 0.9521
Mortality (%)
Cu Concentration (□g l⁻¹)
CuSO4 (no added HA)
CuSO4 (10 mg l⁻¹ HA)
0
20
40
60
80
100
120
0
100
200
300


(b)
R² = 0.9539
R² = 0.9859
Mortality (%)
Cu Concentration (□g l⁻¹)
CuNPs (no HA)
Cu-NPs (10 mg l⁻¹ HA)
0
20
40
60
80
100
0
500
1000
1500

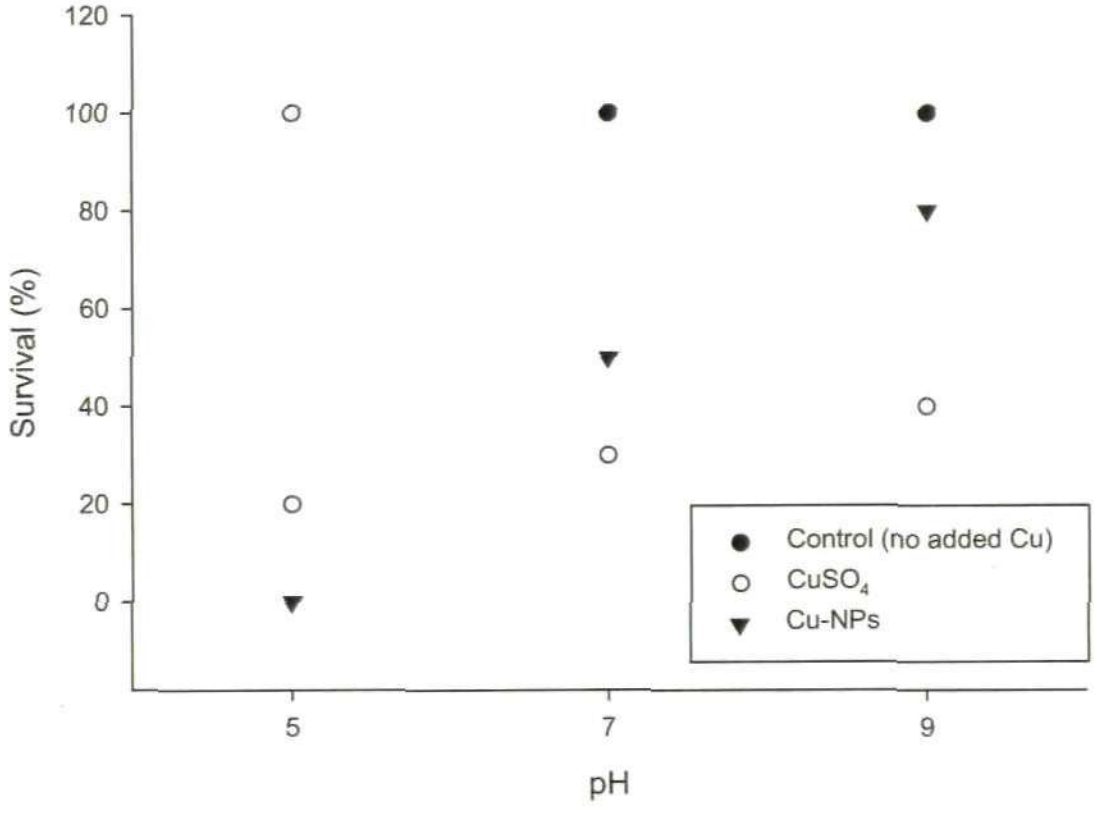


Fig. 6.7. The effects of pH on the toxicity to larvae of 100 $CuSO_4$ or 500 µg l^{-1} Cu-NPs respectively. Control fish were exposed to varying levels of pH, but with no added Cu. Data are mean of three replicates per treatment. As there no significant differences between triplicates of each treatment (Students *t*-test, $P > 0.05$)) error bars have been omitted for clarity. No curves were fitted as $n = 3$ pH data points per treatment only.

6.4 Discussion

Due to the importance of ELS fish for the long term survival of stocks (including commercially important species), it is imperative that toxicity tests with new contaminants are carried out using these organisms, particularly as pollution avoidance may be more difficult in the organisms. In the current study, the effects of exposure to Cu-NPs (mortality end point) on the early life stages of zebrafish (*Danio rerio*) were examined along with the effects of $CuSO_4$ to aid a comparison. Cu-NPs were acutely toxic to both larvae and embryos though Cu-NPs were less toxic to larvae than the $CuSO_4$, but equally as toxic as the Cu salt to embryos. There were some notable influences of abiotic factors on Cu-NP toxicity, at times more pronounced than with $CuSO_4$. Copper levels from both the $CuSO_4$ and Cu-NP treatments remained within ± 10 % of the nominal exposure concentration throughout which falls within the 20 % recommend in OECD guidelines (OECD, 1992). Notably, separate experiments using the bulk equilibrium reverse dialysis bag technique showed that the rate of dissolution of Cu ions from the Cu-NPs over 24 h was 10.1 %, at which point dissolution was seen to level off (Boden, 2011, personal communication).

6.4.1 Acute toxicity

Both zebrafish larvae and embryos died following exposure to $CuSO_4$ and Cu-NPs, with larvae generally more sensitive to Cu-NPs than embryos. This phenomenon has been observed in numerous trace metal studies, suggesting that embryos are less sensitive than larval fish (e.g., Eaton et al., 1978; McKim et al., 1978). During the current study there was not a significant difference in the acute toxicity of Cu-NPs to embryos compared to $CuSO_4$ (Fig. 6.1 and Table 6.1). The LC_{50} values for both treatments seen here (between 526-582 µg l^{-1}, Table 6.1) are broadly similar to for fish embryos exposed to soluble Cu in the literature

(e.g., 400 μg l^{-1} 96 h LC_{50}, Giles and Klaverkamp; 1982; 327 μg l^{-1} 24 h LC_{50}, Chan et al., 2006). It is unlikely that Cu ions dissolved from the Cu-NPs contributed significantly to acute toxicity in embryos here, as separate studies showed a dissolution of 10.1 % of total Cu after 24 h (unlikely to rise significantly after this time). This represents a relatively low concentration compared to that of $CuSO_4$ required to kill 50 % of the population (i.e., ~ 60 μg l^{-1} free Cu present in Cu-NP treatments compared to LC_{50} values of approximately 500 μg l^{-1} in $CuSO_4$ only treatments). In one of the few studies exposing zebrafish embryos to Cu-NPs, (Bai et al., 2010) observed 100 % mortality in embryos exposed to 1000 and 500 μg l^{-1} Cu-NPs at 8 and 24 hpf respectively. In that study exposure to 300 and 600 μg l^{-1} Cu (as $CuSO_4$) also resulted in 100 % mortality at the same time points. Similar effects have been seen with other nano-metals when compared to their bulk counterparts. For example, zebrafish embryos exposed to ZnO-NPs experienced similar effects to embryos exposed to bulk ZnO with LD_{50} concentrations of 1.793 and 1.550 mg l^{-1} respectively (Zhu et al., 2008). In another study exposing zebrafish embryos to ZnO-NPs and bulk ZnO it was discovered that the NPs exerted far more toxic effects than the bulk powder, with hatching rates and development end points (Zhu et al., 2009). Though many studies have not reported uptake of NPs in to fish embryos it is possible that the barrier and subsequent protection afforded by the chorion is susceptible to NP exposure. This has been demonstrated by Lee et al. (2007) who developed a method for imaging single Ag-NPs (5–46 nm) through zebrafish embryos. In their study, the transport of Ag-NPs in and out of the developing embryos was observed, with movement through chorion pore canals by diffusion. The adsorption of fluorescent NPs onto the chorion has also been observed (latex NPs, Kashiwada, 2006; silica NPs, Fent at al., 2010), with larvae hatched from exposed embryos in the study by Kashiwada (2006) exhibiting highly concentrated fluorescent NPs in the yolk and gallbladder.

The 96 h LC_{50} values for $CuSO_4$ exposure to larvae seen here (Table. 6.1) fall within the range of values reported for a range of different ELS fish species in the literature (e.g., 5-1780 $\mu g\ l^{-1}$, Scudder et al., 1988; Hamilton and Buhl, 1990; Welsh et al., 1996; Shyong and Chen, 2000; Griffitt et al. 2008). As with embryos it is unlikely that free Cu dissolved from the NPs played a significant role in acute toxicity to larvae. In one of the few studies exposing ELS zebrafish to Cu-NPs, Griffitt et al. (2008) observed a 48 h LC_{50} of 710 $\mu g\ l^{-1}$ compared to 1780 $\mu g\ l^{-1}$ for Cu exposed fish. However, this increased sensitivity to Cu-NPs compared to soluble Cu was not seen in the current study with $CuSO_4$ more than three times toxic to larvae than Cu-NPs (Table 6.1). However, the considerably higher Cu-NP LC_{50} values reported by Griffitt et al. (2007, 2008; all life stages of zebrafish) were from tests conducted in hard water and at a pH in the alkaline range, compared to the moderately soft and slightly less than pH 7 water used here. However, some notable variations were seen in the acute toxicity of Cu-NPs in the present study (Figs. 6.2 and 6.8 (whole stock data) with differences in acute toxicity possibly due to the difficulty in maintaining reproducible solutions (Handy et al., 2008a). It is possible that this was the case here, though every attempt was made to ensure there were no differences between Cu-NP stock preparation and dosing between experiments. Differences could also occur between different batches of larvae or if the dissolution rate of Cu ions from the NPs changed between experiments. A study by Heinann et al. (2008) showed that the toxicity of CuO-NPs to bacteria (*Vibrio fischeri*) and an anostracan crustacean (*Thamnocephalus platyurus*) was largely attributable to Cu^{2+} dissolved from the NPs, although Griffitt et al. (2007) say that only a small fraction of Cu-NP toxicity to zebrafish was due to Cu^{2+}.

6.4.1 Acute toxicity with varying abiotic factors

The influence of abiotic factors on the bioavailability and toxicity of Cu has been recognised for many years, with the substantial amount of research carried out culminating in exposure prediction models such as the BLM. The advent of metal NPs and their likely release into the environment has raised the question as to whether these new materials are subject to similar effects as their bulk counterparts and how this affects toxicity. Several paradigms have been established with dissolved metals such as reduced bioavailability and toxicity with differing abiotic factors and the effects of $CuSO_4$ on larvae seen here reflect that described in the literature (e.g., Ca^{2+} effects, Erickson et al., 1996; dissolved organic matter, Brown et al., 1974).

6.4.1.1 The effects of elevated Ca^{2+} on toxicity

Increased water hardness has long been associated with decreased Cu bioavailability and subsequent toxicity, with Ca^{2+} and Mg^{2+} offering protection by competing with Cu^{2+} for binding sites on the gills (Meyer et al., 2007). Hardness attributed to Ca^{2+} has been seen to attenuate metal uptake and toxicity more than Mg^{2+} (Meyer et al., 2007 and references therein) and as such was used in the present study to assess the effects on Cu-NP toxicity. In the first calcium experiment (Fig. 6.3), increased Ca^{2+} elevated the $CuSO_4$ LC_{50} to larvae (*i.e., reducing acute toxicity*) with statistically significant correlations ($P < 0.05$, Table 6.2). The three concentrations of Ca^{2+} used here (0, measured as 17 mg l^{-1} (background levels; no further addition of Ca^{2+}), 40 and 80 mg l^{-1}) represents three different levels of water hardness (as $CaCO_3$); moderately soft, slightly hard, and hard. Larvae exposed to 0-150 µg l^{-1} $CuSO_4$ and 80 mg l^{-1} Ca^{2+} did not even register a 96 h LC_{50} (Fig. 6.3). However, larvae exposed to

Cu-NPs at the same three concentrations of Ca^{2+} experienced a reversal of effects with mortality inversely related to Ca^{2+} concentration (Fig. 6.3). Exposure to increasing concentrations of Cu-NPs caused significantly correlated increases in mortality at each level of Ca^{2+}, resulting in decreased LC_{50} values (i.e., increased acute toxicity, Table 6.2). It is unlikely that dissolved Cu from the NPs contributed greatly to toxicity as firstly levels would have been relatively low, at least in larvae exposed to ≤ 400 µg l^{-1}, and also the elevated Ca^{2+} would presumably have been sufficient to counteract free Cu at these concentrations. In a second experiment to assess the effects of increasing Ca^{2+} levels (0-2000 mg l^{-1}) on the acute toxicity of 100 or 500 µg l^{-1} $CuSO_4$ or Cu-NPs respectively, both treatments saw an increase in survival with increased Ca^{2+} which was significant in the former, but not for Cu-NPs (Table 6.3). A transient decrease in survival was seen in larvae exposed to Cu-NPs, but the overall trend was for a Ca^{2+} dependent increase in survival (Fig. 6.4). It is quite possible that dissolved Cu could have partially contributed to mortality here, with 10 % of the 500 µg l^{-1} Cu-NPs approximately representing the 96 h LC_{25} for larvae exposed to $CuSO_4$ in the current study. However, if this was the only source of toxicity then one would expect mortality in the Cu-NP treatment to be approximately half that seen in the $CuSO_4$ treatment, but this was clearly not the case (Fig. 6.4). Handy et al. (2008b) state that some NPs will aggregate in the presence of Ca^{2+} and in high ionic strength seawater, as was seen with TiO_2 NPs and Ag-NPs (Mukherjee and Weaver, 2010). The high levels of Ca^{2+}used in the second Ca^{2+} experiment here reached that typically found in seawater (e.g., > 400 mg l^{-1}), and it is likely that the Cu-NPs aggregated with possible affects on toxicity. Handy et al. (2008a) argue that elevated water hardness may increase NP aggregation due to specific sorption and/or compression of the electrical double layer (EDL). The research conducted so far suggest that NPs follow the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory of colloidal stability in that, as electrolyte concentrations increase, the attachment efficiency increases due to increased

screening of particle surface charge, thus reducing the energy barrier to aggregation (Chen et al., 2007; Gao et al., 2009). Also, aggregation may induce changes to particle size and shape and that these changes could themselves result in transport potential, as well as NP reactivity and toxicity (Klaine et al., 2008). In the absence of stabilising organic matter, aggregated NPs will most likely fall out of suspension (Handy et al, 2008a,b), with the tendency of NPs to aggregate previously shown to be inversely proportional to their stability (Mackay et al., 2006). As such aggregates of NPs could pose less of a (or at least a different) toxic risk to pelagic species. However, after hatching larvae will adhere to hard surfaces (Laale, 1977), most likely at the bottom of beakers/tanks, before inflating their gas bladder over the following days during which time they are restricted in their swimming capabilities (Lawrence, 2007). Therefore at the early stages of larval development these animals could be at particular risk to aggregated and sedimented NPs. Furthermore the differences seen in response to Cu-NPs with different batches of larvae could be due to differences in larvae quality and time to develop, with larval growth varying widely (Lawrence, 2007). For instance, poor quality larvae that take longer to inflate their swim bladder and begin swimming may be more susceptible to NP aggregates sedimented on the base of the beaker or tank compared to those who develop more quickly and can swim away.

6.4.1.2 Effects of a metal chelator (EDTA)

Significantly reduced Cu uptake has previously been seen in fish exposed to Cu and EDTA (e.g., James et al., 1998) and therefore, experiments were conducted with exposure to $CuSO_4$ or Cu-NPs in the presence of EDTA with the aim of chelating any free Cu ions present, to render them non-bioavailable to the larvae and thus less toxic (Fig. 6.5, Table 6.3). As expected $CuSO_4$ toxicity was reduced (almost 2-fold) by increasing the levels of EDTA

from 0 to 800 nmol l^{-1}, whilst Cu-NP exposed larvae experienced an overall decrease in mortality with increased EDTA (Fig. 6.5). However, the correlation, although positive, was much weaker than with $CuSO_4$ and was non-significant ($P = 0.107$, Table 6.4). A contribution towards mortality of Cu ions dissolved from the NPs is a distinct possibility here with a potential dissolved Cu concentration of 50-60 µg l^{-1}. Interestingly though, survival in Cu-NP exposed larvae was greatest in fish exposed concurrently to 100 nmol l^{-1} EDTA, whilst larvae exposed to higher concentrations of EDTA experienced decreased survival (Fig. 6.5) and a more linear relationship would be expected if toxicity was directly correlated to the concentrations of free Cu and EDTA only (as in $CuSO_4$ exposed larvae, Fig. 6.5). Mortality in fish exposed to low waterborne Cu (4.27 µg l^{-1}) along with EDTA (1 g l^{-1}) has been seen previously (exact data not reported, James et al., 1998), with no deaths in fish exposed to the same concentration of Cu only. Notably however, the concentration of EDTA used by James et al. (1998) is far higher than used here (1 g l^{-1} there compared to 800 nmol l^{-1}, approximately 0.000234 g l^{-1} here), with no mortality reported in fish exposed to 0.5 g l^{-1} EDTA in that study. Data regarding the effects of EDTA on Cu-NP behaviour in water is currently lacking and it remains to be seen what effect, if any, these interactions have on the bioavailability and toxicity of Cu-NPs to fish. It is possible that the presence of EDTA could result in an increase in the rate dissolution of Cu ions over that which would occur without the addition of a metal chelator. Although there is unlikely to be a strong enough attraction of Cu ions to the EDTA to effectively pull them from the NPs, it is possible that as Cu ions are chelated by the EDTA, their absence from the water column may then encourage more from the NPs (diffusion gradient effect). In a study looking at the effects of EDTA on Ag-NPs Chappell et al. (2011) saw increase in the mean Ag-NP size in the presence of EDTA (at least in part explainable by EDTA sorption), and some evidence that EDTA acts as a mild dispersant to Ag-NPs (at 1 mM $NaNO_3$). The authors also report that, predictably, free Ag

ions in the water were reduced by the presence of increasing EDTA. However, total dissolved Ag (i.e., Ag ions both free in the water and complexed with EDTA, but with Ag-NPs removed) increased due to the complexation of Ag^{+} ions from solution (Chappell et al., 2011), though notably this data contained large error bars. The Cu-NPs themselves may also be affected by the presence of EDTA. For example, a complete coverage of the NP surface could shield the particle surface from van der Waals forces, thus potentially reducing particle aggregation (Chappell et al., 2011).

There is also the possibility that EDTA becomes more toxic to zebrafish larvae at the higher concentrations used here when in the presence of Cu-NPs, particularly as no mortality was observed in larvae exposed to EDTA only in the present study. The phenomenon of increased uptake of a contaminant in fish in the presence of a NP has been seen previously (Trojan horse effect; e.g., Zhang et al., 2007) with the potential for increased toxicity. Notably, with their widespread use and poor biodegradability, chelating agents have become persistent organic pollutants and EDTA is among the most concentrated anthropogenic compound in European surface waters (Yuan and VanBriesen, 2006 and references therein). Therefore it would be prudent to conduct further studies to assess the interaction between Cu-NPs and organic solutes such as EDTA and subsequent effects to aquatic biota.

6.4.1.3 The influence of humic acid on Cu-NP toxicity

Dissolved or particulate organic matter is ubiquitous in almost every natural water body and as such will almost certainly be present in areas where NPs are released. Humic acid (HA) constitutes 30-50% of surface water organic matter (Woodwell et al., 1978), and is possibly the most abundant naturally occurring organic macromolecule (Thurman and Malcolm, 1981). Concentrations of 1-20 mg l^{-1} HA have been reported in water (Shapiro,

1969), and HA is a known modifier of acute Cu toxicity to fish (review on DOC; Wood et al., 2011) with interactions with NPs of particular interest to ecotoxicologists (review; Aiken et al., 2011). In the present study mortality was reduced in larvae exposed to both $CuSO_4$ and Cu-NPs in the presence of 10 mg l^{-1} HA (Fig. 6.6), with a consequential increase in LC_{50} values (significantly correlated, Table 6.4). However, this effect was far more pronounced in the Cu-NP treatment with an almost 4-fold reduction in the 96 h LC_{50} of larvae exposed in the presence of HA, compared to a 2-fold reduction in those exposed to $CuSO_4$ (Table 6.4). However, at the higher concentrations of Cu-NPs (i.e., > 400 µg l^{-1}), it is difficult to discern the effects of Cu-NPs to that of Cu ions dissolved from the particles. For example at the highest concentration of Cu-NPs (1600 µg l^{-1}), the potential concentration of dissolved Cu ions at 10 % dissolution would be approximately 50 % greater than that the LC_{50} for $CuSO_4$ to larvae (Table 6.1). However, as seen previously, mortality is often not wholly or even partly attributable to free Cu in the Cu-NP treatments and that is likely the case at higher concentrations, though perhaps to a far lesser degree. Therefore the effects of HA in reducing toxicity are still of interest.

Natural organic matter such as HA may form a coat on the NPs which could either reduce or enhance aggregation depending upon certain other factors (Christian et al., 2008), and this in turn could affect toxicity similarly to ionic strength. A similar trend of decreasing toxicity with increasing DOM as seen here, was observed with an aquatic invertebrate (*Ceriodaphnia dubia*) exposed to Cu-NPs in the presence of DOM, with size of Cu-NP aggregates also effected (by the presence of DOM as well as its source) suggesting a link between the two. Humic acid has also been observed to induce disaggregation in iron oxide NPs (Baalousha, 2009) and C_{60} fullerenes (Xie et al., 2008) in water, whilst stabilising multi-walled carbon nanotubes (also in water, Hyung et al., 2007). The stabilisation of NPs due to steric repulsion following coating by NOM has also been seen (TiO_2 NPs with fulvic acid,

Domingos et al., 2009; C_{60} with HA, Chen and Elimelech, 2007). Silver-NPs were seen to stabilise in the presence of humic substances, which could result in long residence times in aquatic systems potentially increasing bioavailability (Cumberland and Lead, 2009). The (few) effects reported so far suggest that NP and Ha interactions are complex and dependent on certain factors (Ju-Nam and Lead, 2008). Such effects are important in ecological terms as in most aquatic environments where NPs may be released, concentrations of natural colloids such as HA will far outweigh that of NPs ($\mu g\ l^{-1}$ predicted NP concentrations compared to mg l^{-1} colloidal concentrations) and as such NP aggregation and behaviour will be dominated by these interactions (Klaine et al., 2008).

6.4.1.4 The effects of pH on Cu-NP toxicity

Dissolved Cu toxicity is also affected by pH, whereby at low pH the more toxic free Cu ion (Cu^{2+}) dominates (Bury and Handy, 2010), with increased Cu toxicity in fish seen with pH in the acidic range (e.g., Howarth and Sprague, 1978, Cusimano et al., 1986) including fish embryos (Stouthart et al., 1996). Conversely, fish exposed to Cu at pH levels over 7 have seen a reduction in toxicity (e.g., Erickson et al., 1996), although there have been exceptions to the rule (see Meyer et al., 2007 for review). Here, larvae exposed to 100 $\mu g\ l^{-1}$ $CuSO_4$ experienced similar effects with an almost 2-fold increase in acute toxicity at pH 5, compared to pH 7 and pH 9 (Fig. 6.7). This is to be expected, as at pH 5 the dominant species would be the toxic Cu^{2+}, whilst the less bioavailable and less toxic $Cu(OH)_2$ and $Cu(CO_3)_2$ would dominate at pH 9 (Leckie and Davis, 1979). The trend seen with $CuSO_4$ was also seen in larvae exposed to 500 $\mu g\ l^{-1}$ Cu-NPs (Fig. 6.7), though far more pronounced in the latter than for $CuSO_4$. At pH 5, larvae experienced 100 % mortality, with just 50 % mortality at pH 7. However, at pH 9, larvae exposed to an equal concentration of Cu-NPs suffered just over

20 % mortality by 96 h (Fig. 6.7). Notably, no control larvae died at any pH over 96 h. It is possible that changes to pH may alter aggregation of NPs, and although it remains to be seen if this occurs with Cu-NPs, it could be argued that increasing or decreasing Cu-NP aggregates (i.e., from that found at circumneutral pH) may affect toxicity. As argued above for the effects of water hardness, increased aggregation and a subsequent deposition of the NPs at the bottom of the beaker may results in closer contact with the contaminant in ELS fish. Conversely, if disaggregation occurred then the larvae could be exposed to smaller particle aggregates or perhaps single NPs which could also affect toxicity. Furthermore, free Cu ions dissolved from the NPs would be more toxic to fish at acidic pH levels, again enhancing toxicity. Dissolution could partially explain the toxicity seen in the current study with a potential 50 $\mu g\ l^{-1}$ dissolved Cu present in the water column (approximately the 96 h LC_{25} for larvae exposed to $CuSO_4$ in earlier studies (e.g., Table 6.1). It is unknown if the rate of dissolution is altered by changes to pH, yet this remains a possibility and requires further consideration. Zhang et al. (2010) assessed the dissolution rate of zinc sulphide NPs and found greater dissolution at lower pH levels and smaller particle sizes. The surface potential of NPs may also be altered by pH (Handy et al., 2008a) with low pH most likely resulting in a positively charged surface and negatively charged at high pH. It is thought that when there is no net charge (i.e., at the point of zero charge (PZC)) there is no repulsion and the NPs will aggregate, whereas more stable solutions are formed at pH levels ± the PZC. However, Guzman et al. (2006) also report that TiO_2 NPs aggregate over a wide range of surface potentials so aggregation may not be directly correlated. They discovered that over 80 % of suspended particles and aggregates were mobile between pH 1–12, except as the pH approaches the point of zero charge (PZC) where high levels of aggregation occurred, an effect also seen with magnetite NPs (Hu et al., 2010). The behaviour of Cu-NPs in water could therefore be somewhat dependent on where (along the pH scale) the PZC lies. For

example, FeO-NPs have a PZC in the pH region of natural waters (i.e., pH 6 to 8, Handy et al., 2008a), which infers that these NPs would readily aggregate in most natural waters. Alternatively, many oxide NPs have a PZC at low pH levels and will thus aggregate at these pH levels. However, the presence of humic acid induced a shift in the PZC of magnetite NPs due to partial neutralisation of the positive charges on the particles (Hu et al., 2010). Chang et al. (2010) found that Cu-NPs in solution had an isoelectric point (i.e., no net surface charge) at pH 9.1, so that decreasing pH would result in a better suspension (less aggregation). Therefore although further pH experiments are required in order to assess these phenomena with Cu-NPs, it is obvious that the aggregation, and environmental fate and behaviour of NPs cannot be predicted by knowledge of water pH alone.

6.4.2 Conclusions

The acute toxicity of Cu-NPs to embryos was similar to that of $CuSO_4$, whilst larvae were more sensitive to $CuSO_4$ than the NPs. Exposure to embryos was not commenced within 1 hpf, so it remains to be seen if exposing these organisms prior to the hardening of the chorion would increase acute toxicity (potentially important in terms of ecotoxicology as it is quite possible that eggs would be laid in NP contaminated water). Clearly, there needs to be some standardisation in acute toxicity bioassays using NPs, though the results from identical larvae experiments seen here indicate that even under controlled conditions there may be extraneous factors that influence toxicity that are perhaps out of the control of the experimenter. Notably, the acute toxicity of Cu-NPs was in the $\mu g\ l^{-1}$ range, rather than the $mg\ l^{-1}$ range which has been predicted for some NPs. Abiotic factors were seen to influence Cu-NP toxicity though not always correspondingly to $CuSO_4$. Toxicity was seen to increase with elevated Ca^{2+}, whilst HA had a much greater effect in reducing toxicity to Cu-NP

exposed larvae than for $CuSO_4$. The influence of pH was also greater in Cu-NP exposed larvae with higher mortality at lower pH and higher survival at a high pH than $CuSO_4$ exposed animals. It is quite possible that dissolved Cu ions played at least a partial role in some of the toxicity seen in some Cu-NP exposed fish, particularly at the higher concentrations, though in no experiment was this considered the sole cause.

Chapter 7. General Discussion.

The results observed throughout this study confirm that metal-NPs are acutely toxic to fish following waterborne exposure and exert sublethal effects when exposure is through both the water and the diet. Effects in fish included disturbances to ionoregulation and osmoregulation, and oxidative stress. Some of these effects can be considered generic responses to contaminants, particularly metals, though some, such as effects to the brain, may be particular to NPs (or their aggregates) and may require further investigation. Generally metal-NPs are not as acutely toxic as their bulk metal counterparts; though experiments with early life stage (ELS) fish showed little difference in the acute toxicity of $CuSO_4$ and Cu-NPs to embryos. Further experiments showed that changes to abiotic factors resulted in similar effects to dissolved metals, whereby toxicity was either reduced or increased depending upon the manipulation of a particular factor. The degree of these effects was sometimes more pronounced in Cu-NP exposed fish than in those exposed to $CuSO_4$.

The experiments and investigations contained within this thesis began when nanotoxicology (of engineered NPs) was in its infancy and only a small number of reports concerned fish, with very little known of the behaviour, bioavailability, and effects of NPs in aquatic systems. Since then, several more studies have emerged as well as some key concepts regarding the use of NPs in bioassays, particularly in relation to how NP characteristics may affect toxicity. This has materialised through a combination of experimental trial and error, as well as the shared expertise from several fields of science, technology and policy making. As such the work carried out and presented here underwent a similar pathway, whereby a great deal of experimentation was required to achieve the aims of comparing NPs to their bulk metal counterparts. This included, for example, painstaking attempts to successfully disperse the NPs in solution to enable homogeneous dosing, and subsequent quantification in water and fish tissues, as well as the assessment of toxicological effects on fish. Therefore the work contained here significantly contributes to the field in terms of our understanding of NP,

water and fish interactions, and can also contribute towards the necessary 'data bank' required to begin risk assessment and making informed policy decisions.

7.1 Considerations for NP exposure and quantification

Fish have long been adapted to natural NPs in the environment and one issue was whether they were adapted to cope with engineered NPs. In order to achieve this it was necessary to add the NPs to solution to be able to dose the fish. One challenge here is whether the NPs should be added and allowed to aggregate naturally in stock solutions prior to the addition to aquaria, or whether it is better to disperse the NPs as best as possible in order to expose the fish to more homogenous NP solutions. It was decided that the latter was the preferred option as effects obtained from a uniform solution are more easily described. Also if you wish to repeat the experiment you can then start each successive trial with a solution that matches the previous as much as possible. If no attempts were made to disperse NP stocks then the rate of aggregation may differ between stocks meaning that when dosing, the fish are exposed to different size ranges of aggregated NPs and comparisons are difficult. Nevertheless, it was also decided that no dispersants or sonication would be used for Cu-NPs due to risks associated with adding in unknown variables and the risk that sonication may aid and increase dissolution. Laban et al., (2010) saw an approximate 10-fold increase in acute toxicity in fathead minnow (*Pimephales promelas*) embryos when Ag-NP stocks were sonicated compared to those stirred. The Cu-NPs used here were instead stirred for several hours prior to each use, though it remains to be seen if this in itself affects any NP characteristics. With this approach it is likely that the NPs will quickly aggregate when added to the experimental aquaria, but this may be seen as a positive due to its environmental significance.

Another issue faced was that of the effects of stock age on NP toxicity. Using the same Cu-NPs as used in these experiments, a trial investigating the acute toxicity of a 6 month old stock compared to a freshly made stock was carried out on zebrafish larvae (Calver, 2010, personal communication). In that experiment the 6 month old stock was almost twice as lethally toxic as the fresh one. This may have been due to ongoing dissolution of Cu ions from the NPs with Kittler et al. (2010) noting that aged stocks of Ag-NPs were much more toxic to human mesenchymal stem cells than freshly prepared Ag-NPs. In that study, Ag-NPs lost up to 90 % of their weight when stored for up to 125 days, with the rate and degree of dissolution depended upon surface functionalisation and the temperature at which the NPs were stored. Murdock et al., (2008) showed that over time Cu-NPs of varying sizes all experienced changes to shape and size (from more solid, spherical particles to smaller particles with spherical centres, but with crystalline spikes emanating from the surface), aggregation (increases over time), and zeta potential (initial decrease followed by an increase in all NPs). The changes to individual NP size and shape, and subsequent changes to zeta potential were as a result of dissolution of Cu ions from the surface of the NPs (Murdock et al., 2008). Therefore all experiments in the current study were carried put with freshly made stocks.

Of note from the study by Murdock et al. (2008) was their realisation that additional dispersants (high in Na and S) had been used on Ag-NPs by the manufacturers, which was not disclosed to the researchers (subsequently discovered by XPS analysis). This raises the importance of knowing the full life history of NPs prior to use in toxicology assays, especially as dispersants have been proven to be the root cause of toxicity rather than the NPs themselves in previous studies with fish (see Henry et al., 2007).

When formulating fish feeds contaminated with TiO_2 NPs it was decided to disperse the NPs in water by sonication with subsequent spraying onto the surface of the feed pellets.

This was carried out before evidence was raised regarding concerns as to the effects of sonication on NPs and a more prudent method may have been that which was adopted in our laboratory in the study by Merrifield et al., (2011), whereby NPs were added as dry powder to base feed ingredients and mixed, with a subsequent formation of pellets containing relatively well dispersed NPs. However, it is quite possible that only some NPs are affected by sonication in this manner, and that TiO_2 NPs are not amongst them.

The second technical issue raised in the current study was that of the quantification of NPs in water, fish issue and fish feeds. Preliminary experiments (spike recovery tests) showed that Cu-NPs were able to be measured using the methods routinely established for traditional Cu analysis. That is water samples were acidified with a small volume of concentrated nitric acid and fish tissue sample digested in nitric acid and subsequently diluted with ultrapure water, and both analysed by ICP-OES. Spike recoveries were good with over 90 % recovery of nominal concentrations in both water and tissue. However, similar experiments with TiO_2 NPs were not as successful. Attempts, as detailed in Chapter 3, were made to improve recovery of Ti from fish tissue containing TiO_2 NPs (along with experimental diets). Whilst recovery was greatly improved for most tissue types by the addition of Triton X-100, work is still ongoing in order to improve the method further. This includes assessing the efficacy of single particle counting ICP-MS. A variation of this method has been employed for the quantification of gold (Degueldre et al., 2006a) and uranium (Degueldre et al., 2006b) colloids in water and it remains to be seen if the recovery of TiO_2 NPs in fish tissue can be improved by this method.

7.2 Nanoparticle fate, behaviour and bioavailability

The transport, persistence, and bioavailability of NPs in the environment are essential aspects to consider when assessing and managing risks (Wiesner et al., 2009). When NPs undergo aggregation they will most likely precipitate to the sediment, thus becoming far less mobile than would single particles (Petosa et al., 2010). This could result in reduced contact with pelagic species and less bioavailability (as suggested in the current hypothesis). They could also enter the food chain by interacting with benthic organisms (Wiesner and Bottero, 2007). The uptake of polystyrene-NPs has already been seen in two species of suspension-feeding bivalves (Ward and Kach, 2009), and a food chain effect has been shown, with zebrafish consuming TiO_2 NP contaminated *Daphnia magna* (Zhu et al., 2010). Nanoparticle aggregates could also adsorb to the surface of animals (Handy et al., 2008c), with surface acting toxicants previously seen in ecotoxicology (e.g., Handy and Eddy, 1991). In studies using species such as rainbow trout, increased aggregation and sedimentation could theoretically result in reduced exposure to NPs in the water. However, in the waterborne Cu-NP study detailed in Chapter 5, Cu levels from samples taken mid-water remained high over 12 hours suggesting that trout were exposed to close to nominal concentrations. This was in clean experimental conditions though and it remains to be seen, for example, if the presence of a sediment substrate (potentially resulting in sediment-trapped NPs) in the tanks would alter the mid-water concentrations observed in that study.

In experiments with Cu-NPs it is possible that in some instances the dissolution of Cu ions from the NPs contributed to toxicity, particularly where high Cu-NP concentrations were used (i.e., $> 500\ \mu g\ l^{-1}$). However, in experiments where lower concentrations were used this contribution would have been negligible and the effects seen can be attributed to Cu-NPs or more likely their aggregates, though the specific mechanism of this requires further

investigation. This is particularly relevant where Cu-NP concentration were 100 $\mu g\ l^{-1}$ or less, with separate dissolution experiments showing that over 12 hours the concentration of dissolved Cu in the water would be approximately 6.1 $\mu g\ l^{-1}$ when a Cu-NP concentration of 100 $\mu g\ l^{-1}$ was used. Notably, these dissolution experiments were conducted in pure water and it remains to be seen if the rate of dissolution differs when in tank water (with the influence of abiotic factors not present in pure water).

Figure 1.2 of the hypothesis in Chapter 1 showed some proposed differences in *bioavailability of NPs to fish, with Cu-NPs and $CuSO_4$ as examples*, whilst Fig. 1.3 illustrated differences in ADME along with some known or suggested toxic effects (again using Cu as examples). Following the experiments conducted and presented throughout this thesis, it is now possible to formulate some conclusions regarding these concepts. Figs. 1.2 and 1.3 have been redrawn following the work carried out and are presented in Figs. 7.1 and 7.2 respectively (the latter discussed in the next section).

In view of the results obtained from the waterborne Cu-NP exposures seen in Chapters 5 and 6, along with separate dissolution studies and NP stock analysis on NanoSight LM_{10}, it is evident that most of the Cu-NPs in solution will aggregate with some dissolution (approximately 6 % over 12 h). This is reflected in Figure 7.1, though bioavailability is still described as limited as there is no definitive evidence that intact Cu-NPs are bioavailable to fish. The other noticeable difference between the initial Fig. 1.2 and the revised Fig. 7.1 is the interaction with abiotic factors. It has been well established that differences in water chemistry can affect the fate and behaviour of contaminants such as trace metals, which in turn affects bioavailability to fish. In experiments exposing larvae to Cu-NPs or $CuSO_4$ with varying abiotic factors (Chapter 6), it was seen that acute toxicity was reduced or enhanced similarly between Cu treatments, but that in some cases the effect was more pronounced in NP exposed fish. As seen in Chapter 6, humic acid (HA) had the effect of reducing the acute

toxicity of Cu-NPs. It is perhaps not surprising that toxicity was reduced as organic matter such as HA can stabilise NPs in the water column (Lead & Wilkinson, 2006), with NPs tending to form agglomerates with organic matter, something already seen with carbon nanotubes visible on fish mucous following exposure (Smith et al., 2007). Here this had the effect of reducing mortality in Cu-NP exposed larvae more than 3-fold, compared to the 2-fold reduction in acute toxicity seen in $CuSO_4$ exposed larvae (Chapter 6). This was most likely as a result of the NPs being coated by the HA, thus reducing contact with fish epithelial tissue, probably through steric hindrance. The specific interaction between Cu-NPs and HA remains unknown in terms of whether the organic matter does actually increase stability or possibly aggregation. However, mortality still occurred from Cu-NP exposure in the presence of HA (albeit reduced), and the relationship requires further investigation in order to elucidate the actual mechanism of this interaction.

Calcium was used as an example of a competing cation and the results obtained were somewhat variable. Initially Ca^{2+} did not affect acute Cu-NP toxicity (in fact slightly increasing it), but in a following experiment, Ca^{2+} appeared to alleviate the acute effects of the NPs. As such the relationship is presented in terms of limited bioavailability in Fig. 7.2, but that there is an effect. The effects of pH on Cu-NPs were similar to those on $CuSO_4$, but more pronounced in the NP exposed fish. Figure 7.1 presents this as limited bioavailability (and thus reduced mortality) at high pH (i.e., $< H^+$), with increased bioavailability or at least $>$ contact with fish epithelial tissue at low pH (i.e., $> H^+$). Of course, it is possible that the dissolution of Cu ions from the Cu-NPs may be affected by changes to pH, and certainly at the acidic pH 5 used in these experiments (Chapter 6), free (dissolved) Cu would have predominantly been the toxic Cu^{2+} species. Further experiments at lower concentrations of Cu-NPs and dissolution experiments at various levels of pH would overcome this issue.

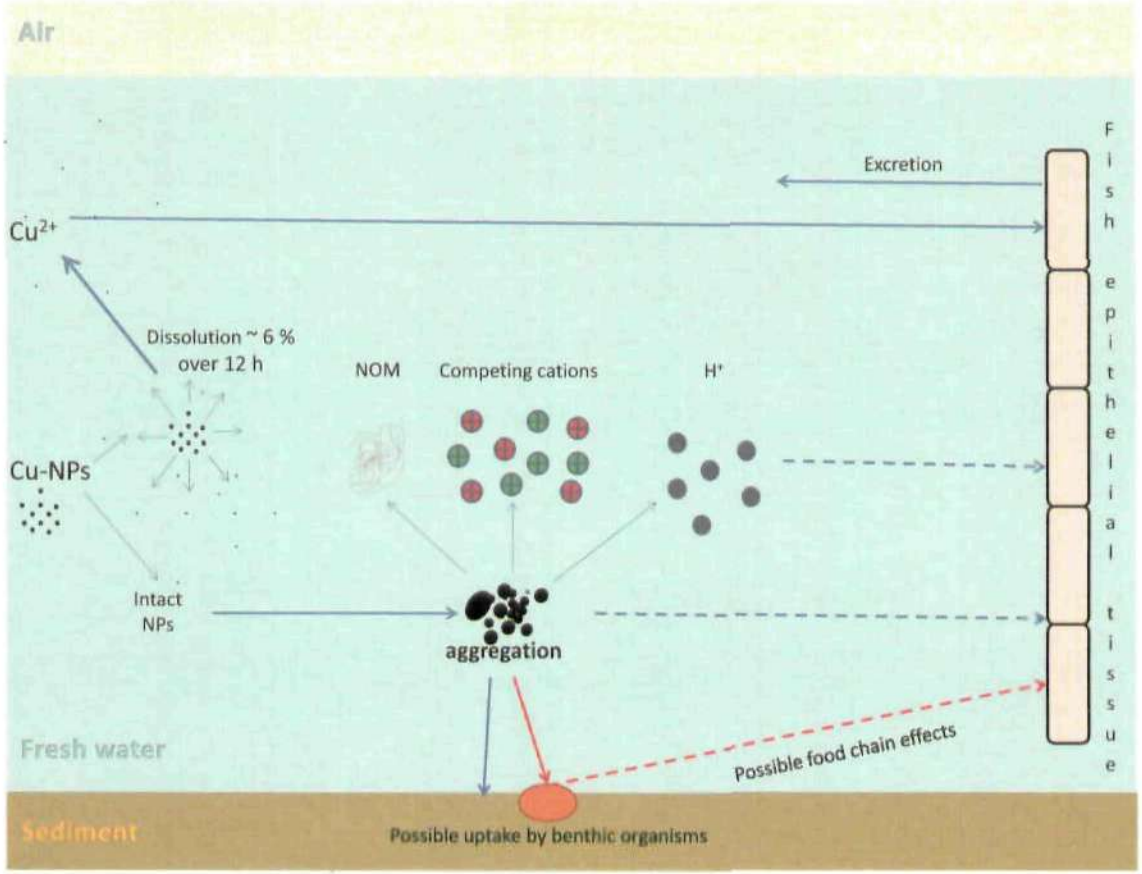


Figure 7.1. An updated version of the conceptual diagram found in Chapter 1 (Fig. 1.2), following the results obtained throughout this study. The diagram shows the current understanding of the differences in bioavailability of Cu-NPs to fish with some example abiotic factors. Dashed lines indicate limited bioavailability, with unbroken lines showing high bioavailability or contact with epithelia. Blue lines represent known effects (from work presented in this thesis), whilst red lines show possible interactions. The arrow linking Cu-NP aggregates and HA is bi-directional as it us unknown if HA stabilises Cu-NPs or affects aggregation. Low pH results in a greater bioavailability or contact with epithelial tissue, whilst the opposite is true for high pH. Note that compared to the corresponding Fig. 1.2, the affects of abiotic factors for dissolved Cu are not included here.

Some differences were observed between the hypothesised effects of NPs on fish (Fig. 1.3) and what was actually seen (Fig. 7.2), although some effects were as suggested (or not assessed in some cases). For example, no evidence of cutaneous uptake was presented throughout any study, and no skin lesions were noted on any fish. As predicted there were some affects on the brain, and no accumulation of Cu-NPs, though TiO_2 NPs (as Ti), showed some signs of accumulation in this organ, albeit at very low concentrations. The liver did not accumulate Cu-NPs, though fish exposed to dietary TiO_2 NPs did initially, along with the spleen. The spleen was a notable absentee from Fig. 1.3, but subsequent analyses showed that this organ may play a role in NP ADME.

In experiments using a waterborne exposure route, 100 $\mu g\ l^{-1}$ $CuSO_4$ was more toxic than the same mass concentration of Cu-NPs to juvenile rainbow trout with 80 % and 15 % mortality respectively after 96 h (LC_{50} not calculable due to a lack of Cu concentrations in the exposures). Zebrafish larvae were similarly affected by exposure to $CuSO_4$ with 96 h LC_{50} values around 70-100 $\mu g\ l^{-1}$. If you accept that the 96 h LC_{25} for larvae was approximately 150 $\mu g\ l^{-1}$, then it appears that these fish were of similar sensitivity to juvenile trout, as the latter fish experienced 14 % mortality following exposure to 100 $\mu g\ l^{-1}$ Cu-NPs for 96 h. As in traditional trace metal toxicity, the gills were the target organ for waterborne Cu-NP exposure (some accumulation and ionoregulatory disturbances), though as with some other NPs the gut was also affected with elevated Cu levels in Cu-NP exposed fish (but not from waterborne $CuSO_4$). Sublethal effects were seen in the gut and brain (< sodium pump activity, changes to TBARS), though there was no evidence of Cu-NP uptake in these organs (or the spleen or muscle). Notably, many of these sublethal effects were on a (severity) par with those seen in $CuSO_4$ exposed fish, though fish from the 100 $\mu g\ l^{-1}$ $CuSO_4$ treatment did

not survive until the final sampling point at day 10, making a like for like comparison of sublethal effects with the 100 $\mu g\ l^{-1}$ Cu-NP treatment impossible.

Further hypothesise included sublethal effects following chronic dietary exposure, and conjecture that these could possibly lead to death. No treatment related mortality occurred when rainbow trout were fed diets containing TiO_2 NPs, though some sublethal affects were observed. Notably, TiO_2 NPs did not affect growth or nutritional performance and compensatory processes may have protected this. The dietary TiO_2 NP experiments here showed that fish will eat food contaminated with NPs, and this has also been seen in fish exposed to C_{60} fullerenes and carbon nanotubes through the diet (Fraser et al., 2010). However, although the target organ, Ti accumulation in the gut of exposed fish was somewhat limited, with a greater difference in Ti levels seen between the liver, spleen and later the gills of control and exposed fish, and to a lesser extent the brain. As Ti is not an essential micronutrient and that bulk TiO_2 is used as an inert marker in fish dietary studies, it is not surprising that uptake was relatively low in all organs (nmol g^{-1} levels from $\leq$ 100 mg kg^{-1} dietary inclusion). The pathologies observed in the gut of fish exposed to waterborne TiO_2 NPs (Federici et al., 2007) did not manifest to the extent expected in dietary exposed fish (Ramsden et al., 2009). Similarly to several dietary metal studies (e.g., dietary Cu, Handy et al., 1999; Shaw and Handy, 2006; Hoyle et al. 2007), growth was not affected in fish fed TiO_2 NP contaminated food. This may be due to the fish adopting a strategy whereby growth is protected, but with consequential sublethal effects on other physiological processes (Handy et al., 1999; Clearwater et al., 2002).

In the current study (Chapter 5), fish exposed to waterborne Cu-NPs experienced an elevation in gut Cu along with ionoregulatory disturbances. However, it remains to be seen if feed contaminated with Cu-NPs would firstly be palatable to fish, and secondly whether they

would be bioavailable and exert toxic effects. During the course of these studies such a trial was commenced, but was abandoned due to technical difficulties. In view of the effects seen in the gut of waterborne exposed fish, it remains prudent to carry out a dietary exposure with Cu-NPs.

7.4 Nanoparticle toxicity to ELS fish

The early life stage (ELS) of fish is a critical period for recruitment into fish populations and disruptions can have dire consequences for both commercial and non-commercial wild stocks. As such it is critical for these animals to be a considered in ecotoxicological research and subsequent risk assessment. Experiments with ELS fish showed that Cu-NPs followed a similar pattern to soluble Cu (as $CuSO_4$) in that embryos were less sensitive than larvae. Copper sulphate was also considerably more acutely toxic to larvae than Cu-NPs, though this was not the case with embryos. In experiments exposing zebrafish embryos to both forms of Cu, there was not a significant difference in the 96 h LC_{50} values. This is in agreement with Zhu et al. (2008) who found no differences in the toxicity of TiO_2 NPs, ZnO-NPs and Al_2O_3 NPs to zebrafish embryos. The embryo exposure experiments described in Chapter 6 commenced 2-4 hours post-fertilisation (hpf) and it remains to be seen if exposing embryos prior to the hardening of the chorion would result in an increase in acute toxicity. The chorion presents a formidable barrier to soluble metals, effectively preventing free passage to the embryo following its hardening after contact with water (von Westernhagen, 1988; Weis and Weis, 1991). With few reports of NPs crossing the chorion of fish embryos it is suggested that the chorion may offer some protection from waterborne exposure, but once the fish has hatched the internal organs can become contaminated (Handy et al., 2011). However, Lee et al. (2007) observed the transport of Ag-NPs in and out of

developing zebrafish embryos, with movement through chorion pore canals by diffusion. Clearly Cu-NPs caused acute toxicity to fish embryos in the current study and further work is required to investigate the mechanism by which this occurs.

Copper-NPs were acutely toxic to zebrafish larvae in the current study. Griffitt et al. (2008) also observed acute toxicity of Cu-NPs to zebrafish larvae, though in their study the Cu-NPs were more toxic than dissolved Cu (Cu-NP 48 h LC_{50} of 710 $\mu g\ l^{-1}$ compared to 1780 $\mu g\ l^{-1}$ for Cu exposed fish). This may be indicative of water quality differences between the two studies, with Griffitt and workers using relatively hard water, compared to the moderately soft water used here and notably they also report dissolution of less than 1 % of total mass of the original dose of Cu-NPs over 48 h. This is considerably less when compared to the approximate 10 % dissolution over 24 h here. This illustrates the difficulties faced by nano-ecotoxicologists and highlights the need for standardised test materials and procedures in order to progress our understanding of nanotoxicology.

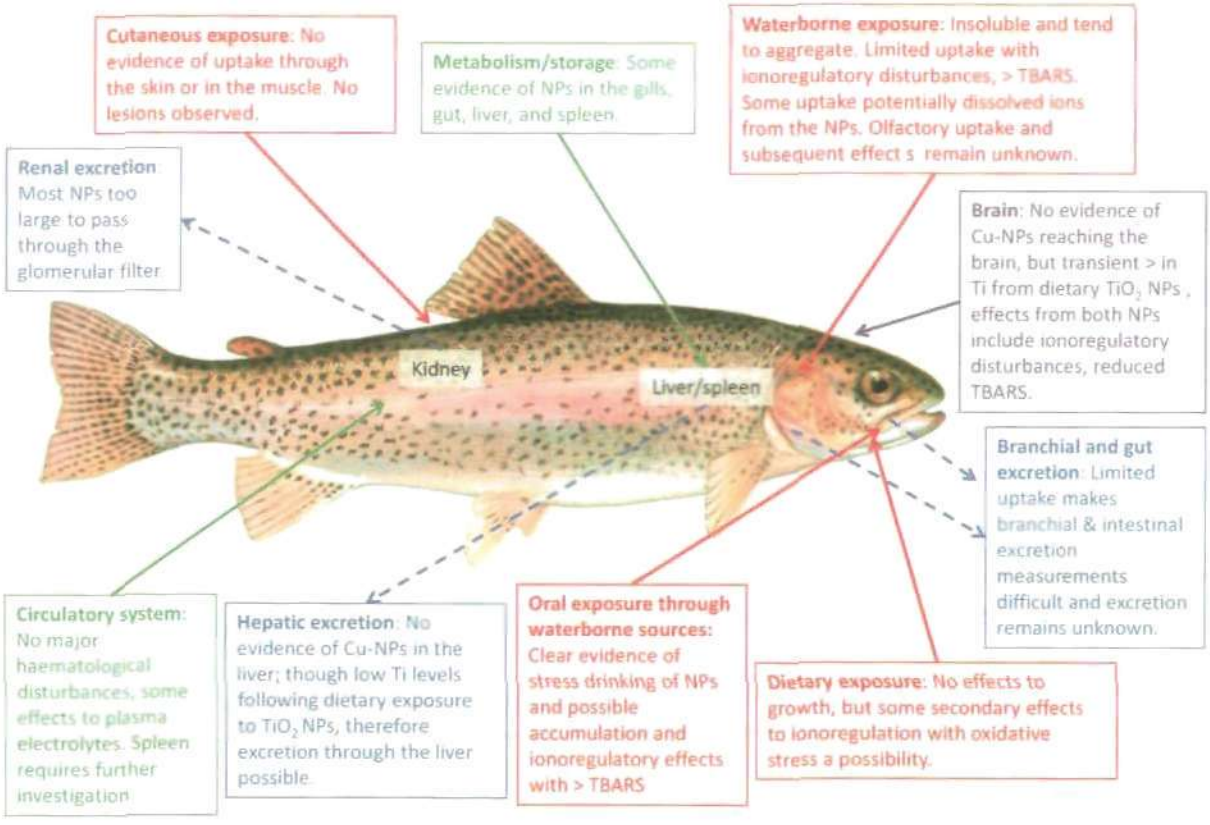


Figure 7.2. An updated version of the conceptual diagram found in Chapter 1 (Fig. 1.3, panel a), showing absorption, distribution, metabolism, and excretion (ADME) and toxicity of TiO_2 NPs and Cu-NPs in rainbow trout following the results obtained throughout this study. Note that compared to the corresponding Fig. 1.3, the diagram for dissolved Cu is not included here. Diagram conception based on Handy et al. (2008b).

7.5 Is there evidence of a nano effect?

The initial hypothesis of this thesis considered that metal-NPs would be both acutely and sublethally toxic to fish and impart unique toxicological effects due to their specific characteristics (particle size, shape, surface area and surface charge etc). Whilst acute and sublethal toxicity was observed, throughout these experiments there was no direct evidence of a 'nano' effect. Observations of Cu-NPs by NanoSight LM_{10} in pure water showed mean aggregates of 216 ± 122 nm, with aggregation expected to increase in certain water chemistries (see Chapter 6 for discussion). Therefore it is extremely unlikely that fish were exposed to single particles, however, technical limitations do not aid this assessment, with no techniques available to irrefutably demonstrate that intact NPs are present in fish after exposure.

In an attempt to address the issue of acute toxicity being related to particle and aggregate size, an experiment was conducted to assess differences in toxicity between larger aggregates of Cu-NPs compared to smaller NPs and filterable Cu (data not presented in an experimental chapter, see below). Briefly, a 3 mg l^{-1} stock solution of Cu-NPs was prepared as described in Chapter 5 and filtered using a 250 ml Millipore filter tower with a 0.2 μm pore membrane. Larvae were then exposed in triplicate to either the filtered fraction, or the unfilterable particles (removed into solution from the filter), or an identical (unfiltered 3 mg l^{-1} stock) for 96 h, with similar mortality between each treatment (16.7 ± 5.5, 10.0 ± 1.7, and 17.8 ± 3.9 % respectively, data mean ± SEM). However, subsequent analysis of the Cu content of each fraction revealed vastly different values (2730 ± 127, 1576 ± 69, and 92.9 ± 17 μg l-1 in the whole stock, particulate, and filtered Cu-NP fractions respectively). Therefore, the smaller particles, aggregates and free Cu in the filtered fraction was equally as toxic as the larger aggregates (particulate faction, > 0.2 μm), even though there was almost

30 times more Cu in the latter treatment. Particle size-dependent toxicity has been seen with NPs before, whereby equal concentrations of Ag-NPs of different sizes (5.9, 15.3, 51.2, and 108.9 nm) produced 80, 64, 36, and 3 % mortality in zebrafish embryos respectively (Bar-Ilan et al., 2009). However, concerns over the low toxicity seen in the filtration experiment described here (i.e., less than 20 % mortality in animals exposed to 2730 $\mu g\ l^{-1}$, compared to LC_{50} values around 200-300 $\mu g\ l^{-1}$ seen previously) prohibited the inclusion of this data in an experimental chapter, but it could prove a valuable experiment to rerun.

7. 6 Implications for risk assessment

With current legislation for metal exposure taking decades to research and implement it would be prudent, if possible, to apply these to metal-NPs. This is only feasible however, if the metal-NPs are less or similarly toxic compared to their soluble metal counterparts. From the data presented in Chapter 4 and by previous work (i.e., Federici et al., 2007) it can be seen that TiO_2 NPs are more toxic to fish than bulk TiO_2 from both dietary and waterborne sources (note: effects of bulk TiO_2 not directly assessed due to their use as inert biomarkers in fish nutrition and lack of toxicity, along with major differences in structure, see Chapter 4). In the experiment described in Chapter 5, although not as acutely toxic, Cu-NPs were clearly capable of producing sublethal effects in trout on a par with soluble Cu at similar exposure concentrations. This signifies that current legislation for safe environmental limits of Cu could be applied to Cu-NPs, though this is clearly not acceptable for TiO_2 NPs and legislation for Ti metal. However, there is a danger, for example, that chronic exposure or exposure in conditions not assessed in Chapter 5 could result in increased Cu-NP toxicity. Furthermore, differences in specific characteristics of the same NP can affect their behaviour in water. For example, NP size can affect aggregation with 5 nm TiO_2 NPs forming larger aggregates

compared to 32 nm TiO_2 NPs particles under the same conditions of pH and solid concentrations (Pettibone et al., 2008).

Another consideration is can we use current bioavailability and toxicity prediction models for metal-NPs? Although Cu-NPs caused similar sublethal effects to rainbow trout as $CuSO_4$ (Chapter 5), the differences in fate and behaviour in the water column raises the issue of the appropriateness of current models for NPs. Several factors may influence the behaviour of NPs in solution including, aggregation chemistry and the ability to form stable dispersions in liquids, NP characteristics, the adsorption of manufactured NPs onto surfaces (including the exterior surfaces of organisms), and the effects of abiotic factors (Handy et al., 2008c). However, the fate and behaviour of most, if not all, NPs in water is not currently fully understood. There are indications that certain abiotic factors, such as humic acids (HA), stabilise NPs (Klaine et al., 2008), though depending upon certain other conditions HA could also promote aggregation (Ju-Nam and Lead, 2008). Other physicochemical factors can also increase or decrease aggregation such as changes to pH (Petosa et al., 2010). The results seen in Chapter 6 demonstrate the influence that abiotic factors can have on acute toxicity of Cu-NPs. Clearly the issue of aggregation plays an important role in NP ecotoxicology, having a significant impact upon transport, fate, reactivity, and bioavailability. Therefore due to the (probable) differences in bioavailability, fate and behaviour of metal-NPs in water compared to soluble metals, current toxicity prediction models such as biotic ligand models are inadequate in their present form. Consequently new, empirically derived models or at least updated versions of current ones would be required to predict metal-NP uptake and toxicity. However, the NPs themselves remain the biggest problem in developing such models. Differences in characteristics such as size and surface charge between NPs of the same basic material (e.g., Cu), can influence aggregation, and subsequent bioavailability and toxicity, with these factors in turn influenced by water chemistry. Therefore, the characterisation of

each individual type or batch of NP used and potentially released into the environment would be required for modelling. However, this is a challenging and monumental task and clearly there is a need for established standard test materials and standard tests to act as a starting point. It may then be possible for toxicologists and regulators to modify the model in accordance with each specific NP of interest, perhaps adding functionalised groups etc. This is particularly pertinent as new generations of NPs are undoubtedly being developed with, for example, new forms of organic and inorganic functionalisations currently not present.

Current prediction models are well equipped to deal with the speciation of metals such as Cu which can alter depending upon water chemistry, and remain useful if dissolution of metal-NPs was solely responsible for toxicity to fish. However, the results presented in this thesis and the work by Griffitt et al. (2007), suggest that for Cu-NPs at least, dissolution is not a major factor in a many of the effects seen, although cannot be discounted at higher Cu-NP concentrations. Yet another facet of NP toxicity that may need to be included in future models is the interaction with other contaminants. Some evidence of increased uptake of other contaminants in the presence of metal-NPs is emerging in the literature (e.g., Zhang et al., 2007; Sun et al., 2007, 2009). There is also the possibility that the non-linear mortality response of larvae exposed to Cu-NPs in the presence of EDTA (Chapter 6) is a co-contaminant effect.

Also of concern is the appropriateness of a pelagic fish species as test models for Cu-NP toxicity. If Cu-NPs settle out of the water column after forming aggregates a more ecologically relevant test species may be a flat fish or a sedimentary organism (e.g., molluscs or polychaetous annelids), though mid-water column Cu levels remained high following the addition of Cu-NPs in Chapter 5.

7. 7 Nanoparticle hazard versus risk

One important aspect to consider is the hazard risk versus the environmental risk of exposure in relation to environmentally realistic scenarios. Many laboratory based toxicity studies are conducted in clean, controlled conditions with artificially high concentrations of contaminant, including NPs. These studies are quite relevant as proof of principle experiments where the researcher is attempting to assess whether or not a NP (in this instance) is toxic and at what level the toxicity manifests at. However, this needs to be placed into context when considering what, if any, effects will occur within the environment. The association between toxic effects and dose/concentration of NP is another issue awaiting resolution (Savolainen et al., 2010) and caution must be advised when extrapolating the effects seen with high NP concentrations in the laboratory to those likely within the environment. Toxicologists have a duty to responsibly interpret and report nanotoxicology data in order that a potentially beneficial and valuable industry might progress and fulfil its potential. However, this is made all the more difficult where NPs are concerned as there is very little data regarding the release of these particles into the environment and techniques for environmental monitoring are either inadequate for NPs (e.g., CNT) or are unable to distinguish between the nano and bulk forms of a material (e.g., Cu). However modelling techniques have predicted low $\mu g\ l^{-1}$ concentrations of NPs releases into the environment (dependent upon the NP, Boxall et al., 2007). For example, the estimated environmental concentrations of TiO_2 NPs in Nordic aquatic ecosystems range from 0.0007 to 0.0245 $\mu g\ ml^{-1}$ (Mueller and Nowack, 2008; Pérez et al., 2009). The issue of risk assessment and regulation is further compounded as NPs are not a uniform group of substances (Borm et al., 2006) and generalisations are difficult.

However, several studies have now shown deleterious effects from exposure to NPs, although there is currently no general consensus regarding routinely observed effects with many disparities between different studies. Coupled to that are that many responses are generic in nature (to numerous contaminants) and may not necessarily be as a result of actual NP exposure, rather exposure to aggregates which effectively clog the gills with toxicity a result of secondary effects. Regardless, that effects have been observed signifies that there is a hazard risk associated with NPs, but the risk of exposure via the aquatic environment remains relatively low (when considering the literature). Several factors will effect potential exposure including target species (pelagic/sedimentary etc); NP type and its characteristics; environmental concentration, fate and behaviour of the NPs; bioavailability and the mechanisms of absorption, distribution, metabolism, and excretion (ADME) for NPs in fish; and perhaps the ability of the animal to cope with exposure and/or remove itself from the area of exposure (pollution avoidance). Therefore at present there appears to be some disparity between NP hazard and risk of exposure to NPs in the aquatic environment, but further work is required to elucidate this relatively unknown area of ecotoxicology.

Notably, in terms of the risk of human exposure via consumption of contaminated fish fillets, Cu and Ti levels in the muscle of NP exposed fish remained low; suggesting that inadvertent ingestion of NPs would be negligible, at least from fish exposed to NPs similarly to the exposure scenarios used here (concentrations, route and duration of exposure etc).

7.8 Conclusions and future work

Metal-NPs are both acutely and sublethally toxic to fish depending upon the route of exposure and NP type. Copper-NPs were acutely toxic to early life stage fish, and caused

some mortality in juvenile rainbow trout (though the latter were only exposed to concentrations intended to cause sublethal effects). Sublethal effects from waterborne exposure included generic responses to contaminants (such as dissolved metals) including disturbances to ionoregulation and indications of oxidative stress. However, these effects often manifested without significant accumulation of the NPs in the affected organ. Dietary exposure to TiO_2 NPs did not result in treatment related mortality and no affects were seen on growth or nutritional performance.

In terms of hazard assessment, TiO_2 NPs are evidently more toxic to fish than their bulk counterparts and as such current safe limit regulations are not adequate. The situation for Cu-NPs is more complicated as although not as acutely toxic as soluble Cu, the NPs induce equal, if not greater, sublethal toxicity to fish. Abiotic factors affect Cu-NPs similarly to $CuSO_4$ in some cases, whilst causing more extreme or different reactions in others. From the results seen here and those presented in the literature it is clear that the fate, behaviour, and ADME of Cu-NPs is complicated and requires further elucidation, though in the short term at least current legislation is probably adequate in order to protect the environment. However, in terms of protecting human health, NP levels measured in edible fish fillets (muscle) remained low throughout; indicating that consumption of contaminated fish would not be a concern for TiO_2 NPs and Cu-NPs.

It is clear that the technical ability to successfully investigate the effect of NPs on aquatic organisms is currently hampering efforts. Current techniques such as ICP-OES analysis do not allow differentiation between different versions of the same basic material (e.g., Cu-NPs and Cu^{2+}), with high naturally occurring environmental levels of these elements making NP quantification extremely difficult. This could be overcome if the NPs were comprised of an unusual Cu isotope not commonly found in appreciable levels, but this does not appear to be the case. More rare elements may be easier to quantify in the environment,

but in terms of common usage, NPs based on elements rarely found in measurable quantities in the environment is unusual. Furthermore, many NPs have proven difficult to measure in fish tissue due to limitations of the instruments used in analytical chemistry.

7.8.1. Future work and recommendations

- The role, if any, of the spleen in NP ADME requires further investigation following its implication in a few studies to date.
- A dietary Cu-NP experiment would still be a necessary project in order to make comparisons with waterborne Cu-NP exposure (considering the effects seen to the intestine of fish exposed to waterborne Cu-NPs here), as well as a comparison between a NP with an essential metal base and one without (i.e., Ti from TiO_2 NPs).
- During dietary exposures it may be prudent to prepare diets in different ways in order to assess potential differences in ADME and toxicity. These would include biologically incorporated NPs (i.e., in prey items), NPs bound to the surface of feed items, and NPs incorporated into fish feeds during the manufacturing process.
- Future studies on embryo toxicity should start at < 2 hpf in order to assess the role of the chorion in NP toxicity, or perhaps expose dechorionated embryos, though this is a less attractive proposal.
- Standard reference materials and tests are a necessity in order to make some informed decisions on the future direction of nanotoxicology and risk assessment. This would then enable a range of test conditions and abiotic factors to be analysed with the standardised tests enabling effective comparison.
- The affects of temperature on NP toxicity are needed, particularly as some studies have shown dissolution to be temperature related. The studies on abiotic factors here

were conducted at 28 °C, and it may be that colder water temperatures will yield different results, although the acute toxicology of Cu-NPs did not appear dissimilar between trout exposed at 16 °C and zebrafish at the higher temperature.

- If aggregation and subsequent precipitation of NPs is a common effect in water (particularly under a range of conditions), then further toxicity tests with flatfish, for example, may be important, as well as assessing food chain effect with, for example, benthic filter feeders.
- Further studies using the filtration method of separating different size factions of a NP stock (described previously in section 7.5) would be an interesting way of assessing the issue of particle size affects on toxicity. However, in order to negate further dissolution of NPs over time following filtration it may be necessary to limit the exposure time (e.g., 24 h exposure rather than 96 h).
- Studies assessing the effects on fish from brackish water habitats may be valuable as, for example, increased aggregation and decreases in particle numbers can occur through small increases in salinity (e.g., 2.5 ppt, Stolpe and Hassellöv, 2007), meaning the interface between fresh and estuarine water may produce different toxicity results, with data currently lacking.
- In order to further assess the effects of pH on Cu-NP toxicity it would be valuable to expose the fish at lower concentrations of Cu-NPs than were used in pH experiments here (Chapter 6) and also to carry out dissolution rate experiments at various levels of pH. This would then allow a more comprehensive understanding of the effects of pH, the role of dissolved Cu ions in toxicity and whether pH influenced dissolution.
- In view of the issues of NP aggregation and/or dissolution over time it is worthwhile comparing the effects of static versus semi-static exposure bioassays in order to assess possible affects on toxicity. The question remains however, do we leave the NPs in an

experimental aquaria without agitation or renewal in order to be environmentally realistic or do we refresh daily in order to see potential nano effects and reduce the possibility of dissolved ions affecting toxicity? Arguably, both may be environmentally realistic depending upon the nature of the release of NPs into the environment, but it seems more likely at present that the former (e.g., a single pollution episode) would be the more relevant scenario rather than one of continued NP release.

- Ongoing work is currently focusing on the efficacy of single particle ICP-MS with a view to improving the quantification of TiO_2 NPs in fish tissue. If successful, this would represent a significant breakthrough for nano-ecotoxicologists.

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Appendix

Copies of the published peer reviewed papers associated with this thesis.

1. 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. Permission to reproduce this paper granted by Springer Science and Business Media (License number: 2734250077198).

2. Shaw, B.J., Handy, R.D., 2011. Physiological effects of nanoparticles on fish: A comparison of nanometals versus metal ions. Environ. Int. 37, 1083-1097. Permission to reproduce this paper granted by Elsevier Limited (License number: 2734250455412).

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Dietary exposure to titanium dioxide nanoparticles in rainbow trout, (*Oncorhynchus mykiss*): no effect on growth, but subtle biochemical disturbances in the brain

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Abstract Our laboratory recently reported gut pathology following incidental ingestion of titanium dioxide nanoparticles (TiO_2 NPs) during aqueous exposures in trout, but *there are almost no data on dietary exposure to TiO_2 NPs in* fish. The aim of this experiment was to observe the sub-lethal effects of dietary exposure to TiO_2 NPs in juvenile rainbow trout (*Oncorhynchus mykiss*). Stock solutions of dispersed TiO_2 NPs were prepared by sonication without the use of solvents and applied to a commercial trout diet. Fish were exposed in triplicate to either, control (no added TiO_2), 10, or 100 mg kg^{-1} TiO_2 NPs diets for 8 weeks followed by a 2 week recovery period where all fish were fed the control diet. TiO_2 NPs had no impact on growth or nutritional performance, and no major disturbances were observed in red or white blood cell counts, haematocrits, whole blood haemoglobin, or plasma Na^+. Ti accumulation occurred in the gill, gut, liver, brain and spleen during dietary TiO_2 exposure. Notably, some of these organs, especially the brain, did not clear Ti after exposure. The brain also showed disturbances to Cu and Zn levels (statistically significant at weeks 4 and 6; ANOVA or Kruskal–Wallis, $P < 0.05$) and a 50% inhibition of Na^+K^+-ATPase activity during TiO_2 NP exposure. Na^+K^+-ATPase activity was unaffected in the gills and intestine. Total glutathione in the gills, intestine, liver and brain were not affected by dietary TiO_2 NPs, but thiobarbituric acid reactive substances (TBARS) showed up to 50% decreases in the gill and intestine. We conclude that TiO_2 NPs behave like other toxic dietary metals where growth rate and haematology can be protected during sub-lethal exposures, but in the case of TiO_2 NPs this may be at the expense of critical organs such as the brain and the spleen.

Keywords Titanium dioxide nanoparticles · Rainbow trout · Dietary exposure, Na^+K^+-ATPase · TBARS · Glutathione

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Introduction

Engineered nanoparticles (NPs) are novel materials with at least one dimension <100 nm and there are concerns about the fate and ecotoxicity of these materials in the aquatic environment (reviews, Moore 2006; Nowack and Bucheli 2007; Handy et al. 2008a). There are many different types of manufactured nanomaterials (e.g., nanometals, carbon nanotubes, C_{60} fullerenes, composites, polymers) and the potential benefits to society are vast including applications in environmental remediation, drug delivery, electronics, building materials, textiles, and cosmetics (Aitken et al. 2006). However, data are needed for environmental risk assessment of these new materials; especially on uptake and biological effects (Owen and Handy 2007; Crane et al. 2008; Handy et al. 2008a, b).

TiO_2 NPs are used in a range of commercially available products such as cosmetics, sunscreens, paint, and building materials (Aitken et al. 2006). The bulk form of ordinary TiO_2 powder is not considered toxic and has been used as a negative control in respiratory toxicity studies (e.g., Warheit et al. 1997), and as an inert dietary marker for fish nutrition studies (e.g., Lied et al. 1982). However, several studies with fine and ultrafine (<100 nm) TiO_2 have demonstrated respiratory toxicity in rodents (e.g., Ferin and Oberdörster 1985; Ferin et al.

1991; Oberdörster et al. 1992; Bermudez et al. 2004; Warheit et al. 2005).

Only a few ecotoxicological studies have been carried out using TiO_2 NPs, and most of these have used waterborne rather than dietary exposures. *Daphnia magna* showed mortality during exposure to TiO_2 NPs, depending on the method of NP preparation (Lovern and Klaper 2006). Zhang et al. (2007) showed that exposure to TiO_2 NPs can also influence the uptake of other pollutants, with carp experiencing 146% more Cd uptake in the presence of TiO_2 NPs compared to Cd only controls. Recently, our laboratory exposed juvenile rainbow trout to 0–1.0 mg l^{-1} TiO_2 NPs for up to 14 days and found a range of toxic effects and organ pathologies including evidence of oxidative stress, respiratory toxicity, and erosion of the gut epithelium (Federici et al. 2007). The latter was probably caused by stress-induced drinking, and raised the possibility that TiO_2 NPs might be toxic via the oral exposure route (see Federici et al. 2007).

To our knowledge, there are no detailed reports of dietary TiO_2 NP exposure in fish and only a few reports on oral toxicity in mammals. Wang et al. (2007) exposed mice to either nano-sized (25 or 80 nm) or fine (155 nm) TiO_2 particles by single oral gavage. No acute mortality occurred, but changes to serum biochemistry and liver pathology were observed. Toxic effects have also been seen in mice exposed to oral nano copper or zinc. Chen et al. (2006) used a single oral gavage of Cu–NPs (108–1,080 mg kg^{-1}, 23.5 nm NPs) in mice and reported pathologies in the kidney, spleen and liver. Wang et al. (2006) observed lethargy, vomiting, diarrhoea, and some mortality in mice exposed to nano zinc via the oral route (5 g kg^{-1} body weight, 58 nm NPs). Despite the fact that these oral studies on rodents have used large doses of NPs, there are some concerns about the dietary hazard. Notably, in both rodents and fish metal accumulation in the internal organs raises the possibility that NPs may cross the gastrointestinal barrier. However, the effect of NPs on the nutritional performance of animals is currently unknown.

The aim of the current study was to provide some of the first toxicological observations on sub-lethal dietary exposure to TiO_2 NPs in rainbow trout, and to enable some comparison with our previous experiments on aqueous exposure (Federici et al. 2007). Our goal was simply to establish whether or not this material was toxic via the dietary route compared to an unexposed control, and used a well established nutrition trial experimental design that is identical to our previous work on dietary metals (e.g., Cu, Handy et al. 1999; Shaw and Handy 2006). We adopted a body systems approach similar to Federici et al. (2007) and measured key areas of physiology such as growth, osmoregulation, haematology, biochemical responses of organs, and a range of nutritional parameters. In addition, because we have observed latent toxic effects of dietary metals in fish (e.g., Shaw and Handy 2006), this experiment also includes a period on a control diet at the end of the exposure to look for post-exposure effects.

Methodology

Experimental design

Juvenile rainbow trout ($n = 400$) were obtained from Exmoor Fisheries, Somerset, UK, and held for 10 days in a stock aquaria with flowing, aerated, dechlorinated Plymouth tap water (see below). Fish were then transferred into a recirculation system (with 10% water renewal per day) consisting of nine 120 l experimental fibre glass aquaria (40 fish/tank; identical water conditions) and acclimated for 14 days prior to experiments. Fish were individually weighed (mean ± SEM, $n = 360$, 21.63 ± 0.15 g) and three tanks per treatment were randomly allocated. Fish were exposed in triplicate to one of the following treatments for 8 weeks: control diet (no added TiO_2 NPs), 10 or 100 mg kg^{-1} dry weight feed TiO_2 NPs (see below for diet formulation). This was followed by a 2 week recovery period where all fish were fed the control diet. The TiO_2 NP concentration in the feed was selected after considering TiO_2 NP toxicity in our previous waterborne exposure experiments (Federici et al. 2007) and our previous experience of dietary metal toxicity in fish (e.g., Shaw and Handy 2006). Fish were fed to satiation twice each day (1000 and 1600 hours), and behaviour was monitored during each feeding event. Care was also taken to ensure that all the feed added to the tanks was eaten. The self-cleaning design of the aquarium system also ensured that faecal waste was quickly removed from the tanks. Ti analysis of the water before and after feeding also confirmed that no TiO_2 leached from the food. Background levels of Ti remained low in the water (<25 ng l^{-1}). Separate leaching experiments with food pellets showed no release of Ti from the food (data not shown).

Water samples were taken each day for pH, temperature, and dissolved oxygen (all measured with a HACH HZ40d multi meter). Water samples were also collected three times each week (prior to feeding) for total ammonia, nitrite and nitrate (HI95715, HI 93707, and HI 93728 Hanna Instruments respectively). There were no treatment differences in water quality between tanks (ANOVA, $P > 0.05$). Values were (mean ± SEM, $n = 68$ or 30 samples) pH, 7.09 ± 0.04; temperature, 15.46 ± 0.07°C; oxygen saturation, 90.6 ± 0.48%; total ammonia, 0.29 ± 0.07 mg l^{-1}, nitrite, 0.35 ± 0.06 mg l^{-1}, and nitrate, 1.96 ± 0.18 mg l^{-1}. The photoperiod was set to a 12 h light:12 h dark cycle. The electrolyte composition of the dechlorinated tap water used

for the experiments was 0.3, 0.1, and 0.4 mmol l^{-1} for Na^+, K^+ and Ca^{2+} respectively. Fish were randomly sampled at the start of the experiment (initial fish), and then every 2 weeks during the experiment for haematology, plasma ions, tissue electrolytes, histopathology, biochemistry, nutritional performance and growth.

Titanium dioxide NP stock solution

The titanium dioxide NPs used here was from the same batch that has been previously characterised by our laboratory and are reported in Federici et al. (2007). The preparation of stock solutions, and confirmation of the level of dispersion, was carried out according to Federici et al. (2007). Briefly, dry powder of TiO_2 NPs ("Aeroxide" P25 TiO_2, DeGussa AG, supplied via Lawrence Industries, Tamworth, UK) made of (revised manufacturer's information); crystal structure of approximately 25% rutile and 75% anatase TiO_2, purity was at least 99% TiO_2 (maximum impurity stated was 1% Si), and an average particle size of 21 nm with a specific surface area of 50 ± 15 m^2 g^{-1}. Chemical analysis of stock solutions revealed no metal impurities and the batch purity was high (data not shown), with a measured mean primary particle size of 24.1 ± 2.8 nm (mean $\pm$ SEM, $n = 100$ electron microscope images, see Federici et al. 2007). A 10 g l^{-1} stock solution of TiO_2 NPs was made (no solvents) by dispersing the NPs in ultrapure (Millipore) water with sonication (bath type sonicator, 35 kHz frequency, Fisherbrand FB 11010, Germany) for 6 h.

Diet formulation

The control diet was a commercial fish food; Advanced Fish Feed Trout Excel 18 (2 mm pellets), with fish progressing onto a mixture of this feed and Trout Excel 30 (3 mm pellets) at week 5 as their body size increased. Proximate composition of the diets was (% of dry diet from manufacturer's guidelines, Trout Excel 30 diet in brackets): lipid 18 (21); protein 50 (46); ash 8 (8); fibre 1 (1); phosphorus 1.2 (1.2). In order to obtain experimental diets, the NP stock solutions (above) were sonicated for 8 h, and then either 1 or 10 ml of stock solution was added to 49 or 40 ml of ultrapure water to make a 0.2 or 2.0 g l^{-1} TiO_2 NP dilution that could be sprayed on to the food for the 10 and 100 mg kg^{-1} treatments respectively. The approach of spraying diets with metal solutions is a well established method in nutritional ecotoxicology (e.g., Handy et al. 2005; Shaw and Handy 2006) and is highly relevant to mimic the effects of materials known to adsorb onto the surfaces of prey organisms. The diluted TiO_2 solutions were sonicated for a further 15 min just before spraying to ensure even delivery of the material through the spray nozzle. One kg of commercial feed was place in a commercial food mixer (Kenwood Catering Professional food mixer XKM810) and gradually sprayed with the appropriate TiO_2 NP solution. The TiO_2 NP immediately coated the feed, and was then sealed in by spraying the food with a 10% bovine gelatine (BDH, Poole, UK) solution. The gelatine coat was allowed to dry, after which the feed was transferred into airtight containers for storage. The control diet was prepared in exactly the same way, except that the TiO_2 solution was replaced by an equal volume of ultrapure water. Titanium metal concentrations in the diets were confirmed by ICP-OES following nitric acid digestion (as in tissue ion analysis below) and were 5.4 and 53.6 mg kg^{-1} feed weight of Ti metal respectively. Our calibrations showed that Ti metal forms 59.9% of the TiO_2 (data not shown) and equates to recoveries of 90 and 89% of the nominal TiO_2 NP concentrations in the 10 and 100 mg kg^{-1} TiO_2 NP diets respectively.

Growth and nutritional performance

Growth and nutritional performance were measured according to Handy et al. (1999) with minor modifications. Briefly, food intake was calculated for each tank by weighing food containers before and after feeding. All fish were individually weighed at the start of the experiment, and every 2 weeks thereafter. Individual fish weights were used in growth rate calculations. Specific growth rate (SGR), feed conversion ratio (FCR), and feed conversion efficiency (FCE), were calculated as previously described (Handy et al. 1999) for: (1) the TiO_2 NP exposure phase (weeks 0–8), (2) the recovery phase (weeks 8–10), and (3) the entire experiment (weeks 0–10). Condition factor and hepatosomatic index (HSI) for each fish was also determined (Handy et al. 1999). The spleen index (SI% = spleen weight (g)/body weight (g) $\times$ 100) was also measured. Two fish per tank (6/treatment, $n = 5$ for initial fish) were also terminally anaesthetised with MS222 at weeks 0 and 8, and stored at -20°C for proximate composition of the whole fish (ash, lipid, protein and moisture) according to Handy et al. (1999).

Haematology and analysis of blood plasma

Haematology, plasma ions and osmometry were performed exactly as described in Smith et al. (2007). Briefly, two fish were randomly collected from each tank (six fish/treatment and initial fish) at weeks 0, 2, 4, 6, 8, and 10 and carefully anaesthetised with buffered MS222. Whole blood was collected via the caudal vein into heparinised syringes, and the fish weight and total length was recorded. Haematological measurements included haematocrit (Hct), haemoglobin concentration (Hb), and calculated mean erythrocyte cell volume (MEV), and mean erythrocyte haemoglobin

content (MEH) according to Handy and Depledge (1999). Whole blood (20 µl) was fixed in Dacie's fluid for red and white blood cell counts. The remaining blood was centrifuged (13,000 rpm for 2 min, Micro Centaur MSE), and the serum collected. The serum was stored at −20°C until subsequent analysis of plasma ions and osmometry as described in Smith et al. (2007). Plasma protein was determined using the Bio-Rad (Bradford) protein assay kit II, and plasma glucose was measured according to Sigma Diagnostics procedure No. 315 glucose (Trinder).

Tissue ion analysis

Following blood sampling, fish were terminally anaesthetised with MS222 and dissected for tissue ion analysis. Gill, liver, intestine, spleen, skinned muscle from the flank, and whole brain were harvested, then processed for ion analysis as described in Smith et al. (2007) with minor modifications. Briefly, tissues were oven dried to a constant weight, then digested in 1 or 4 ml of concentrated nitric acid. In order to disperse TiO_2 NPs in the tissue digests, a few millilitres of an appropriate Triton x-100 stock solution (prepared in ion-free ultra pure water) was slowly added to each of the digested tissue samples to achieve a final concentration of 2% Triton x-100 in each tube. Each sample was then diluted to a final volume of either 5 or 20 ml with ultra pure water and analysed for Ti, Cu, Zn, Mn, Ca, Na and K by inductively coupled plasma optical emission spectrometry (ICP-OES, Varian 725 ES). Analytical grade standards and reference materials were used throughout.

Biochemistry

Biochemistry was performed exactly as described in Smith et al. (2007). Briefly an additional two fish were randomly collected from each tank (6 fish/treatment, and initial fish) at weeks 0, 2, 4, 6, 8, and 10 for biochemistry. Gill, liver, intestine, and whole brain were removed and immediately snap frozen in liquid nitrogen, then stored at −80°C until required. Tissues (about 0.5 g or the whole brain) were homogenised (Cat X520D with a T6 shaft, medium speed, Bennett & Co., Weston-super-Mare) in five volumes (2.5 ml) of ice-cold isotonic buffer [in mmol l^{-1}; 300 sucrose, 0.1 ethylenediamine tetra acetic acid (EDTA), 20 (4-(2-hydroxyethyl)piperazine-1-ethane sulfonic acid (HEPES)), adjusted to pH 7.8 with a few drops of Tris (2-amino-2-hydroxylmethyl-1,3-propanediol)]. Crude homogenates were stored in 0.5 ml aliquots at −80°C until required. Tissue homogenates were analysed (in triplicate) for Na^+K^+-ATPase activity (15 µl of homogenate), and thiobarbituric acid reactive substances (TBARS, 40 µl of homogenate) and total glutathione (GSH, 20–40 µl of homogenate) exactly as described in Smith et al. (2007).

Statistical analysis

All data were analysed by StatGraphics Plus version 5.1. No tank effects were observed throughout the experiment, so data was pooled by treatment for statistical analysis. After checking for kurtosis, skewness and unequal variance (Bartlett's test), data were tested for treatment or time effects by ANOVA followed by Fisher's 95% least-squares difference, at 95% confidence limits. For non-parametric data, where data transformation was not effective, the Kruskal–Wallis test was used and differences located by notched box and whisker plots. Results are presented as mean ± SEM unless otherwise specified.

Results

Dietary exposure to titanium dioxide nanoparticles

There was a small background incidence of mortality (2% in total), that is typical of juvenile trout in recirculation sysytems, and not associated with any one treatment. A total of nine mortalities were recorded during the experiment; 2, 4 and 3 fish from the control, 10 and 100 mg kg^{-1} TiO_2 NP treatments respectively. Most of these fish were small and were probably subordinate fish that died as a result of aggression. The remaining fish did not show any visible signs of ill health and retained normal swimming behaviours throughout.

Total Ti concentrations in the tissues of the fish are shown in Fig. 1. Elevated levels of TiO_2 (as Ti metal) were observed in fish from both TiO_2 NP treatments compared to the controls (Fig. 1). The gill, gut and livers from both TiO_2 NP treatments showed statistically significant increases in Ti compared to control from weeks 4 or 6 of exposure, and Ti levels generally remained elevated compared to controls in the post-exposure period; especially in the fish from the highest TiO_2 NP treatment (Fig. 1). Notably, hepatic Ti levels peaked at week 4 in the TiO_2 NP treatments, and at the highest exposure concentration Ti levels showed a gradual but statistically significant decrease over time, suggesting some partial elimination of Ti from the liver (Fig. 1). There was also a time effect in the intestine of control fish, with Ti decreasing over time (Fig. 1). This reflects a reduction in the background dietary Ti intake associated with switching from farm food to our experimental feeds at the start of the experiment. Apart from some background noise, there was no treatment or

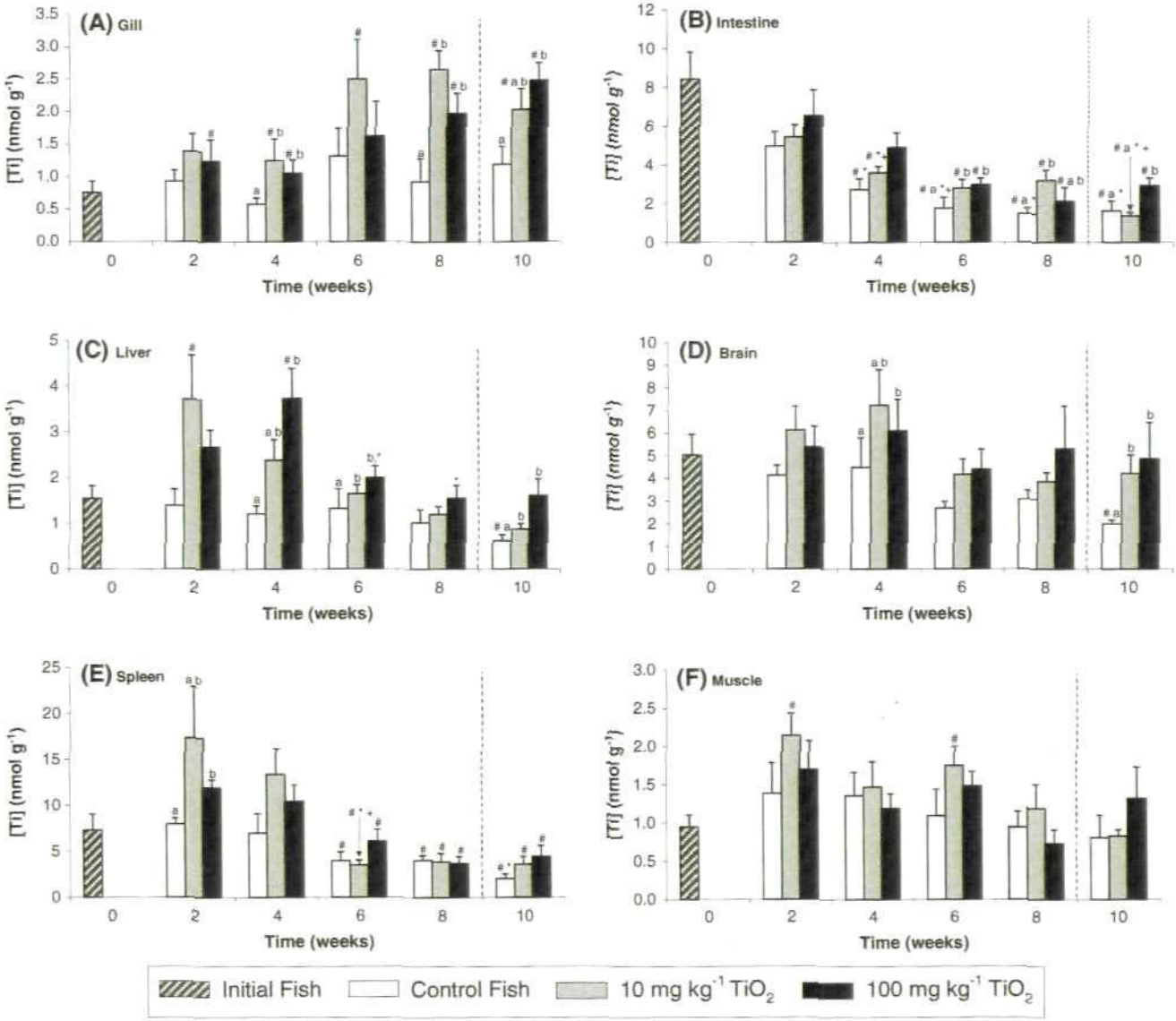

Fig. 1 Titanium metal levels in the gill (**a**), intestine (**b**), liver (**c**), brain (**d**), spleen (**e**) and (**f**) muscle of trout after exposure to 0 (*clear bars*), 10 (*grey bars*) or 100 (*black bars*) mg kg^{-1} TiO_2 NP for 8 weeks, followed by a 2 week recovery period (week 10) with all fish fed on normal food. The *dashed line* indicates the end of exposure and the return of all fish to normal food ("recovery phase"). Diagonal hatched *bars* are initial (day 0) fish. Data are mean ± SEM, nmol Ti g^{-1} dry weight tissue, $n \approx 6$ fish. *Different letters* within a time point indicate significant differences within each tissue (ANOVA or Kruskal–Wallis, $P < 0.05$). # Significant time effect compared to initial fish (ANOVA or Kruskal–Wallis, $P < 0.05$). + Significant time effect within treatment compared to the previous time point (ANOVA or Kruskal–Wallis, $P < 0.05$)

time-related changes in Ti levels in the muscle. The brain of exposed fish showed transient increases of Ti in both TiO_2 NP treatments at week 4 and 10 compared to controls (Kruskal–Wallis, $P < 0.05$, Fig. 1). The spleen showed the highest Ti levels of any tissue, and spleen Ti concentrations increased earlier than other tissues (week 2 instead of week 4 or 6) in the treatments compared to controls (statistically significant, Kruskal–Wallis, $P < 0.05$). However, spleen Ti levels then sharply decreased suggesting that the exposed fish were able to regulate excess TiO_2 in the spleen (Fig. 1).

Growth and nutritional performance

Fish from all treatments gained body weight during the experiment (Fig. 2), with no statistically significant differences in mean final weights (ANOVA, $P > 0.05$), or big differences in specific growth rates (overall SGR by week 10; 2.30, 2.47 and 2.57% day^{-1} for controls, 10 and 100 mg kg^{-1} TiO_2 respectively). No differences were seen in the time to start feeding, or time spent feeding (data not shown), and food refusal or regurgitation of feed was not observed. Mean daily ration size, FCR, and FCE were

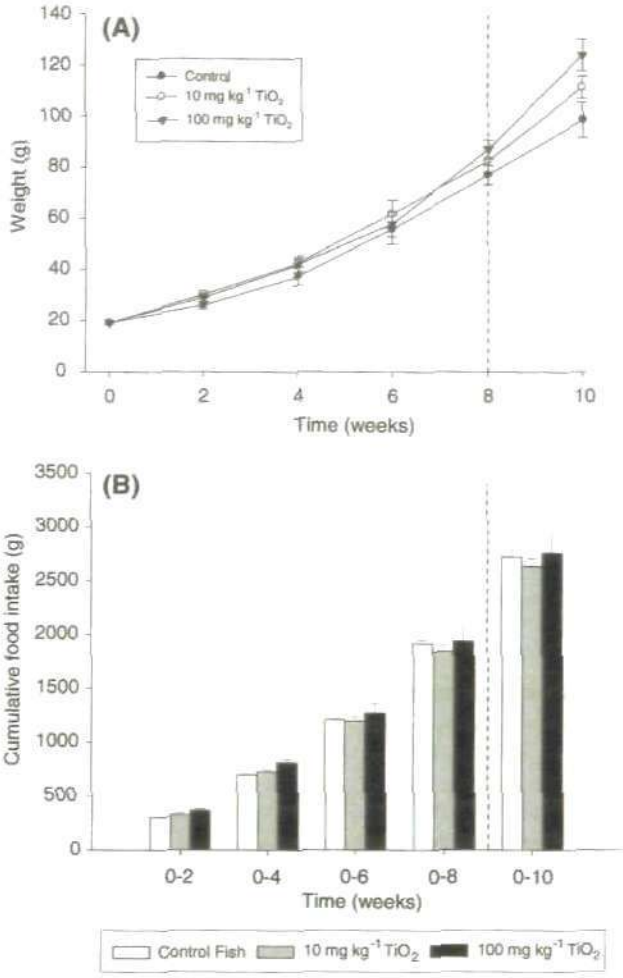

Fig. 2 Body weight (**a**) and cumulative food intake (**b**) in rainbow trout fed 0, 10 or 100 mg kg^{-1} TiO_2 NPs for 8 weeks, followed by a recovery period with all fish fed normal food (no TiO_2 NP) for a further 2 weeks. In panel (**a**) data are mean ± SEM, $n = 18$ fish per treatment at each time point. In panel (**b**) data are means of triplicate tanks for each treatment. The *dashed line* indicates the end of exposure and the return of all fish to normal food ("recovery phase")

similar for all treatments (e.g., overall FCR by week 10; 1.22, 1.16, 1.05 for controls, 10 and 100 mg kg^{-1} TiO_2 respectively). There were no time or treatment effects on condition factor or HSI (data not shown). However, the spleen index increased twofold in fish exposed to TiO_2 compared to controls at week 8 (% of body weight, means, $n = 5$–6 fish); 0.08 ± 0.01, 0.16 ± 0.03, 0.16 ± 0.03 for controls, 10 and 100 mg kg^{-1} TiO_2 respectively (ANOVA, $P < 0.05$), but recovered to control levels by week 10 when all fish were fed the control diet. Carcass proximate composition was unaffected by TiO_2 exposure (protein remained between 53 and 54%; ash, 7% for all treatments), except for a statistically significant decrease in lipid in the carcasses of fish fed the 100 mg kg^{-1} TiO_2 diet at week 8 compared to the controls or the 10 mg kg^{-1} TiO_2 treatment (ANOVA, $P < 0.05$). The % lipid was (means, $n = 5$–6 fish); 24.72 ± 1.38, 23.61 ± 1.30, and 20.42 ± 1.23 for controls, 10 and 100 mg kg^{-1} TiO_2 respectively.

Haematology and blood plasma analysis

Dietary exposure to TiO_2 NPs did not cause any major haematological disturbances, with values remaining within the normal range for trout. For example, whole blood haemoglobin levels remained between 5 and 7 g dl^{-1}, haematocrits between 30 and 35%, and red cell counts between 0.4 and 0.6 cells × 10^3 mm^3 throughout the experiment. Total white blood cell counts were more variable, and ranged between 13 and 30 cells × 10^3 mm^3 with no statistically significant treatment effect; apart from a transient increase in white blood cells at week 2 in the fish exposed to 10 mg kg^{-1} TiO_2 NPs (ANOVA, $P < 0.05$). In the blood plasma, there were no treatment-dependent differences in osmolarity, glucose or Na^+ concentrations (ANOVA, $P > 0.05$) with values remained between 252 and 342 mOsm kg^{-1}, 2.9 and 4.9 mmol l^{-1}, and 128 and 169 mmol l^{-1} respectively. However plasma K^+ showed a small, but statistically significant increases at week 8 in both TiO_2 treatments compared to controls (means, $n = 5$–6 fish); 4.3 ± 0.1, 4.5 ± 0.1, 4.9 ± 0.2 mmol l^{-1} for controls, 10 and 100 mg kg^{-1} TiO_2 NP treatments respectively. This effect on K^+ was lost by week 10.

Tissue electrolytes, trace metals and moisture content

Fish tissues (gill, intestine, liver, spleen, muscle, whole brain) were analysed for the major tissue electrolytes (Na^+, K^+, Ca^{2+}) and some trace elements (Cu, Zn, Mn). There were no time or treatment effects on tissue K^+ or Ca^{2+} (data not shown; ANOVA or Kruskal–Wallis, $P > 0.05$). Tissue Na^+ did exhibit some transient changes which were statistically significant in the gill, liver and spleen (ANOVA or Kruskal–Wallis, $P < 0.05$). However no clear treatment-dependent trends were observed overall, with the Na^+ data remaining within the normal range for rainbow trout. For example, Na^+ concentrations for controls and 100 mg kg^{-1} TiO_2 NP treatment were (means, $n = 6$, μmol g^{-1} dry weight); 206.4 ± 38.0 and 361.2 ± 117.6 (gill); 93.2 ± 4.0 and 152.1 ± 47.8 (liver); 66.4 ± 5.9 and 141.1 ± 67.1 (spleen).

Exposure to dietary TiO_2 NPs caused some statistically significant decreases in Cu levels in the intestine, brain and spleen of both TiO_2 treatments at some time points (Kruskal–Wallis, $P < 0.05$, Fig. 3). Notably, Cu depletion in spleen (Fig. 3) was coincident with the Ti peak in the tissue (Fig. 1). Some treatment-dependent and transient elevations in tissue Zn were also noted. Zinc levels in spleen of the 10 mg kg^{-1} TiO_2 NP treatment at week 4 were elevated to almost twofold that of control and the highest TiO_2 treatment

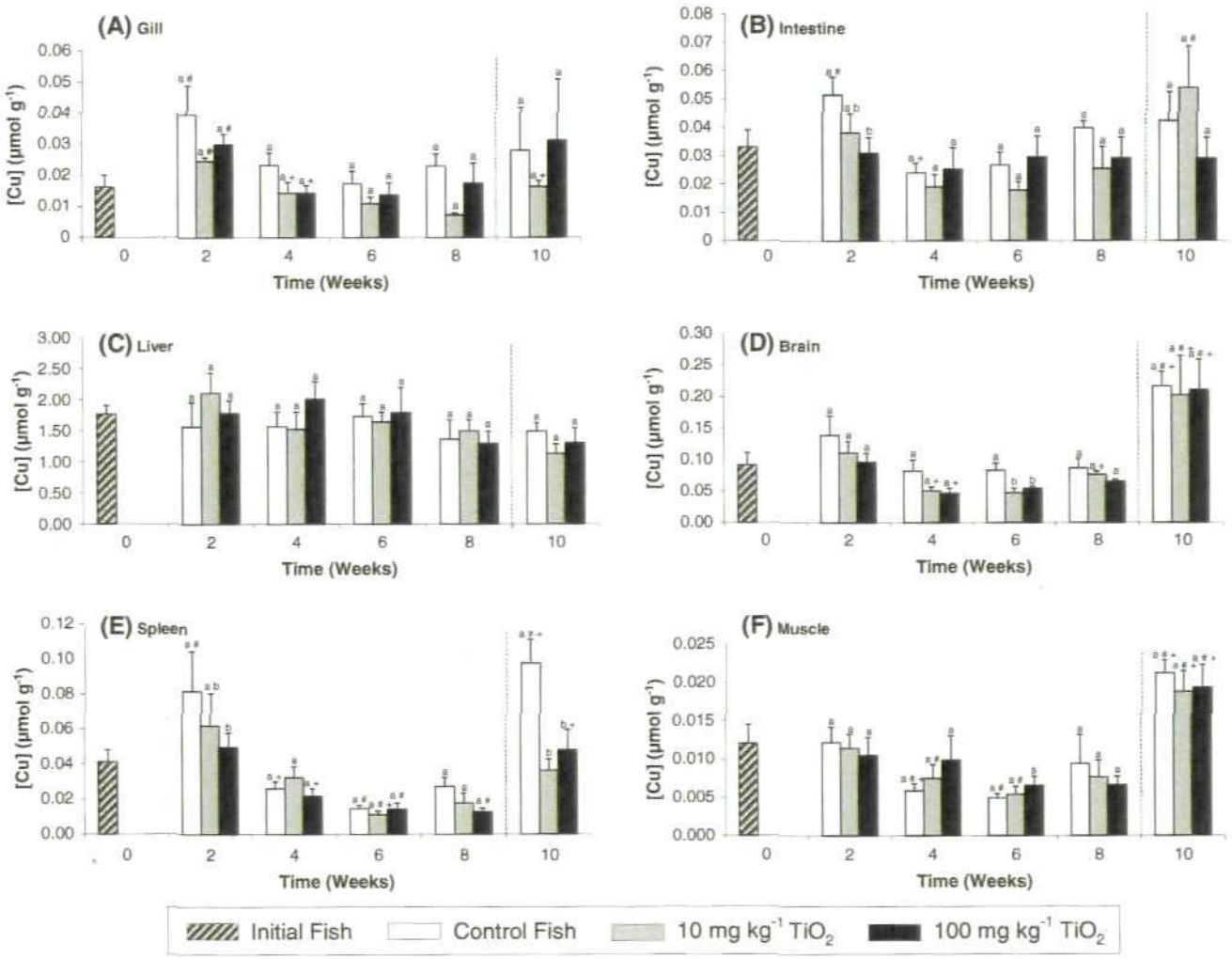

Fig. 3 Copper levels in the gill (**a**), intestine (**b**), liver (**c**), brain (**d**), spleen (**e**) and (**f**) muscle of trout after exposure to 0 (*clear bars*), 10 (*grey bars*) or 100 (*black bars*) mg kg^{-1} TiO_2 NP for 8 weeks, followed by a 2 week recovery period (week 10) with all fish fed normal food. The *dashed line* indicates the end of exposure and the return of all fish to normal food ("recovery phase"). Diagonal hatched *bars* are initial (day 0) fish. Data are mean ± SEM, µmol Cu g^{-1} dry weight tissue, $n = 6$ fish. *Different letters* within a time point indicate significant differences within each tissue (ANOVA or Kruskal–Wallis, $P < 0.05$). # Significant time effect compared to initial fish (ANOVA or Kruskal–Wallis, $P < 0.05$). + Significant time effect within treatment compared to the previous time point (ANOVA or Kruskal–Wallis, $P < 0.05$)

(Kruskal–Wallis, $P < 0.05$; means, $n = 6$, µmol g^{-1} dry weight; 1.76 ± 0.19, 3.16 ± 0.71; 2.45 ± 0.69 for control, 10 and 100 mg kg^{-1} TiO_2 respectively). Also in week 4, the brain tissue of fish from both TiO_2 treatments showed statistically significant increases in Zn compared to controls (Kruskal–Wallis, $P < 0.05$, means, $n = 6$, µmol g^{-1} dry weight; 0.89 ± 0.02, 1.02 ± 0.02; 1.03 ± 0.11 for control, 10 and 100 mg kg^{-1} TiO_2 respectively), although the effects in both these organs was lost by the end of the exposure phase. Tissue Mn was unaffected by exposure to dietary TiO_2 NPs (data not shown), apart from a transient increase in the Mn content of spleens from fish at the highest TiO_2 treatment at week 6 (statistically significant compared to controls, Kruskal–Wallis, $P = 0.00659$, means, $n = 6$, µmol g^{-1} dry weight; control, 0.016 ± 0.002; and 100 mg kg^{-1} TiO_2 NP treatment, 0.028 ± 0.010). Tissue moisture was not affected by dietary exposure to TiO_2 NPs (data not shown), apart from a transient decrease in spleen moisture content in the highest TiO_2 treatment at week 2 (statistically significant compared to controls, Kruskal–Wallis, $P < 0.0001$, means, $n = 6$, as %; control, 71.8 ± 2.9; 10 mg kg^{-1} TiO_2 NP, 65.5 ± 5.2; 100 mg kg^{-1} TiO_2 NP, 58.4 ± 3.2).

Na^+K^+-ATPase, TBARS and total glutathione

Na^+K^+-ATPase activities in the gill and intestine were unaffected by TiO_2 exposure, but the brain showed around

50% inhibition of Na^+K^+-ATPase activity at the end of the exposure phase that did not recover (ANOVA, $P < 0.05$, Fig. 4). There was no apparent TiO_2 dose-effect within the brain Na^+K^+-ATPase inhibition, with the 10 and 100 mg kg^{-1} diets causing the same level of inhibition.

Fish exposed to TiO_2 NPs generally showed a decrease in TBARS compared to controls at the end of the experiment (Fig. 5). Significant differences were seen in the gills and intestine of TiO_2 NP exposed fish at week 8 with maximum decreases of 49% (gill) and 50% (intestine) in the 100 mg kg^{-1} TiO_2 NP treatment compared to the control. TBARS in both gill and intestine did not recover. TBARS in the liver and brain were unaffected by exposure to TiO_2 NPs (ANOVA, $P > 0.05$).

Total glutathione (GSH) levels were measured in the gill, intestine, liver and brain homogenates with only the gill showing statistically significant changes in GSH. Levels of total glutathione in the intestine, liver and brain remained stable throughout with no treatment-dependent effects (ANOVA, $P > 0.05$). Values ranged between 0.49 and 2.13; 1.68 and 3.73 and 0.68 and 1.92 μmol g^{-1} wet weight tissue for intestine, liver and brain respectively. Following exposure to 100 mg TiO_2 NPs for 8 weeks, the gills displayed a statistically significant decrease in GSH compared to all other treatments and the initial fish (ANOVA, $P < 0.05$). Glutathione levels in the gills at week 8 were (means, $n = 6$): 1.28 ± 0.13, 1.46 ± 0.13, 1.24 ± 0.12 and 0.92 ± 0.08 μmol g^{-1} wet weight tissue for initial fish, control, 10 and 100 mg kg^{-1} TiO_2 NP treatments respectively. This treatment-effect was lost after fish were returned to the control diet, and by week 10 no statistical differences were observed between treatments (ANOVA, $P > 0.05$; means, $n = 6$): 1.30 ± 0.12, 1.24 ± 0.16, and 1.20 ± 0.10 μmol g^{-1} wet weight tissue for control, 10 and 100 mg kg^{-1} TiO_2 NP treatments respectively.

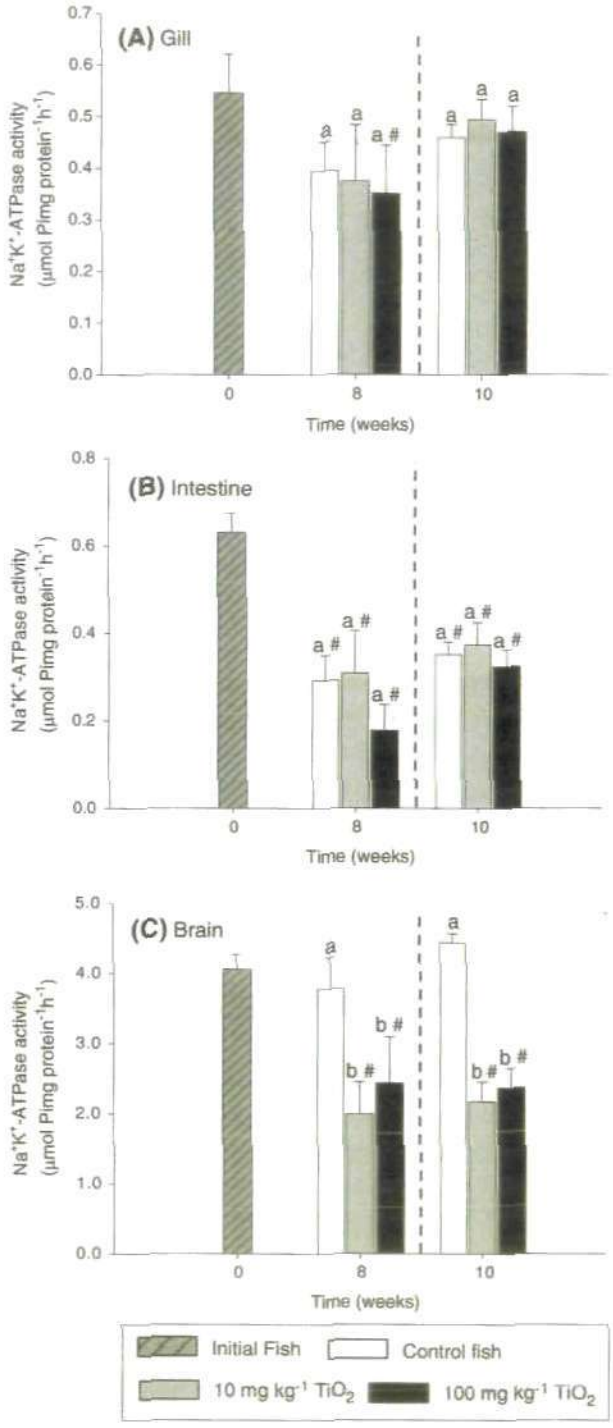

Fig. 4 Na^+K^+-ATPase activity in crude homogenates of the gill (**a**), intestine (**b**), and brain (**c**) of rainbow trout fed 0 (*clear bars*), 10 (*grey bars*) or 100 (*black bars*) mg kg^{-1} TiO_2 NPs for 8 weeks, followed by 2 week recovery (week 10). The *dashed line* indicates the end of exposure and the return of all fish to normal food ("recovery phase"). Data are mean ± SEM, $n = 6$ fish. *Different letters* within a time point indicate significant differences within each tissue (ANOVA or Kruskal–Wallis, $P < 0.05$). # Significant time effect compared to initial fish (ANOVA or Kruskal–Wallis, $P < 0.05$). Diagonal hatch *bar* are the initial fish at time zero collected immediately prior to starting the experimental diets

Discussion

This study is one of the first reports of dietary exposure to NPs in fish, and we show that juvenile rainbow trout will eat diets containing TiO_2 NPs, and can accumulate the Ti in the gut and other internal organs. Despite Ti accumulation, the fish had relatively normal growth rates, suggesting that nutritional performance was protected even though biochemical disturbances occurred in other organs such as the brain.

Dietary exposure protocols for TiO_2 NPs

In our experiment we used a commercially available NP ("Aeroxide" P25 TiO_2 NPs) because of the practical value to hazard assessment of using a material that is found in

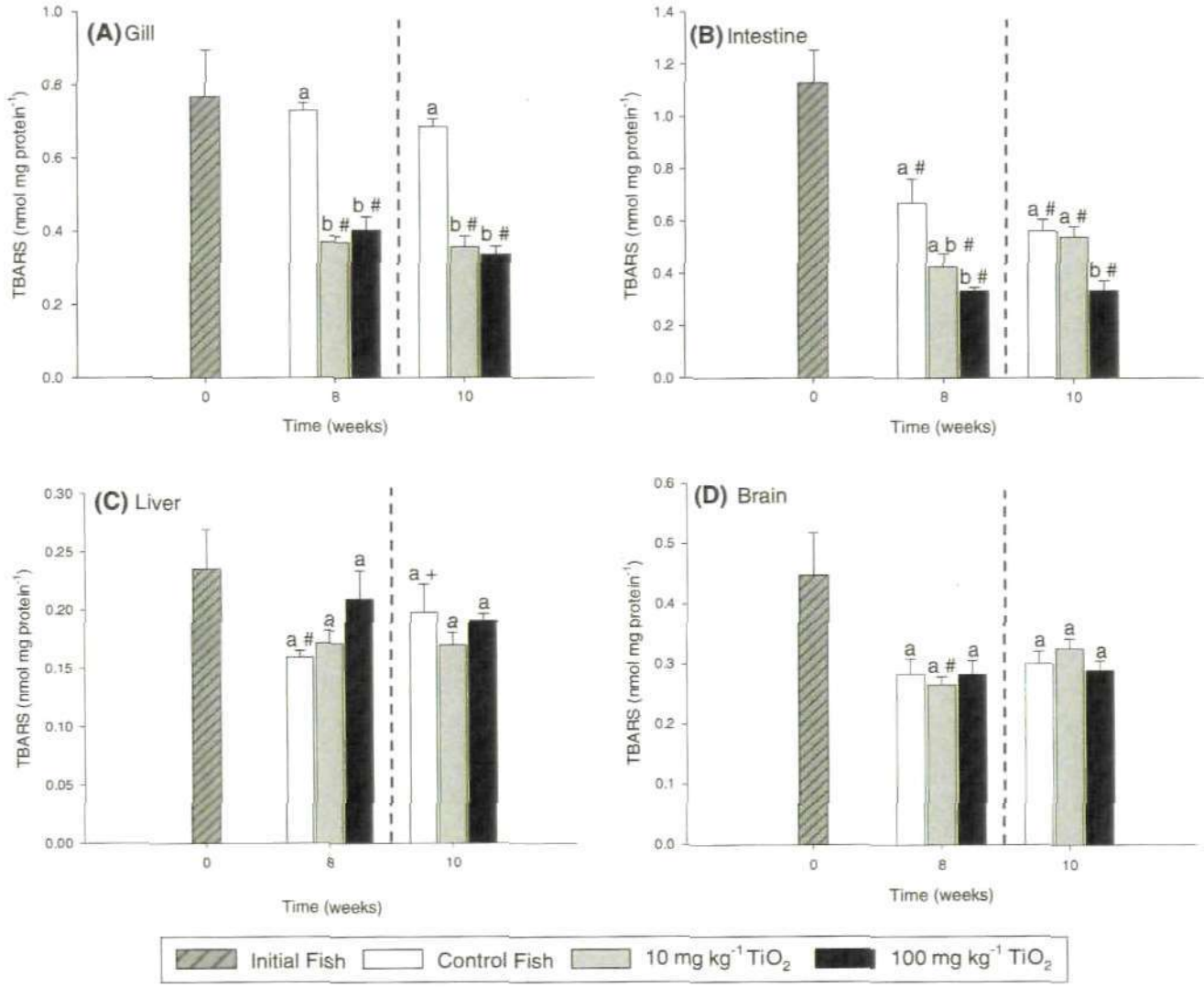

Fig. 5 Thiobarbituric acid reactive substances (TBARS) in the gill (**a**), intestine (**b**), liver (**c**) and brain (**d**) of rainbow trout fed 0, 10 or 100 mg kg^{-1} TiO_2 NPs for 8 weeks, followed by 2 week recovery period (week 10). The *dashed line* indicates the end of exposure and the return of all fish to normal food ("recovery phase"). Data are mean ± SEM, $n = 6$ fish. *Different letters* within a time point *indicate significant differences within each tissue (ANOVA or* Kruskal–Wallis, $P < 0.05$). # Significant time effect compared to initial fish (ANOVA or Kruskal–Wallis, $P < 0.05$). + Significant time effect within treatment compared to the previous time point (ANOVA or Kruskal–Wallis, $P < 0.05$). Diagonal hatch *bar* are the initial fish at time zero collected immediately prior to starting the experimental diets

many commercial products. We decided not to include a bulk TiO_2 (ordinary TiO_2 powder) as a "particle size" control (see discussions in Federici et al. 2007; Crane et al. 2008) because the proportions of different crystal structures, stoichiometry, zeta potential, surface area, aggregation kinetics, chemical reactivity, the presence of different impurities associated with manufacturing processes, and many other properties between the P25 TiO_2 particles we used and ordinary TiO_2 powders are different (e.g., Štengl et al. 2007; Warheit et al. 2007; Behnajady et al. 2008; Uzunova-Bujnova et al. 2008). It would therefore be extremely difficult to identify a particle size effect from all these other differences between commercially available bulk TiO_2 powders and the commercial TiO_2 NP used in our experiment. In addition, animal feeds already contain a myriad of different particulate matter (the food matrix) and it is not possible to control the particle size of the food itself.

There are also special ethical considerations for dietary TiO_2 experiments, which are different from aqueous studies. Ordinary TiO_2 powders have been used for many years as an inert marker in fish nutrition studies (e.g., Lied et al. 1982; Weatherup and McCracken 1998; Mamun et al. 2007), and are considered to be non-toxic in the food. A typical TiO_2 powder inclusions of 1% of dry matter (i.e., 10 g kg^{-1} food) is used in fish foods; this is orders of

magnitude higher than the TiO_2 NP inclusion in our experiment. It would therefore seem to be ethically questionable to repeat experiments with bulk TiO_2 powders (and use more live fish), when the nutritional safety of the bulk material is already established.

Furthermore, a bulk TiO_2 control may also be uninformative in a dietary study because of the background of natural titania already in the animal feed ingredients. This type of problem is well known for studies on dietary iron where the metal is so abundant in the earth's crust and in all the feed ingredients (e.g., fish meal) that it is technically impossible to make an iron-free basal diet (e.g., Carriquiriborde et al. 2004). The same situation applies to TiO_2, and we must accept that the experimenter has limited control over the titania levels in the basal diet. Attempts to dialyse or otherwise remove the background Ti are also problematic, as any harsh treatment would compromise the nutritional quality of the food (e.g., accidental removal of other trace metals and vitamins).

Dietary TiO_2 NP exposure and titanium accumulation in the tissues

The exposure can be regarded as sub-lethal, with a background 2% mortality (9 out of 400 fish died, no treatment-effect) typical of spontaneous losses of juvenile trout in aquaria (e.g., Handy et al. 1999). Dietary Ti exposure was verified by the measured Ti in the feed, the fact that the fish ate the food (Fig. 2), and that measurable increases in Ti metal were found in the tissues of the fish (Fig. 1), but not in the water.

There are almost no reports of background Ti levels in juvenile rainbow trout. In this study values in the control fish ranged between about 0.8–7 nmol g^{-1} dw, depending on the tissue examined (Fig. 1), and are broadly within the wide range reported for fish and shellfish (nmol–μmol g^{-1} levels, Bustamante and Miramand 2005; about 0.2 nmol g^{-1} in Atlantic salmon, *Salmo salar*, Dubé et al. 2005). The values in controls (Fig. 1) are lower than those we previously reported for trout (Federici et al. 2007), but this is easily attributed to differences in the supply of stock trout and the natural background of titania. In the latter study, the fish were obtained from a farm with higher natural Ti levels in the environment, and the fish were eating a commercial farm food containing more natural Ti. Nonetheless, trout showed measurable Ti accumulation in the gill, gut, liver, brain and spleen during dietary TiO_2 exposure compared to the controls in this study (Fig. 1). The levels of accumulation remained in the nmol g^{-1} range, despite the large mg levels in the food, suggesting that only a small fraction of the dietary dose was absorbed. This is consistent with other dietary metal studies (reviews, Clearwater et al. 2002; Handy et al. 2005). For Ti this is perhaps no surprise, given the use of bulk TiO_2 powder as an inert digestibility marker where only 1% or less of the ingested dose is accumulated (Vandenberg and De La Noüe 2001; Richter et al. 2003). The behaviour of the NPs during the diet preparation also suggests low bioavailability. The solutions used to spray the NPs onto the food were initially dispersed (as reported in Federici et al. 2007), but the material (not surprisingly) quickly aggregated onto the surface of the food matrix.

Dietary metals often give a characteristic accumulation pattern, with metal uptake into the gut mucosa (i.e., the route of entry), and then transfer via the hepatic portal vein to the liver, and finally to other internal organs (review, Handy et al. 2005; e.g., dietary Cu, Shaw and Handy 2006; Hoyle et al. 2007). Dietary TiO_2 NP exposure also seems to fit this general pattern (Fig. 1). However, Ti did not clear quickly from all of the tissues after exposure (Fig. 1), with the brain, liver, intestine and gill also showing elevated Ti concentrations in treated animals compared to controls at the end of the experiment. This is similar to the findings in mice which do not clear Ti from the tissues 2 weeks after a single oral exposure to TiO_2 NPs (Wang et al. 2007).

Growth and nutritional performance

Fish from all treatments showed a steady weight gain and cumulative food intake, with no adverse effects of either TiO_2 NP inclusion (Fig. 2). There were also no treatment effects on mean ration size, SGR, FCR, FCE, condition factor or HSI throughout the experiment. These observations indicate that dietary TiO_2 NPs do not adversely affect growth or nutritional performance in rainbow trout at the inclusion levels and exposure times used here. This is similar to our previous studies on dietary metals where many sub lethal toxic effects can occur, but are not necessarily reflected in a loss of growth (e.g., Cu, Handy et al. 1999; Shaw and Handy 2006; Hoyle et al. 2007). Indeed, Handy et al. (1999) and Clearwater et al. (2002) argue that fish will often adopt a strategy where growth is protected, with consequent sub-lethal effects on other physiological processes. The loss of carcass lipid from the fish fed 100 mg kg^{-1} TiO_2 NPs is consistent with this hypothesis (e.g., utilised more lipid than controls as part of a metabolic strategy to maintain growth, Handy et al. 1999), rather than as a result of lipid peroxidation (not observed) .

Several studies have reported the potential for TiO_2 NPs to cause oxidative stress (e.g., fish cells, Reeves et al. 2008; trout in vivo, Federici et al. 2007). However, TBARS did not increase, but instead showed statistically significant decreases in the gill and intestine during TiO_2 NP exposure (Fig. 5). This phenomenon has also been observed during waterborne SWCNT exposure in trout (Smith et al. 2007), and in the absence of changes in the total glutathione pool, suggested that the fish were probably up regulating other

anti-oxidant defences to cause a fall in TBARS. For fish, much of this anti-oxidant capacity comes from the food (Baker et al. 1998) and the continuation of food intake during this study probably enabled some critical protection from the oxidising effects of TiO_2.

Haematology and ionic regulation

Dietary TiO_2 NP exposure had no effect on haematology, or plasma ions (apart from a small transient increase in plasma K^+), and the tissue electrolytes (Na^+, K^+, Ca^{2+}) were normal. Na^+K^+-ATPase activity was also normal in the gill and intestine (Fig. 4). Taken together these findings indicate that TiO_2 NPs are not potent ionoregulatory toxicants via the dietary route, and this is consistent with the findings for waterborne exposure to the same NPs (Federici et al. 2007).

Effects on the brain

In this study, Ti accumulated in the brain of trout (Fig. 1), and this has also been reported in the brain of mice following gut gavage (Wang et al. 2007). Notably in this study (Fig. 1), and in Wang et al. (2007), the Ti accumulation in the brain persisted 2 weeks after the exposure; suggesting that the brain does not clear Ti or does so very slowly. In terms of brain as a target organ, this experiment on Ti is similar to dietary Hg exposure in fish where the brain tissue also accumulates metal during the exposure (Berntssen et al. 2003).

Brain tissue showed transient, but statistically significant depletions of Cu at week 6 in fish from both treatments compared to controls (Fig. 3). Federici et al. (2007) also noted transient depletion of tissue Cu during TiO_2 NP exposure, especially in the brain. The cause of the Cu depletion remains uncertain, but effects of TiO_2 NPs on Cu transporters in the brain cannot be excluded (e.g., inhibition of Cu-ATPases) given that the closely related Na^+-K^+-ATPase is also inhibited (Fig. 4). Notably, the Na^+-K^+-ATPase activity in the brain showed large decreases (around 50% inhibition, Fig. 4), and this effect was much greater than that observed with aqueous exposures to NPs in trout (TiO_2, Federici et al. 2007; carbon nanotubes, Smith et al. 2007).

Zinc levels in the brain also showed some small, but statistically significant, transient increases. This was also noted in the brain after 14 days of waterborne exposure to TiO_2 NPs (Federici et al. 2007). Elevations of brain zinc levels are implicated in many processes in the brain, including neuro-endocrine functions (e.g., Su et al. 1997) and memory formation (Takeda et al. 2008). We did not measure these neurological processes, but it is clear that Ti exposure may cause neurological effects via interference with Zn homeostasis. The mechanism of Ti effect on tissue Zn (and Cu) remains unclear, but the fact that two different routes of exposure (via the water or the food) can produce similar trace element disturbances in the brain requires further investigation.

Does the spleen protect the internal organs from TiO_2 exposure?

The spleen showed a rapid rise in Ti in week 2, prior to Ti increasing in the other internal organs (Fig. 1), and spleens from exposed animals also increased in size during the exposure. One of the main functions of the spleen is to filter damaged cells and foreign material from the blood. The normal haematology in this study suggests the spleen continued to function. However, Ti levels in the spleens of treated animals returned to control levels by week 4, and this was coincident with Ti elevations in other internal organs (Fig. 1); suggesting the spleen was not able to protect the other internal organs from Ti exposure. Wang et al. (2007) also noted increased Ti levels in the spleen of mice following a single dose (gut gavage 5 g kg^{-1} of either 25 or 80 nm diameter TiO_2 NPs).

There was also some transient depletion of Cu in the spleen (Fig. 3). Copper depletion has long been implicated in the alteration of cell surface markers on splenocytes, and therefore the modulation of spleen function (Flynn 1984). The effect of NPs on the function of the spleen and immunity in fish clearly requires further investigation.

Hazard assessment implications

This study demonstrates that fish can accumulate Ti from a dietary TiO_2 NP exposure, and that a number of subtle physiological and biochemical disturbances occur. This information is collected against a long historic use of ordinary TiO_2 powder (bulk TiO_2) in fish nutrition (and the food industry generally) where no such toxic effects have been observed. This at least provides circumstantial evidence that there may be a different hazard from the commercially available nano TiO_2 product used in this study compared to ordinary TiO_2 powders used in foods.

Perhaps a more important question for risk assessors interested in ecological food chains, is whether or not the hazard presented by TiO_2 NPs is more or less than other dietary metals (review, Handy et al. 2005). If this experiment is compared against dietary metal concentrations in other studies on salmonids where growth rate was maintained, but subtle biochemical disturbances occurred (e.g., Cu, 500 mg kg^{-1}, Handy et al. 1999; Zn, 590–1,520 mg kg^{-1}, Clearwater et al. 2002; Inorganic Hg, 100 mg kg^{-1}, Berntssen et al. 2003); then TiO_2 NPs might be considered more toxic than dietary Cu and Zn, and at least as toxic as Hg. The

human health hazard from eating contaminated fish is also a concern, but the risk from accidental ingestion of TiO_2 NP contaminated trout may be limited, because the Ti does not appear to accumulate in the edible muscle at the concentrations and time scales used here.

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Physiological effects of nanoparticles on fish: A comparison of nanometals versus metal ions

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ABSTRACT

The use of nanoscale materials is growing exponentially, but there are also concerns about the environmental hazard to aquatic biota. Metal-containing engineered nanoparticles (NPs) are an important group of these new materials, and are often made of one metal (e.g., Cu-NPs and Ag-NPs), metal oxides (e.g., ZnO and TiO_2 NPs), or composite of several metals. The physiological effects and toxicity of trace metals in the traditional dissolved form are relatively well known and the overall aim of this review was to use our existing conceptual framework of metal toxicity in fish to compare and contrast the effects of nanometals. Conceptually, there are some fundamental differences that relate to bioavailability and uptake. The chemistry and behaviour of nanometals involves dynamic aspects of aggregation theory, rather than the equilibrium models traditionally used for free metal ions. Some NPs, such as Cu-NPs, may also release free metal ions from the surface of the particle. Biological uptake of NPs is not likely via ion transporters, but endocytosis is a possible uptake mechanism. The body distribution, metabolism, and excretion of nanometals is poorly understood and hampered by a lack of methods for measuring NPs in tissues. Although data sets are still limited, emerging studies on the acute toxicity of nanometals have so far shown that these materials can be lethal to fish in the mg–$\mu g\ l^{-1}$ range, depending on the type of material. Evidence suggests that some nanometals can be more acutely toxic to some fish than dissolved forms. For example, juvenile zebrafish have a 48-h LC_{50} of about 0.71 and 1.78 $mg\ l^{-1}$ for nano- and dissolved forms of Cu respectively. The acute toxicity of metal NPs is not always explained, or only partly explained, by the presence of free metal ions; suggesting that other novel mechanisms may be involved in bioavailability. Evidence suggests that nanometals can cause a range of sublethal effects in fish including respiratory toxicity, disturbances to trace elements in tissues, inhibition of Na^+K^+-ATPase, and oxidative stress. Organ pathologies from nanometals can be found in a range of organs including the gill, liver, intestine, and brain. These sublethal effects suggest some common features in the sublethal responses to nanometals compared to metal salts. Effects on early life stages of fish are also emerging, with reports of nanometals crossing the chorion (e.g., Ag-NPs), and suggestions that the nano-forms of some metals (Cu-NPs and ZnO NPs) may be more toxic to embryos or juveniles, than the equivalent metal salt. It remains possible that nanometals could interfere with, and/or stimulate stress responses in fish; but data has yet to be collected on this aspect. We conclude that nanometals do have adverse physiological effects on fish, and the hazard for some metal NPs will be different to the traditional dissolved forms of metals.

1. Introduction

The ecophysiology and toxicology of trace metals in fish has been the focus of extensive research for many years (Henry and Atchison, 1991; Spry and Wiener, 1991; Wendelaar Bonga and Lock, 1992; Handy, 1996; Clearwater et al., 2002; Kamunde and Wood, 2004; Handy et al., 2005). This has led to a consensus view on the main effects of metals on fish, as well as the associated environmental hazards and risks (review, Campbell et al., 2006). It is well established that fish have essential nutrition requirements for trace metals (e.g. copper, zinc, and iron; Bury et al., 2003), and that some trace metals are "non-essential" with no known or important biological role (e.g., mercury and silver). The bioavailability and biological uptake of trace metals by fish has been given special attention by researchers. These studies include detailed models of trace element speciation in water (e.g., free ion activity models, van Leeuwen et al., 2005) and investigations on the uptake of particular trace metal species by fish (e.g., Playle et al., 1993). The effects of abiotic factors in water chemistry, such as pH and water hardness, on metal uptake and toxicity are well known (e.g., Cusimano et al., 1986; Erickson et al., 2008) and models have been constructed to predict metal toxicity (e.g., biotic ligand models; Paquin et al., 2002; Niyogi and Wood, 2004). The target organs, mechanisms of sequestration and storage of metals in the biological systems of fish, and excretion have also been studied (e.g., Grosell et al., 1997), as well as the effects upon fish behaviour (review, Scott and Sloman, 2004).

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In recent years, novel chemical engineering techniques have produced nanomaterials, and the application of these materials in products and processes is known as nanotechnology. Engineered nanomaterials comprise of numerous different physical forms and some of these materials have adverse effects on fish including; carbon nanotubes (e.g., Smith et al., 2007), carbon spheres called fullerenes (e.g., C_{60}, Zhu et al., 2007) and nanoparticles (NPs) made from metals (e.g., Cu, Griffitt et al., 2007), metal oxides (e.g., TiO_2, Federici et al., 2007), or composites made of several metals (e.g., quantum dots, King-Heiden et al., 2009). Engineered NPs can also be made into many different shapes (e.g., wires, rods or spherical particles), and can be functionalised with almost any type of surface chemistry. Thus even carbon-based nanomaterials can be functionalised with metals (e.g., metal-coated carbon nanotubes (Meng et al., 2009) and metal encapsulated fullerenes, known as endohedral metallofullerenes, (Śliwa, 1996)). Also in development are hybrid versions of (nano) materials containing more than one NP or metal (e.g., ZnS:Cu, ZnS nanoparticles co-doped with Pb^{2+} and Cu^{2+}, Yang et al., 2001; Cu-doped CdSe nanocrystals, Meulenberg et al., 2004; CdS–TiO_2 nano-composite, So et al., 2004; Ag–Cu nanoparticle-doped dental amalgams, Chung et al., 2008; Cu–TiO_2 nanocomposite coatings, Ramalingam et al., 2009). This has led to considerable debate on how to classify these new materials, but engineered nanomaterials are generally considered to be materials with at least one dimension of less than 100 nm (Roco, 2003). This definition has been adopted by the British Standards Institution, the American Society for Testing Materials, and the Scientific Committee on Emerging and Newly-Identified Health Risks (see reviews on NPs, Handy et al., 2008a; Klaine et al., 2008). However, it is important to note that this is a somewhat arbitrary size cut off from the view point of ecotoxicity, and it may be prudent to also consider aggregates of NPs that can be a few hundred nanometres wide (Handy and Shaw, 2007) or that have a distribution of particles around the nanoscale; but may have some primary particles larger than 100 nm (Handy et al., 2008a).

The focus of this review is on nanomaterials that contain metals, e.g., nanoparticles made of metal or metal oxides (hereafter referred to as nanometals). Some of the types of nanometals and their product applications are outlined in Table 1, and although these uses will likely be continuously changing and evolving with commercial markets. It is clear that these new materials can include particles made from different types of metals, including metals that are consider to be toxic in the traditional dissolved form (e.g., Ag, Hogstrand and Wood, 1998; Cu, Handy, 2003; Zn, Hogstrand and Wood, 1996). It is therefore important to determine whether or not the alternative "nano" forms of metals are also toxic. Moreover, nanotechnology is a rapidly expanding industry. In 2006 the nanotechnology sector had achieved a multibillion dollar market, with predictions that it will grow to a trillion US dollars by 2015 (Aitken et al., 2006). Lux business forecast also suggests that nanotechnology products are worth about 1500 billion dollars in 2010 (http://www.nanowerk.com/spotlight/spotid=1792.php). The Woodrow Wilson International Centre for Scholars product inventory of nanomaterials, indicted that 1015 different nano-containing products were on the market in 2009, and these products mostly involved metal-based nanomaterials (http://www.nanotechproject.org/inventories/consumer/analysis_draft). Peer reviewed scientific reports also show that products containing nanometals are well represented (Hansen et al., 2008). It is therefore likely that nanomaterials are already being released into the environment (Owen and Handy, 2007; Ju-Nam and Lead, 2008; Gottschalk et al., 2009). Modelling techniques have predicted low $\mu g\,l^{-1}$ concentrations of NPs in the environment over the long term (Boxall et al., 2007), and this has been confirmed by field measurements for at least one nanometal (TiO_2, Kaegi et al., 2008). It is clear that the environmental hazard of NPs, and especially nanometals, needs to be addressed. The overall aim of this review is therefore to use our existing conceptual framework of metal toxicity in fish, and to compare and contrast this with emerging information on nanoforms of metals. The issue of hazard is approached from the view point of body systems physiology of the fish, starting with water chemistry and uptake routes, and consideration of the toxicological principles of absorption, distribution, metabolism, excretion (ADME). Lethal toxicity and sub-lethal toxic effects are described with some speculation on toxic mechanisms likely to be of concern for nanometals, as well as the likely effects on the stress response of fish. Finally, consideration is given on the interpretation of physiological effects data on nanomaterials in the context of hazard assessment and whether or not the current approaches used for dissolved metals would also be "fit for purpose" for nanometals.

Table 1
Nanoparticles in production and examples of current or proposed use.

Nanoparticle or nanomaterial	Application
Aluminium oxide	Optical polishing, cosmetics, and clothing
C_{60} fullerenes	Hydrogen storage, drug delivery, therapeutics, coatings and pigments, lubrication, and cosmetics
Carbon nanotubes (single or multi walled)	Hydrogen storage, drug delivery, textiles, electronics, water purification, and sporting equipment
Ceramics	Electronics, anti-oxidants, and car polish
Copper or copper oxide	Lubrication oil additive, electronics and computer processors, conductive coatings, printer inks, sintering additives, 'anti-ageing' cream and skin conditioner, and mineral supplements
Gold	Drug delivery, labels for immunocytochemistry, and biological hazard detection e.g., ricin, *E. coli*, and mineral supplements
Iron oxide	Ultrafiltration and oxidation reduction catalyst
Iron sulphide	Removal of organochlorine pesticides from drinking water
Nanocrystals	Insulators and drug delivery
Nano-rods	Electronics, sensors and sensing devices
Polymers	Therapeutics, coatings and pigments, lubrication, absorbents
Quantum dots	Medical imaging, photonics
Silica	Photovaltaics, optics and optical devices, anti-graffiti paint, and cosmetics
Silver	Antibacterial uses in water treatment, fabric softener, clothing, soft toys, wound dressing, kitchen utensils and appliances, computer keyboards, food storage containers, and baby products (e.g. cups) and uses in contraception and toothpaste
Titanium dioxide	Paint, sunscreen, cosmetics, capacitors, building materials, catalyst, air clearance, anti-bacterial, viral, algal, fungus and mould coating for domestic baths, and sporting equipment
Nano-vitamins (some vitamins encapsulated in nano-delivery vehicles)	Vitamin E; food, beverages and cosmetics, vitamins B12 and E; cosmetics
Zinc oxide	Sunscreen, cosmetics, cosmetic remover, foot deodorant, and car polish

Data source: The Project on Emerging Nanotechnologies, The Woodrow Wilson International Centre for Scholars; http://www.nanotechproject.org/inventories/consumer/.

2. Water chemistry and bioavailability

The importance of water chemistry in the bioavailability of metals has been extensively investigated over many years. The main paradigm is that the free metal ion is the bioavailable chemical species, and therefore research has focused on identifying metal speciation in water and how this is influenced by abiotic factors such as pH, and the presence of ligands in the water that may remove free ions (e.g., dissolved organic matter), anions that can complex metals or form anionic metal species (e.g., chloride and hydroxyl ions), or cations (e.g., H^+, Ca^{2+}, Mg^{2+}, and Na^+) that may be competing for biological uptake with trace metals (e.g., Cusimano et al., 1986; Campbell, 1995; Bervoets and Blust, 2000; Wood, 2001; Pyle et al., 2002; van Leeuwen and

Galceran, 2004; van Leeuwen et al., 2005; Erickson et al., 2008). For elements that have different redox states, the level of oxygenation and/or redox potential of the natural water may also be a consideration in calculating the metal speciation (e.g., the metalloid arsenic, Cheng et al., 2009). However for most of the simple equilibrium models (e.g., WHAM speciation model, MINEQL), the main factors are the total concentration of the metal, pH, concentrations of divalent cations (or total hardness of the water), and the electrolytes contributing to ionic strength (e.g., bulk NaCl concentration in freshwater or seawater) (review, Wilkinson and Buffle, 2004). These simplified models are intended for practical use in predicting free metal ion concentrations, and have been applied successfully in hazard assessment for many years (e.g., corrections for water hardness or pH in metals legislation) and to predictive models of acute metal toxicity (e.g., biotic ligand models, BLM, Playle et al., 1993; Hollis et al., 1997; Paquin et al., 2002).

The environmental chemistry and ecotoxicity of engineered NPs has recently been reviewed (see Handy et al., 2008a). This chemistry shares some superficial similarities with metal chemistry in those abiotic factors such as pH, the presence of divalent ions, and ionic strength can influence the colloidal behaviour (aggregation) of NPs. However, the reasons for these interactions are often fundamentally different to those for dissolved metals. Current metal speciation models are equilibrium models, whereas the behaviour of NPs is described in a very different way by dynamic process where the system is dependent on the amount of energy added to the NP dispersion and the physico-chemical properties of the particles (called DLVO theory, see Handy et al., 2008a for discussion). These physico-chemical properties include particle size, shape (aspect ratio), and surface charge (often measured as zeta potentials). The ability of a particle to colloid and aggregate with another particle will depend on these properties, as well as the kinetic energy of the particles, viscosity of the water and any drag on the particles, and the presence of other materials in natural waters such as small peptides (e.g., bacterial exudates) or macromolecules (e.g., humic acids) that may provide steric hindrance of particle–particle interactions. Clearly, this is a fundamentally different chemistry to the metal speciation models that are more familiar to the fish ecotoxicologist.

However, there is also some common ground. One concept in NP toxicity is that the toxicity may be driven by the surface chemistry of the particles. The aggregation chemistry will be useful in predicting the effective surface area of the material in the context of potential interaction with the organism, but the surface chemistry (reactivity) may inform on toxicity. These ideas are not yet proven by experimental data, but for example, one might expect a particle with an oxidising metal surface to cause oxidative stress on/in the organism. This is certainly the case for at least one type of TiO_2 NPs which caused oxidative stress in trout (Federici et al., 2007). So, perhaps metal reactivity rather than the concentration of the free metal ion will be more important in the case of NPs. Nonetheless there is a scenario where existing free metal ion models may apply, with some modifications. Some types of nanometals will eventually dissolve (albeit slowly over several hours or days) by dissolution of metal ions from the surface of the particle. This is probably the case for nano-silver and nano-copper in freshwater. In a study comparing the effects of three nanometals (nano-Cu, nano-Ag, and TiO_2 NPs) with dissolved metals (salts of Cu and Ag at concentrations to match estimated dissolved ions from each NP), Griffitt et al. (2009) found that both dissolved and nanoparticulate forms of Cu and Ag increased metal levels in the gill tissue after 48 h. Copper accumulation in the gill was similar following exposure to both soluble and nanoparticulate Cu; and the authors interpreted this as uptake of dissolved Cu from the Cu-NP. Conversely, branchial Ag levels were much greater in fish exposed to Ag-NPs compared to dissolved Ag, indicating that the NPs themselves (not dissolved Ag from the NPs) were contributing to the branchial Ag burden (Griffitt et al., 2009). As the authors do not mention rinsing excised gills in, for example, ion-free water prior to analysis, it is possible that Ag-NPs associated with the gills, but not actually taken up into the cells, contributed to total burden. Though they do acknowledge the possibility of Ag-NPs being trapped in the mucous layer, this is not gill Ag burden *per se*, although it does suggest that nano-Ag could act as a respiratory irritant similar to other NPs (e.g., SWCNT, Smith et al., 2007). Scown et al. (2010) also observed increased Ag burden in the gills and liver of fish exposed to different sized Ag-NPs (10 and 35 nm particles compared to bulk Ag and $AgNO_3$), though similarly no mention was made of rinsing tissue samples to remove associated, but not internally localised, NPs (particularly relevant to gill samples) prior to analysis. However, increased hepatic Ag (twice that of the gills) does indicate that there was some uptake and transport of Ag from the NPs, which the authors suggest, cannot be explained by dissolution of Ag ions alone. As gut Ag was not assessed the possibility of hepatic uptake via the gut (stress induced drinking and incidental NP ingestion) cannot be excluded.

In situations involving dissolution, the NP is acting as a "delivery vehicle" for the metal ions, and when the NP sticks to gill surface (for example) it could release locally high concentrations of metal ions, or provide a sustained slow release of metal ions onto the epithelia. In this scenario the free metal ion concentration would be driving toxicity, and it would be relatively straight forward to add an empirical correction factor (measured experimentally) for the dissolution rate of free metal ions for a known mass concentration of particles in a particular water quality.

There is also evidence of "delivery vehicle" effects when the metal is present as a co-contaminant with a NP. This may be related to the ability of metals to adsorp to the surface of some negatively charged NPs (Handy et al., 2008b). For example, Zhang et al. (2007) exposed carp to TiO_2 NPs and found that the fish accumulated 146% more Cd in the presence of the TiO_2 NPs compared to when fish were exposed to Cd alone. Similarly, carp exposed to the metalloid, arsenic (As(V)), in the presence of TiO_2 NPs accumulated 132% more As than fish exposed to an equal concentration of As only (Sun et al., 2007). In a follow up study, Sun et al. (2009) investigated the impact of TiO_2 NPs on arsenic speciation and the uptake in carp of the more toxic form; As (III). However, in separate speciation experiments (no fish), when introduced to TiO_2 NP containing water under natural light, As (III) rapidly oxidised to the less toxic As (V), although experiments without sunlight resulted in less than 3% of As (III) changing to As (V). Therefore in the experimental conditions used (i.e., under sun light), fish were not exposed to great amounts of As (III), although it remains to be seen if increased toxicity would manifest in fish living in conditions with less natural light. Many NPs have a net negative charge (anionic surface) in natural water and the possibility of NPs increasing metal toxicity via a vehicle or mixtures effect clearly requires further study.

3. Absorption of nanoparticles compared to metal ions

The mechanisms of metal uptake across biological membranes involve carrier-mediated transport on metal ion transporters (reviews, Bury et al., 2003; Handy and Eddy, 2004; Bury and Handy, in press). The biological uptake and unstirred layer chemistry of NPs in fish gills has been recently reviewed in detail (see Handy et al., 2008b), and the main differences between metal ions and NPs are summarised (Fig. 1). The central issue is that NPs are too big to use ion transporters, or paracellular diffusion pathways, and that the most likely route of uptake is by endocytosis pathways (Fig. 1). If this is true, then the scientific community will need to reconsider the basic assumptions in metal uptake, and revise them for nanometals. For dissolved metals, bioavailability is assumed to be a function of metal speciation since only certain metal species will fit the relevant ion transport pathway, and diffusion of metal ions through the hydrophobic core of membranes is excluded (Campbell and Stokes, 1985; Campbell, 1995; Escher and Sigg, 2004).

For very small NPs (<20 nm) that may obtain a hydrophilic surface coat (e.g., steric effects coating the particle with phospholipids), or

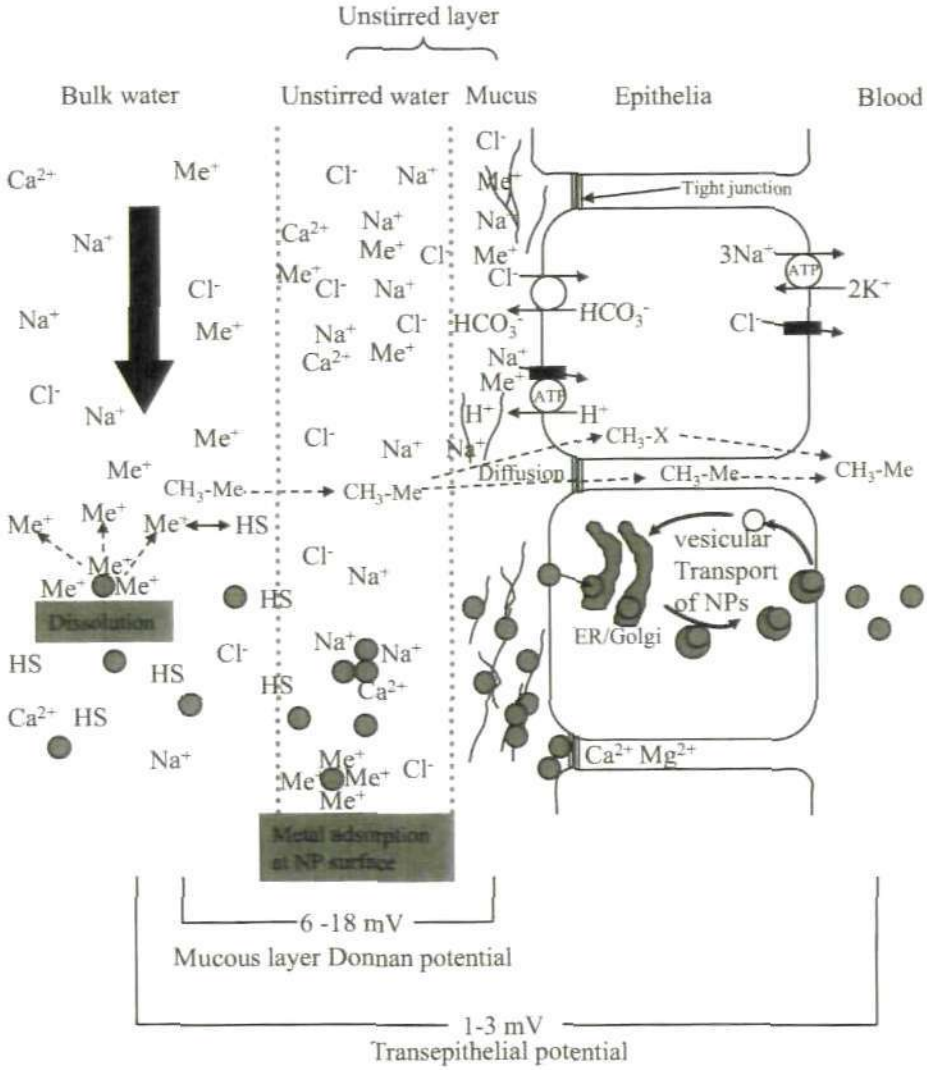

Fig. 1. An idealised diagram of the freshwater fish gill showing the mechanisms of uptake for electrolytes, toxic metal ions (Me^+), and electroneutral diffusion of some small organo-metals (CH_3-Me), compared to nanoparticles (NPs, filled circles). Modified from Handy et al. (2008b), and Handy and Eddy (2004). The substances in the bulk solution (the freshwater) must diffuse into an unstirred layer (USL) comprising of water/mucus secretions, prior to transfer across the gill epithelium. The upper portion of the diagram shows electrolytes and toxic metals ions which diffuse into the USL, and may bind to strands of mucus (mostly polyanionic) where the exclusion of free anions like Cl^- from the mucus layer contributes to the Donnan potential at the apical surface. Electrolytes and toxic metal ions usually move through the cell using ion transport pathways (Na^+ transporters are illustrated here). In contrast, small electroneutral organo-metals may diffuse into the USL and then through the cells (transcellular diffusion), or between the cells via the tight junctions (paracellular diffusion). The situation for NP uptake across the gill will be a little different. NPs will diffuse into the USL, albeit at a slower rate than smaller molecules or solutes, and may be influenced by humic substances (HS). Cationic NPs will bind to strands of mucus (electrostatic attraction, fundamentally similar to other cations), but regardless of surface charge, may also become entangled in the mucoproteins (steric hindrance) to prevent uptake by the epithelial cells. NPs are too large to be taken up by ion or other transporters on the cell membranes, and although diffusion cannot be excluded for lipophilic NPs. The Ca^{2+} and Mg^{2+} rich environment in the tight junctions suggest that NPs would aggregate rather than diffuse through the paracellular route. In addition, some nanometals may release free metal ion (Me^+) by dissolution of ions into the bulk solution. In contrast, nanomaterials can also show surface adsorption of metals, and this is likely to be faster in the higher ionic strength of the USL. Diffusion of charged NPs into the USL will be affected by the Donnan and transepithelial potentials, in a similar way to other charged substances. NP uptake through vesicular transport seems likely.

achieve no net charge to become hydrophobic (e.g., positively charged nanometals that are incidentally coated with biological anions such as those found in mucus, Handy and Maunder, 2009); it remains theoretically possible for these materials to diffuse through the cell membrane. We should therefore add a diffusional component for nanometal uptake that is both a function of particle size and the hydrophobicity of the particle surface. Endocytosis should also be modelled, and here evidence may be drawn from trace metals that are known to use endocytosis pathways for uptake (e.g., Cu, Handy et al., 2000). However unlike dissolved metals, it will not be the activity of a metal ATPase controlling the loading of intracellular metal-containing vesicles (e.g., Cu ions via the Cu-ATPase), but instead, the bulk endocytosis of particles at the apical membrane. Clearly, for the latter the control of the cytoskeleton, membrane turnover, and related aspects of cell volume control will be critical, and metals are known to influence regulatory volume decrease (e.g., Cu, Kramhøft et al., 1988). The rate limiting step in NP uptake may not be the basolateral step as so often is the case for metal ions (Handy et al., 2008b). However, if the nanometal dissolves to release free ions then the current uptake models would also apply. Some of the apical events, such as the protective role of mucus, would also seem appropriate for NPs (Handy et al., 2008b). Smith et al. (2007) have already observed precipitation of carbon nanotubes in the gill mucus of trout. Similar to metal ions, the uptake site (e.g., gill and intestine) could also be a target organ for toxic effects. Some NPs are respiratory toxicants in fish and produce gill pathologies which are similar to those for metal ions (Federici et al., 2007; Smith et al., 2007). Similar to surface acting metals (e.g., Al, Handy and Eddy, 1989), it remains possible that some NPs can exert toxic effects without appreciable uptake of the metal into the

internal organs (e.g., short waterborne exposures to TiO_2, Federici et al., 2007).

4. Distribution, metabolism and excretion of nanometals

Handy et al. (2008b) reviews the possible mechanisms of absorption, distribution, metabolism, and excretion (ADME) for NPs in fish. This review identifies numerous knowledge gaps on the ADME of NPs, and most of this is centred on a lack of routine methods for the direct measurement of NPs in tissues in order to establish body distributions and target organs. Current approaches involve electron microscopy of dissected tissues, which is very labour intensive way of determining the presence or absence of nanometals (see Handy et al., 2008a for discussion of NP detection methods). The development of radiolabelled metal NPs is needed, but even here there are technical concerns. For example, if the exterior of the NP is labelled with a metal isotope, it may be possible for the label to become detached by dissolution of the NP in the tissue. Clearly, such methods will need thorough validation. The detection of metal in fish tissues remains possible using acid digestions followed by ICP-MS or ICP-OES for the metal of interest (e.g., Federici et al., 2007; Ramsden et al., 2009). However, nanometals do not behave the same way as metal ions in ICP-MS, and standard protocols for metal digestion and analysis may not work for NPs. Substantial modifications and revalidation of spike recovery tests, etc., are needed for nanometals. Some researchers report very poor recoveries of total tissue metal contents from nanometals (e.g., 28%, Scown et al., 2009). Such poor recoveries typically generate large error bars on mean data values for the metal content of individual tissues, and it is likely that important differences in tissue metal content are lost due to poor resolution in the method. However careful adaptation of methods, involving correcting instrument calibrations for NPs, the addition of surfactants or dispersants, and careful stirring of samples while they are being drawn into the instrument can result in much improved metal recoveries (e.g., Ramsden et al., 2009). It is possible to identify target organs on the basis of biological effects, but as Federici et al. (2007) point out for TiO_2 NPs at the gills, surface acting toxicants can generate injury to internal organs through secondary means (e.g., diffusion of oxygen radicals). There is a concern that some oxidising nanometals could be potent surface acting toxicants, and therefore, injury to an internal organ does not necessarily confirm the presence of the nanometal in that organ. Nonetheless on the basis of biological effects, a number of target organs have been suggested for nanometals including the gills, gut, liver and brain (see below). These are also well known target organs for metal ions.

Studies on the metabolism and excretion of nanometals are limited for similar technical reasons to those outlined above. Fish normally excrete trace metal ions via the liver as the central compartment in metal metabolism, with urinary losses usually being small (e.g., Grosell et al., 1998). The gills may also be able to excrete metal ions from the systemic circulation by active efflux on branchial ion transport pathways, but this would not apply to nanometals. The molecular weight cut off in the vertebrate kidney (circa 60 kDa) would suggest that NPs would not pass through the glomerular filter. This leaves the liver as the most likely route of excretion for nanometals (see discussion in Handy et al., 2008b). Fish are known to deposit metal granules in the liver (e.g. Lanno et al., 1987), and it would seem logical that nanometals could also form hepatic deposits. It remains unknown if such deposits of nanometals exist, or whether or not they would remain as inert storage granules in the liver, or be excreted via the bile in fish.

5. Lethal toxicity of engineered nanoparticles in fish

Only a few lethal toxicity tests have been conducted using nanometals in fish (Table 2). The literature on fish and invertebrates indicate that acute lethal concentrations are in the mg l^{-1}, rather than μg l^{-1} range. This suggests that metal-containing NPs, such as poorly soluble metal oxides, may have low toxicity. Concerns remain that nanometals that show dissolution of metal ions will show acute lethal toxicity in the μg l range. This is especially worrying for toxic metals where the nanoform may dissolve. Griffitt et al. (2007) reported 48-h LC_{50} concentrations of 250 μg l^{-1} and 1.56 mg l^{-1} in adult zebrafish (*Danio rerio*) exposed to dissolved and nano-copper (80 nm primary particle size) respectively. However, following its addition to water 50–60% of added nano-Cu mass was observed to fall out of suspension due to rapid aggregation of the NPs. Dissolution of Cu ions from the particles (defined as Cu present in the supernatant of samples centrifuged at 100,000 × *g* for 30 min) showed that dissolution was minimal over 48 h with concentrations of dissolved Cu not exceeding 0.19 ± 0.05 mg l^{-1}, representing less than 0.1% of the initial Cu added. Notably these dissolution experiments were conducted in the absence of fish. The relatively low rate of dissolution caused the authors to conclude that observed toxicity was not due to dissolved Cu alone. Subsequent work by Griffitt et al. showed nano-Cu to be more acutely toxic to juvenile zebrafish than dissolved Cu, although this trend was reversed in adult fish (primary particle size 26.7 nm, Griffitt et al., 2008). In the same study nano-Ag was seen to be less toxic than dissolved Ag to adult zebrafish (nano-Ag 48-h LC_{50} was more than 300-fold higher than that of dissolved Ag, Griffitt et al., 2008). Perhaps somewhat unexpectedly, 10 mg l^{-1} dissolved Ag was not elevated enough to kill 50% of juvenile (larval) zebrafish (compared to an LC_{50} of 0.0222 mg l^{-1} in adults and a nano-Ag LC_{50} of 7.20 mg l^{-1}). The latter data may emphasise life stage differences in the toxic responses of fish to nanometals with fathead minnow (*Pimephales promelas*) embryos proving more sensitive to Ag-NPs than zebrafish larvae (96 h LC_{50} values of 1.25 and 1.36 mg l^{-1} for two different commercially available Ag-NPs respectively) (Laban et al., 2010).

6. Sub-lethal effects of nanometals compared to aqueous metal ions

The sub-lethal physiological effects of waterborne metal exposure are well known (reviews, several metals: Wood, 2001; Ag: Hogstrand and Wood, 1998; Wood et al., 1999; Cd: Sprague, 1987; Cu: Taylor et al., 1996; Zn: Hogstrand and Wood, 1996). In contrast, the scientific community is in the early stages of collecting data on nanometals (Table 2). The typical scenario for waterborne metal exposure involves some direct effects on gill functions (reviews, McDonald and Wood, 1993; Perry and Laurent, 1993). These can include alterations to the osmoregulatory (e.g., Cd, McCarty and Houston, 1975; Giles, 1984), acid–base (e.g., Cu, Taylor et al., 1996), or respiratory functions of the gill (e.g., nickel, Pyle et al., 2002). Sub-lethal pathologies in the gill can be reflected by changes in mucous secretions reflecting an increase in mucous cells and chloride cells in the epithelium (e.g., Mallatt, 1985), or evidence of oedema in the epithelium (e.g., Sola et al., 1995; Campbell et al., 1999). Histological changes in the gill epithelium are also associated with adaptive biochemical change in response to metal exposure (e.g., metallothionein induction in the gill, Dang et al., 1999). Metals can also inhibit the branchial Na^+/K^+-ATPase (e.g., Cu, Li et al., 1998). Following this initial disruption, the fish can then experience a general loss of branchial ionoregulatory control, efflux of electrolytes from the blood over the gill epithelium, then subsequent cardiovascular collapse and death (review Handy, 2003 and references therein). Notably, rainbow trout display physiological responses influenced by social status. Schools of rainbow trout develop a social hierarchy where the more aggressive fish in the school are dominant over often smaller, less aggressive fish in the group. When rainbow trout are exposed to Cu, the subordinate fish accumulate more waterborne Cu (and Na) than dominant animals (Sloman et al., 2002). A similar phenomenon has also been seen in fish exposed to waterborne Ag and highlights the

Table 2
Summary of studies carried out into the toxicity of organic and inorganic nanoparticles to fish and fish cell lines.

Nanomaterial and additional information[a]	Exposure conditions	Fish species	Effects	Study
Ag-NPs (5–20 nm particle size)	5–100 $\mu g\ l^{-1}$ for 72 h	Zebrafish (*Danio rerio*) embryos	Dose-dependent increases in mortality and decreases in heart rate. Hatching only affected at highest treatment. Most NPs deposited in the cell nucleus, but uniform distribution of NPs was seen throughout the embryo. Body malformations in embryos exposed above 50 $\mu g\ l^{-1}$.	Asharani et al. (2008)
Ag-NPs (dry powder mean primary particle size of 26.6 ± 8.8 nm) stabilised with 0.5% sodium citrate solution, sonicated with a probe sonicator for six 0.5 s pulses prior to dosing	0–10 $mg\ l^{-1}$ followed by experiments with fish exposed to 0.6–2.78-fold the LC_{50} (both for 48 h)	Zebrafish (*Danio rerio*)	Ag-NP LC_{50} of 7.07 $mg\ l^{-1}$ in adult fish and 7.20 $mg\ l^{-1}$ in larvae; less toxic than soluble Ag	Griffitt et al. (2008)
Ag-NPs (particle size of 10–20 nm; stock diluted with municipal water and dechlorinated)	0–20 $\mu g\ l^{-1}$ from 2.5 to 72 hpf	Zebrafish (*Danio rerio*) embryos	Dose-dependent effects included slow hatching rates, abnormal development, and increased catalase activity suggested by the authors as possibly linked to effects on gene expression from NPs entering the cell nucleus and ROS initiated DNA damage.	Yeo and Kang (2008)
Ag-NPs (5.9, 15.3, 51.2, and 108.9 nm measured mean particle sizes)	0–250 μM for 120 hpf	Zebrafish (*Danio rerio*) embryos	Exposure to 250 μM of each Ag-NP treatment resulted in particle size dependent mortality after 24 h (80, 64, 36, and 3% mortality respectively) rising to 100% at 120 hpf. Uptake of Ag into the embryo not seen in control fish.	Bar-Ilan et al. (2009)
Ag-NPs (49.6 nm mean particle size, 99% purity), stock *sonicated for 1 h in 2 min bursts followed by stirring* for 14 days and filtration (200 nm)	0–50 $\mu g\ l^{-1}$ for 96 h and 0–25 $\mu g\ l^{-1}$ for 10 days	Japanese medaka (*Oryzias latipes*) *4–5 months old*	Similar 96 h LC_{50} values for Ag-NPs and $AgNO_3$ (34.6 *± 0.9 and 36.5 ± 1.8 μg* l^{-1} *respectively*). Expression of stress related genes indicated different modes of toxicity between Ag-NPs and soluble Ag ions ($AgNO_3$).	Chae et al. (2009)
Ag-NPs (dry powder mean particle diameter 26.6 ± 8.8 nm), sonication for 6 s in 1 s bursts prior to dosing	1000 $\mu g\ l^{-1}$ (representing the NOEC) for 48 h	Zebrafish (*Danio rerio*)	Gill filament width not affected by Ag-NPs, though dissolved Ag increased width by approximately 2-fold. Branchial Ag levels in NP exposed fish almost 20× higher than fish exposed to a Ag dose that matched the *concentration of dissolved Ag from the NPs, indicating* that intact Ag-NPs were associated with the gill. Similar pattern observed in whole body Ag measurements.	Griffitt et al. (2009)
Ag-NPs (commercial name of Nanocid, particle size of 4.5 nm, data taken from manufacturers website)	0–20 $\mu g\ l^{-1}$ for up to 96 h	Rainbow trout (*Oncorhynchus mykiss*)	48, 72, and 96 h LC_{50} concentrations of 3.5, 3.0 and 2.3 $\mu g\ l^{-1}$ respectively.	Shahbazzadeh et al. (2009)
Ag-NPs (spherical particles, 5–20 nm in diameter), *stock deionised using ion exchange resin and* sonicated for 30 s prior to dosing	24 h LC_{50} (concentration range not reported), *followed by 0–120 mg* l^{-1} *for 24 h*	Zebrafish (*Danio rerio*)	24 h LC_{50} of 250 $mg\ l^{-1}$, concentration dependent *increases in hepatic malondialdehyde and total* glutathione, DNA damage and apoptosis in liver.	Choi et al. (2010)
Ag-NPs (two commercial Ag-NPs with nominal particle sizes of 35 nm and ≤100 nm, both with 99.99% purity) sonicated at 15 kHz for 1.5 h	0–25 $mg\ l^{-1}$ for 96 h	Fathead minnow (*Pimephales promelas*) embryos	96 h LC_{50} values of 9.4 and 10.6 g $mg\ l^{-1}$ for the 35 and ≤100 nm Ag-NPs respectively. Concentration dependent increase in larval abnormalities. TEM image analysis showed that both types of Ag-NPs were present within embryos following 24 h exposure.	Laban et al. (2010)
Ag-NPs (49, 114, and 137 (bulk) nm mean particle sizes) with sonication for 30 min prior to exposure	10 or 100 $\mu g\ l^{-1}$ for 10 days	Juvenile rainbow trout (*Oncorhynchus mykiss*)	Low uptake with the smallest particles most concentrated in gill tissue and liver (the latter along with the bulk Ag particles). No effects on lipid peroxidation amongst any treatment (TBARS assay), although possible evidence of oxidative metabolism in gills of fish exposed to 49 nm Ag-NPs.	Scown et al. (2010)
Ag-NPs (25 nm spherical particles)	0–8 $mg\ l^{-1}$ for 48 h (adults) and 0–4 $mg\ l^{-1}$ for 168 h (embryos), followed by 0–1000 $\mu g\ l^{-1}$ for 60 days from embryo stage 10	Japanese medaka (*Oryzias latipes*) adults and embryos	48 h LC_{50} of 1.03 $mg\ l^{-1}$ for adult fish, whilst 100% embryo mortality was induced at 2 $mg\ l^{-1}$. Retarded development and pigmentation seen in embryos exposed to ≥400 $\mu g\ l^{-1}$ Ag-NPs with morphological malformations seen all Ag-NP treatments.	Wu et al. (2010)
Al-NPs (dry powder mean primary particle size of 41.7 ± 8.1 nm) sonicated with a probe sonicator for six 0.5 s pulses prior to dosing	0–10 $mg\ l^{-1}$ for 48 h	Zebrafish (*Danio rerio*)	Highest dose (10 $mg\ l^{-1}$) not elevated enough to establish an LC_{50} for adults or larvae; dissolved Al gave LC_{50} concentrations of 7.92 $mg\ l^{-1}$ and over 10 $mg\ l^{-1}$ in adults and larvae respectively.	Griffitt et al. (2008)

Au-nanorods (65 nm length, 15 nm diameter; purified before use and suspended in high purity water)	7.08×10^9 particles ml^{-1} single-dose into estuarine mesocosms for 12 days	Sheepshead Minnow (*Cyprinodon variegatus*)	Au-nanorods did not cause acute toxicology and fish did not accumulate detectable levels of Au in the gills, brain or musculoskeletal tissues (indicating low branchial and cutaneous uptake). Au was detected in combined samples of internal organs and gut content, indicating limited oral uptake/accumulation.	Ferry et al. (2009)
Au-NPs (6.5, 18, 46.1, and 98.1 nm measured mean particle sizes)	0–250 μM for 120 hpf	Zebrafish (*Danio rerio*) embryos	Mortality remained below 3% for all treatments and no evidence of morphological malformations. However, uptake of Au-NPs was observed.	Bar-Ilan et al. (2009)
CdS quantum dots coated in thiol terminated methyl polyethylene glycol (4.2 ± 1 nm) suspended in water without surfactants	5, 50, or 500 μg l^{-1} for 21 days using a flow through system	Three-spined stickleback (*Gasterosteus aculeatus*)	Elevated levels of oxidised glutathione, reduced nest building activities, and 4/6 fish from the 500 μg l^{-1} treatment displayed hepatocellular nuclear pleomorphism.	Sanders et al. (2008)
Carbon NP: C_{60} fullerenes (aggregates of 30–100 μm) dispersed in tetrahydrofuran (THF) with 'overnight' stirring	0.5 or 1.0 mg l^{-1} for 48 h	Largemouth bass (*Micropterus salmoides*)	Reduced lipid peroxidation in gill and liver tissue (0.5 mg l^{-1} treatment) and increased lipid peroxidation in brain tissue (both treatments).[b]	Oberdörster (2004)
Carbon NP: C_{60} fullerenes (10–200 nm aggregates) dispersed in water by stirring >2 months	0.5 mg l^{-1} for 96 h	Fathead minnow (*Pimephales promelas*)	A 42% down regulation of the peroxisomal lipid transporter protein, PMP70.	Oberdörster et al. (2006)
Carbon NP: C_{60} fullerenes (10–200 nm aggregates) dispersed in water with THF or by stirring for 2 months	0.5 mg l^{-1} for 48 h	Fathead minnow (*Pimephales promelas*)	100% mortality in fish exposed to C_{60} with THF between 6 and 18 h compared to none in the stirred C_{60} group. The latter group had elevated branchial lipid peroxidation, possibly in the brain also. Changes in P450 protein expression due to increases in hepatic CYP2-like-iso-enzymes.	Zhu et al. (2006)
Carbon NP: C_{60}, C_{70}, and $C_{60}(OH)_{24}$ fullerenes suspended in DMSO (no primary particle size given, aggregates ranged from ≈50 to 1200 nm).	100–500 μg l^{-1} for C_{60} and C_{70}, and 500–5000 μg l^{-1} for $C_{60}(OH)_{24}$ from 24 hpf to 96 hpf.	Zebrafish (*Danio rerio*) embryos (dechorionated)	Exposure to 200 mg l^{-1} C_{60} and C_{70} caused an increase in malformations, pericardial edema, and mortality, whilst higher concentrations (an order of magnitude) of $C_{60}(OH)_{24}$ produced less pronounced effects.	Usenko et al. (2007)
Carbon NP: C_{60} fullerenes (aggregates between 50 and 300 nm) dispersed in water by either stirring for 7 days with sonication or by use of THF	0–25% (v/v) for 72 h	Larval Zebrafish (*Danio rerio*)	Survival reduced in THF C_{60} and THF control treatments, but not in the water stirred treatment. Minimal gene expression changes in the latter treatment compared to control, whilst changes seen in THF C_{60} and THF control fish deemed to be linked to a THF degradation product (γ-butyrolactone).	Henry et al. (2007)
Carbon NP: C_{60} fullerene aggregates in suspension	0–1.5 mg l^{-1} for 96 h	Zebrafish (*Danio rerio*) embryos (dechorionated)	Embryonic development delayed and reduced hatching success.	Zhu et al. (2007)
Carbon NP: C_{60} fullerenes suspended in DMSO (no primary particle size given, aggregates ranged from ≈50 to 1200 nm)	0–500 μg l^{-1}, with or without H_2O_2, DEM, BSO, NAC or reduced light. Exposed at 24 hpf for 5 days	Zebrafish (*Danio rerio*) embryos (dechorionated)	Embryos exposed in decreased light conditions generally experienced significantly less mortality and malformations than other treatments. A dose dependent increase in toxicity seen in embryos exposed with GSH inhibitors (i.e., DEM and BSO).	Usenko et al. (2008)
Carbon NP: CNT (single-walled, 1.1 nm mean outside diameter, 5–30 μm length) dispersed in sodium dodecyl sulphate with 2 h sonication	0.1–0.5 mg l^{-1} for 10 days	Rainbow trout (*Oncorhynchus mykiss*)	Dose-dependent rise in ventilation rates, gill pathologies (oedema, altered mucocytes, hyperplasia), and mucus secretion (with CNT precipitation on gill mucus). Some dose-dependent changes in brain and gill Zn or Cu occurred along with statistically significant increases in Na^+/K^+-ATPase activity in the gills and intestine.	Smith et al. (2007)
Carbon NP: CNT (single-walled, same batch as used above in Smith et al., 2007) and C_{60} fullerenes (as used above in Henry et al., 2007)	0 or 500 mg SWCNT or C_{60} kg^{-1} diet for 6 weeks	Rainbow trout (*Oncorhynchus mykiss*)	Transient elevation (week 4 only) in brain TBARS from fish exposed to SWCNT.	Fraser et al. (2010)
Carbon NP: CNT (various forms (with or without functionalizations) of single- and multi-walled, diameters 10–20 nm, and single-walled nanohorns, diameter 2–3 nm)	In vitro exposure to 0–10 μg ml^{-1} for 6 or 24 h	Rainbow trout (*Oncorhynchus mykiss*) macrophage culture system	Dose dependent increases in inflammatory gene expression (IL-1β) in all treatments, with functionalized nanotubes more stimulatory than nonfunctionalized.	Klaper et al. (2010)
CeO_2-NPs (25 nm particle size)	1000 mg l^{-1} from 24 to 120 hpf	Zebrafish (*Danio rerio*) embryos	No acute toxicity and no intestinal or skeletal defects.	Ispas et al. (2009)
Co-NPs (dry powder mean primary particle size of 10.5 ± 2.3 nm), sonicated with a probe sonicator for six 0.5 s pulses prior to dosing	0–10 mg l^{-1} for 48 h	Zebrafish (*Danio rerio*)	Highest dose of 10 mg l^{-1} was not enough to establish an LC_{50} in adults or larvae exposed to both nano-form and dissolved Co.	Griffitt et al. (2008)
Cu-NPs (80 nm particles in agglomerates >1 μm in diameter)	0.1–1.5 mg l^{-1} for 48 h over two experiments	Zebrafish (*Danio rerio*)	Nanocopper found to be acutely toxic with a 48-LC_{50} of 1.5 mg l^{-1}. Dose dependent gill pathologies (proliferation of epithelial cells and oedema of primary and secondary filaments), inhibition of branchial Na^+/K^+-ATPase activity. Toxic effects suggested to be unexplained by the effects of dissolved Cu alone.	Griffitt et al. (2007)

(continued on next page)

Table 2 (*continued*)

Nanomaterial and additional information[a]	Exposure conditions	Fish species	Effects	Study
Cu-NPs (dry powder mean primary particle size of 26.7 ± 7.1 nm) sonicated with a probe sonicator for six 0.5 s pulses prior to dosing	0–10 mg l^{-1} followed by experiments with fish exposed to 0.6–2.78-fold the LC_{50} (both for 48 h)	Zebrafish (*Danio rerio*)	48 h LC_{50} of 0.94 mg l^{-1} in adult fish and 0.71 mg l^{-1} in juveniles; the latter more toxic than soluble Cu which had a 48 h LC_{50} value of 1.78 mg l^{-1} for larvae.	Griffitt et al. (2008)
Cu-NPs (dry powder mean particle diameter 26.7 ± 7.1 nm), sonication for 6 s in 1 s bursts prior to dosing	100 μg l^{-1} (representing the NOEC) for 48 h	Zebrafish (*Danio rerio*)	Gill filament width in Cu-NP exposed fish increased by approximately 3.5-fold and 1.5-fold between 24 and 48 h compared to control and dissolved Cu treatments respectively. Branchial Cu levels similar in Cu-NP (likely from dissolved Cu from NPs) and dissolved Cu treatments indicating low cellular uptake of Cu-NPs. Gene expression profile different following Cu-NP exposure to dissolved Cu.	Griffitt et al. (2009)
Fe-NPs (30 nm mean particle size)	0–50 μg l^{-1} (100% daily renewal) for up to 8 day (embryos) and 14 days (adults)	Japanese medaka (*Oryzias latipes*), embryos and adult fish	Medaka embryos experienced dose-dependent decreases in SOD and increases in MDA (TBARS assay), whilst adult medaka showed initial decreases in liver and brain SOD and a dose dependent, though transient, decrease in reduced glutathione at the start of the experiment. Adult fish exposed to 5 and 50 μg l^{-1} Fe-NPs displayed histological changes the gill and intestine, along with large amounts of aggregated Fe on *the gills and lesser accumulation within the intestine.*	Li et al. (2009)
Ni-NPs (dry powder mean primary particle size of 6.1 ± 1.4 nm), sonicated with a probe sonicator for six 0.5 s pulses prior to dosing	0–10 mg l^{-1} for 48 h	Zebrafish (*Danio rerio*)	Highest dose of 10 mg l^{-1} was not enough to establish an LC_{50} in adults or larvae exposed to both nanoparticulate and dissolved Ni.	Griffitt et al. (2008)
Ni-NPs (30, 60, and 100 nm particles and dendritic clusters of 60 nm particles) sonicated for at least 30 min	10–1000 mg l^{-1} from 24 to 120 hpf	Zebrafish (*Danio rerio*) embryos	Similar LD_{50} concentrations between the 30, 60, and 100 nm particles and soluble Ni, with dendritic clusters more toxic. Aggregates of Ni seen in gut lumen with concomitant deleterious effects such as thinning of the intestinal epithelium and skeletal muscle fibre separation.	Ispas et al. (2009)
Se-NPs (36 nm particles)	0.2–3.2 mg l^{-1} for 48 h and 100 μg l^{-1} for 10 days followed by 7 days depuration	Japanese medaka (*Oryzias latipes*)	Se-NPs more acutely toxic than selenite (48 h LC_{50} values of 1.0 and 4.7 mg l^{-1} respectively). Exposure to 100 μg l^{-1} Se-NPs increased tissue Se burden significantly compared to fish exposed to an equal concentration of selenite. Overall trend of reduced Se after depuration, though gill burden remained persistent. Decreased hepatic SOD activity and small, though significant elevation in GSH.	Li et al. (2008)
SiO_2 NPs (fluorescent core shell, 200 and 60 nm mean particle sizes) sonicated for 90 min prior to exposure	0.0025–200 mg l^{-1} for 96 hpf	Zebrafish (*Danio rerio*) embryos <6 hpf	Adsorption of both SiO_2 NPs to the chorion of exposed embryos, though no indication of uptake. No effects on development, survival, hatching time or hatching success.	Fent et al. (2010)
TiO_2 NPs (dry powder mean particle diameter 20.5 ± 6.7 nm), sonication for 6 s in 1 s bursts prior to dosing	1000 μg l^{-1} for 48 h	Zebrafish (*Danio rerio*)	No increase in gill filament width after 48 h compared to control. Expression of genes involved in ribosomal *function altered.*	Griffitt et al. (2009)
TiO_2 NPs (75% rutile, 25% anatase, 24.1 ± 2.8 nm mean particle size) dispersed by sonication	0.1–1.0 mg l^{-1} for 14 days	Rainbow trout (*Oncorhynchus mykiss*)	Changes in tissue Na^+/K^+-ATPase activity, TBARS, and total glutathione, the latter two indicating mild oxidative stress. Some organ pathologies, including the gut.	Federici et al. (2007)
TiO_2 NPs (average particle size of 21 nm)	10.0 ± 1.3 mg l^{-1} TiO_2 NPs in presence of 97.3 ± 6.9 μg l^{-1} Cd for up to 25 days	Common carp (*Cyprinus carpio*)	The presence of TiO_2 NPs increased uptake of Cd by 146% after 25 days exposure.	Zhang et al. (2007)
TiO_2 NPs (dry powder mean primary particle size of 20.5 ± 6.7 nm), sonicated with a probe sonicator for six 0.5 s pulses prior to dosing	0–10 mg l^{-1} for 48 h	Zebrafish (*Danio rerio*)	Highest dose of 10 mg l^{-1} was not enough to establish an LC_{50} in adults or larvae exposed TiO_2 NPs, no bulk TiO_2 powder treatments were used.	Griffitt et al. (2008)

TiO_2 NPs (50 nm primary particle size), surface area of 30 ± 10 m^2 g^{-1}, rutile form crystal structure, with a purity of >98.0% sonicated for 30 min	0–250 mg l^{-1} for 20 days	Juvenile Common carp (*Cyprinus carpio*)	No mortalities, though fish experienced dose dependent increases in respiratory distress with changes in SOD, CAT, POD and LPO levels indicating oxidative stress effects (most evident in the liver).	Hao et al. (2009)
TiO_2 NPs (average particle size of 21 nm)	10.0 ± 1.3 mg l^{-1} TiO_2 NPs in presence of 200.0 ± 10.2 μg l^{-1} As for up to 25 days	Common carp (*Cyprinus carpio*)	Accumulation of As in viscera, gills and muscle significantly enhanced by the presence of TiO_2 NPs.	Sun et al. (2009)
TiO_2 NPs (dry powder mean particle diameter of 34.2 ± 26.1 nm); NP injection solution sonicated for 30 min	Single dose of intravenously injected NPs (mean dose 1.3 mg NPs kg^{-1} body weight) and sampled for up to 90 days	Rainbow trout (*Oncorhynchus mykiss*)	Ti accumulation in the kidney which remained for 21 days with significant clearance by day 90. TiO_2 NPs located in tissue vesicles surrounding the kidney tubules, though kidney function not compromised (as indicated by plasma protein and creatinine levels). No accumulation in brain, gills, spleen, or liver. No evidence of lipid peroxidation in blood, liver or kidney.	Scown et al. (2009)
TiO_2 NPs (24.1 ± 2.8 nm mean particle size), dispersed by sonication for 6 h prior to coating dry feed pellets	0–100 mg TiO_2 NPs kg^{-1} diet for 8 weeks followed by 2 week recovery	Rainbow trout (*Oncorhynchus mykiss*)	No effects to growth, nutritional performance or haematology. Ti accumulated in the gill, gut, liver, spleen, and brain (with Ti not clearing in some organs following recovery). Disturbances to Cu and Zn levels and a 50% inhibition of Na^+/K^+-ATPase activity seen in the brain and a 50% reduction in TBARS in the gill and intestine was observed during exposure, though no changes to total glutathione.	Ramsden et al. (2009)
TiO_2 NPs (anatase form, 5 nm mean particle size),	In vitro exposure to 0.1–1000 μg ml^{-1} for 24 h	GFSk-S1; goldfish (*Carassius auratus*) skin cells	TiO_2 NPs, in the absence of photo-activation, induced genotoxic responses most likely as a result of free radical production.	Reeves et al. (2008)
TiO_2 NPs (75% rutile, 25% anatase, 24.1 ± 2.8 nm mean particle size)	In vitro exposure to up to 50 μg l^{-1} NPs between 4 and 24 h depending upon assay	RTG-2 cells; rainbow trout (*Oncorhynchus mykiss*) gonadal tissue	No elevation in DNA damage in the absence of UVA irradiation, though there was a significant reduction in lysosomal integrity over 24 h exposure. UVA exposure significantly increased levels of strand breaks.	Vevers and Jha (2008)
TiO_2 NPs (21 nm particle size), stock solution sonicated for 10 min	Fish fed *Daphnia magna* previously exposed to TiO_2 NPs for 14 days (measured Ti concentrations in 2 *D. magna* groups of 4.52 and 61.09 mg g^{-1} dry weight)	Zebrafish (*Danio rerio*)	Bioconcentration factors of less than 1 in fish fed contaminated *D. magna* show no biomagnification of TiO_2 NPs. However, dietary exposure to TIO_2 NPs resulted in higher body Ti burden than seen in aqueous exposed fish in complementary experiments.	Zhu et al. (2010)
ZnO-NPs (20 nm primary particle size, though aggregates ranged between 1037 and 6823 nm depending on time), stock prepared by vigorous stirring for 2 h immediately prior to dosing	0.1–100 mg l^{-1} until 96 hpf.	Zebrafish (*Danio rerio*) embryos	Dose-dependent decrease in hatching rates with low incidence of pericardial edema observed 72 hpf in 50 and 100 mg l^{-1} treatments, affecting approximately 10% of embryos by 96 hpf. Dissolved Zn was less toxic with no evidence of malformations. Oxidative stress suggested as one possible cause of toxicity following raised intracellular levels of ROS in ZnO-NP exposed embryos.	Zhu et al. (2009)

THF: tetrahydrofuran; Ag-NPs: silver nanoparticles; Al-NPs: aluminium nanoparticles; Au-NPs: gold nanoparticles; BSO: buthionine sulfoximine; CeO_2-NPs: cerium nanoparticles; CAT: catalase; CNT: carbon nanotubes; Cu-NPs: copper nanoparticles; Co-NPs: cobalt nanoparticles; DEM: diethyl maleate; DMSO: dimethyl sulfoxide; Fe-NPs: iron nanoparticles; GSH: reduced glutathione; H_2O_2: hydrogen peroxide; hpf: hours post fertilisation; LPO: lipid peroxidation; MDA: malondialdehyde; NAC: *N*-acetylcysteine; Ni-NPs: nickel nanoparticles; POD: peroxidase; Se-NPs: selenium nanoparticles; SiO_2 NPs: silica nanoparticles; SOD: superoxide dismutase; TiO_2 NPs: titanium dioxide nanoparticles; ZnO-NPs: zinc oxide nanoparticles.

[a] Additional information as provided by the authors.

[b] Subsequent studies have cast doubt on the source of toxicity with the dispersant THF suggested as pivotal to toxic effects seen (e.g. Henry et al., 2007).

possibility that toxic effects seen in fish following metal exposure may differ depending upon social rank (Sloman et al., 2003).

Evidence is now also emerging that some nanometals can also affect the gill in similar ways to dissolved metals. For example, exposure to 1 mg l^{-1} TiO_2 NPs in rainbow trout caused oedema in the gills (Federici et al., 2007). Griffitt et al. (2007) found that exposure to nano-Cu caused concentration-dependent damage to the lamellae characterised by proliferation of epithelial cells and oedema of primary and secondary filaments whilst an increase in gill filament width was observed in adult zebrafish exposed to nano-Cu, but not nano-TiO_2 or nano-Ag following a 48 h exposure (Griffitt et al., 2009). Notably in the later study gills exposed to nano-Cu showed significantly wider filaments than those from soluble Cu exposures. A concentration-dependent inhibition of branchial Na^+/K^+-ATPase activity was also observed in the nano-Cu treatments (Griffitt et al., 2007). Notably, Griffitt et al. (2007) found that the inhibition of Na^+/K^+-ATPase by nano-Cu was not as great as dissolved Cu (inhibition in fish exposed to 0.25 mg l^{-1} $CuSO_4$ approximately 5-fold that exposed to the same concentration nano-Cu). Other mechanisms of respiratory toxicity are also possible with metals, and for example, excess iron (irrespective of bioaccumulation) can result in iron flocs on the gills which can clog the gills resulting in respiratory distress (Peuranen et al., 1994; Dalzell and MacFarlane, 1999). This might also be highly relevant to other particles such as nanometals, but data remains to be collected on this aspect.

Toxic effects of NPs on fish blood remain to be investigated. However, for TiO_2 NPs, there seems to be no major disturbances to blood cell counts or plasma electrolytes in rainbow trout (Federici et al., 2007) and no evidence of lipid peroxidation (TBARS assay) in rainbow trout blood plasma following intravenous administration (Scown et al., 2009). The effects of other nanometals on fish haematology, immune cells, and plasma biochemistry remain to be documented. Biochemical disturbances in internal organs have been noted, and in particular, oxidative stress is emerging as a potential mechanism of NP toxicity. Oxidative stress, and the induction of enzymes involved in anti-oxidant defence is well known for dissolved metals (e.g., Cd induction of glutathione peroxidase (GPx) and superoxide dismutase (SOD) in fish muscle, Almeida et al., 2002; Cu induction of SOD and catalase (CAT), Sanchez et al., 2005). Some nanometals can also cause oxidative stress. For example, Federici et al. (2007) exposed juvenile rainbow trout 0–1.0 mg l^{-1} TiO_2 NPs for up to 14 days, which caused a rise in thiobarbituric acid reactive substances (TBARS, an indicator of lipid peroxidation) in the gills, liver and brain, along with increased total glutathione (GSH) in the gills and depleted GSH in the liver. Following exposure to 50 or 500 μg l^{-1} nano-cadmium sulphide (quantum dots), elevated levels of oxidised glutathione were observed in the gills of three-spined sticklebacks (Sanders et al., 2008). Oxidative stress was also implicated in a study exposing Japanese medaka to nano-Fe (Li et al., 2009). During the latter study both embryos and adult medaka experienced concentration-dependent decreases in the enzyme; superoxide dismutase (SOD). In adult fish, decreases in hepatic and cerebral SOD during the first few days of exposure were accompanied by reduced glutathione (GSH) levels in the brain. However, levels of both SOD and GSH returned to similar to control after 3 days and no differences were seen in levels of malondialdehyde (MDA, as an indicator of lipid peroxidation) in the liver or brain throughout the exposure. This indicates that adult medaka were able to successfully protect against oxidative stress through antioxidant activity, which were then able to replenish following initial utilisation. Japanese medaka embryos experienced a different response. As with adult fish, there was a dose-dependent depletion of SOD within hours of exposure, but this did not recover throughout the 8 days of exposure (most notably in the high, 50 mg l^{-1}, treatment). Lipid peroxidation product MDA was initially depleted following exposure, presumably regulated by antioxidants such as SOD, but as the experiment progressed a dose-dependent increase in MDA was seen, particularly in the 50 mg l^{-1} treatment, which showed an almost 4-fold increase in MDA compared to control and lowest dose Fe-NP (0.5 mg l^{-1}) treatment by day 8. Adult fish also exhibited some histological and morphological alterations in the gills and intestine (cell swelling, hyperplasia and granulomas) which were deemed to be as a direct result of contact with the NPs (as evidenced for example by large aggregates of Fe-NPs on the gills and accumulation in the intestine, Li et al., 2009). Dietary Fe has previously been seen to cause lipid peroxidation in the liver and heart of African catfish (Baker et al., 1997). An increase in intracellular reactive oxygen species (ROS) was observed in zebrafish embryos exposed to nano-ZnO and implemented in some toxic effects (see next section, Zhu et al., 2009). *In vitro* studies also show evidence of oxidative stress from NPs in fish cell lines. Reeves et al. (2008) in a study using goldfish skin cells (GFSk-S1) demonstrated that TiO_2 NPs, in the absence of photo-activation, induced genotoxicity most likely as a result of free radical production. Vevers and Jha (2008) exposed RTG-2 cells to TiO_2 NPs with and without ultraviolet radiation (UVA) and found that whilst there was no elevation in DNA damage in the absence of UVA irradiation, there was a significant reduction in lysosomal integrity over 24 h exposure, suggesting that the nanometal was also capable of damaging the cells without UV activation.

7. Toxicity of nanometals to early life stages of fish

Many studies have shown that the early life stages of fish are especially sensitive to metals (reviews, e.g., Weis and Weis, 1991; Jezierska et al., 2009), and that can lead to the loss of fish populations (e.g., acidic, metal-containing spring snowmelt, Havas and Rosseland, 1995). There is also some evidence that some nanometals can affect the development and survival of fish embryos. Asharani et al. (2008) exposed zebrafish embryos to silver-NPs and found the NPs distributed in the brain, heart, yolk and blood of embryos. Toxic effects included a concentration-dependent increase in mortality and delays in hatching. Developmental abnormalities consisted of twisted notochords, and slow blood flow associated with pericardial oedema and cardiac arrhythmia. Lee et al. (2007) developed techniques for imaging the transport of single silver-NPs (5–46 nm) through zebrafish embryos, and found that silver-NPs could penetrate the chorion via pore canals, with uptake kinetics characteristic of diffusion rather than active transport. In this study, concentration-dependent increases in mortality were observed as well as deformities including finfold abnormalities, tail and spinal cord flexure/truncation, cardiac malformation, also oedema in the yolk sac and head. Silver NPs were also seen to attach to the surface of the chorion and later detected within fathead minnow embryos (though not conclusively determined to be Ag–NPs) after just 24 h exposure to one of two commercial Ag–NP products (see Table 2 for details) (Laban et al., 2010). Yeo and Kang (2008) saw significantly decreased hatching rates in zebrafish embryos exposed to nano-silver. Furthermore, hatched fish displayed abnormal notochords, weak heart beat, curved tails, and damaged or absent eyes. Catalase, an important antioxidant enzyme, was also induced in exposed embryos, possibly indicating oxidative stress as one mechanism of toxicity.

Zhu et al. (2009) conducted a study to investigate the effects of an aggregated nanometal which had settled out of the water column in an attempt to mimic realistic environmental conditions. In their study reduced hatching rates and pericardial edema were seen in zebrafish embryos exposed to nano-ZnO, which was not likely to be attributable to dissolved Zn from the NPs with a positive control group (exposed to dissolved Zn) experiencing far fewer toxic effects. Reactive oxygen species (ROS) generation was higher in nano-ZnO exposed embryos compared to exposure to dissolved Zn, although notably, embryos exposed to the latter showed up regulation of genes for enzymes involved oxidative stress responses. These genes were not up regulated in nano-ZnO exposed embryos, with levels below controls at some time points. The authors concluded that disruptions in the ability of the embryos to counteract effects from ROS cannot be discounted, with nano-ZnO exposed animals experiencing disruptions

to hatching rates and physical development that could possibly have been associated with oxidative stress. Reduced hatching rates were also seen in zebrafish embryos exposed to nano-Zn along with decreased survival, delayed embryo and larval development and tissue damage (ulceration). However, exposure in the same study to bulk ZnO caused comparable effects and similar acute toxicity, with LD_{50} concentrations of 1.793 and 1.550 mg l^{-1} for nanoscale and bulk ZnO respectively (Zhu et al., 2008). In the same study exposure to titanium dioxide and alumina did not cause toxicity.

Size and shape effects of a nanometal on fish embryos were investigated by Ispas et al. (2009). In their study, dechorionated zebrafish embryos were exposed to spherical Ni-NPs of different sizes (30, 60, and 100 nm diameters) and a dendritic structure consisting of aggregated 60 nm particles (particle size distribution of 540 nm) with comparisons made to soluble Ni salts. Nickel NPs were equally, or less toxic than nickel salts (LD_{50}). But, dendritic clusters were markedly more toxic than both NPs and soluble Ni. (LD_{50} of the three NPs between 221 and 328 mg l^{-1}, soluble Ni; 221 mg l^{-1}, whilst dendritic clusters had an LD_{50} of 115 mg l^{-1}). Interestingly, although both nano- and soluble nickel had similar acute toxicity, sub-lethal affects manifested in different ways, with organ defects (similar in embryos exposed to Ni-NPs and dendritic clusters), absent in those exposed to soluble Ni, or at least until much higher concentrations. These effects included a thinning of the intestinal wall and skeletal muscle fibre separation. Analogous to data from nano-Cu experiments (previously detailed), the majority of toxic effects seen from nano-Ni do not stem from dissolved Ni released from the NPs, with Ispas et al. (2009) suggesting that only 2.4–3.8% of the observed toxicity was due to soluble metal. As discussed in the previous section, oxidative stress may be one mechanism of nanotoxicity in fish at the embryonic stage (e.g., Li et al., 2009), however a comparison of these effects in embryos exposed to dissolved metals is difficult due to the sparsity of data in the literature. This is most likely related to the difficulty in making such measurements in individuals due to the small size of embryos.

Numerous studies on the lethal and developmental affects of dissolved metals to early life stage fish, have led researchers to suggest that embryos are less sensitive than larval fish (e.g., Eaton et al., 1978; McKim et al., 1978; Dave, 1985; Shazili and Pascoe, 1986; Scudder et al., 1988; Kazlauskienė and Stasiūnaitė, 1999). However, Johnson et al. (2007) argue that embryos exposed after the chorion has hardened may display reduced sensitivity compared to those exposed beforehand (i.e., <1 h post-fertilisation) due to the protection afforded by this membrane. Therefore, when using fish embryos in (eco)toxicology tests, including those with NPs, exposure should commence within an hour of the eggs being laid in order to enable a more environmentally realistic appraisal of any effects (Johnson et al., 2007), although dechorionation presents an alternative, albeit more unrealistic, option.

8. Dietary exposure to nanometals

The consensus view is that dietary metal exposure is the main mode of chronic exposure of wild fish to metals, and that whilst dietary metals are not acutely toxic, there can be long term sub-lethal effects (review, Handy et al., 2005). These sub-lethal effects can include dose-dependent effects on growth (Clearwater et al., 2005), although fish usually adopt a bioenergetic strategy that preserves growth at the expense of other aspects of metabolism (Handy et al., 1999; Campbell et al., 2005a). The sub-lethal effects include increased costs of aerobic metabolism with subsequent changes in swimming performance (Campbell et al., 2002) or the ability to compete for food (Campbell et al., 2005b). Fish can also show subtle pathological changes in the liver (e.g., fatty change, foci of necrosis, Handy et al., 1999; Shaw and Handy, 2006) and evidence of transient oxidative stress (Baker et al., 1998; Berntssen et al., 2000; Hoyle et al., 2007). The osmoregulatory effects of dietary metal exposure are much less severe than via the aqueous route, although disturbances to tissue electrolytes and Na^+/K^+-ATPase are sometimes recorded (e.g., Hoyle et al., 2007).

To our knowledge, apart from a very recent report from our laboratory (Ramsden et al., 2009), there is very little information on the sub-lethal effects of dietary exposure to nanometals in fish. In mice, acute toxic effects can be observed (often after a single gavage of mg doses of the nanometal). For example, Chen et al. (2006) found that nano-Cu caused some pathology in the kidney, spleen and liver, some of which were classified as being severe or 'deadly severe'. Wang et al. (2006) observed severe symptoms of lethargy, with vomiting and diarrhoea for the first few days, in mice orally exposed to nanoscale zinc. Two mortalities occurred after the first week, with both animals showing aggregations of Zn particles in the intestine (Wang et al., 2006). In a study by Wang et al. (2007) no acute toxicity was seen in mice exposed to either nano-sized (25 or 80 nm) or fine (155 nm) TiO_2 particles by single oral gavage. However, changes to serum biochemical parameters and cellular pathology in hepatocytes were noted. In the only detailed nutritional toxicity study of nanometals in trout, Ramsden et al. (2009) observed no impact on growth or nutritional performance, but some changes to Cu and Zn levels in the brain, along with a 50% inhibition of brain Na^+/K^+-ATPase activity. Statistically significant decreases in TBARS in the gill and intestine compared to control were also seen during the experiment, although no changes to the total glutathione pool were observed. These data support the notion that nanometals could produce similar subtle toxic effects to metal salts in the diet, without compromising growth. Notably, Ramsden et al. (2009) compared their results for TiO_2 NPs against the known hazard from other metals, and argued that based on sub-lethal effects without inhibition of growth, the dietary hazard from TiO_2 NPs was similar to mercury at equivalent oral doses.

The main target organs for the dietary exposure route, and whether or not nanometals will accumulate in the edible flesh of fish to present a human health risk, remains to be established. Absorption efficiencies for essential metals ranges from around 30 to 70% in the diet of fish (Handy, 1996), although values for non-essential toxic metals can be only a few percent or less of the total oral dose (e.g., Cd, Franklin et al., 2005). There are no data on dietary uptake efficiency for nanometals in fish (or rodents), but the data of Ramsden et al. (2009) suggests that the uptake of TiO_2 NPs will be a few % of the total dose and with similar target organs to other dietary metals. The effects of nanometals on luminal chemistry in the gut, and the availability of nutrients from the gut lumen are unknown; as are the effects of nanometals on gut function. Nonetheless, the drinking of contaminated water can result in severe erosion of the gut epithelium in trout (TiO_2 NPs, Federici et al., 2007). This effect was not observed with dietary exposure via the food (Ramsden et al., 2009), suggesting the presence of food or organic matter in the gut lumen will have protective effects on the oral toxicity of some NPs in fish.

9. Stress responses of fish and metal nanoparticles

The stress responses of fish have been extensively investigated and include two generic responses, the adrenergic response and a cortisol-driven stress response. Both these responses are well known during metal exposure (see review by Handy, 2003 for Cu stress). The adrenergic response involves the elevation of adrenaline and/or noradrenaline in the blood following the stimulation of chromaffin cells in the anterior (head) kidney and posterior cardinal vein to release stored catecholamines (Reid et al., 1995; Sumpter, 1997). The noradrenaline is synthesised from dopamine via a Cu-dependent enzyme, dopamine β-hydroxylase, and the release involves an exocytosis pathway from the chromaffin cells (Handy, 2003). The stimuli causing the release of stored catecholamines include stimulation of the sympathetic nervous system, disturbances to local blood chemistry (anoxia/acidosis, high K^+) and various neuropeptides released from non-cholinergic nerves (Sumpter, 1997; Reid et al., 1998; Handy, 2003). For exposures to dissolved metals, it is clear that the

adrenergic response is stimulated by these mechanisms (Brown, 1993; Handy, 2003). Adrenaline and noradrenaline levels have not yet been measured in fish following nanometal exposure to confirm if this is also the case for NPs. It would seem likely, since NPs are respiratory toxicants in fish (Federici et al., 2007; Smith et al., 2007) and this should result in some respiratory acidosis to stimulate the adrenergic response. There is some evidence of brain injury from NPs in trout (Smith et al., 2007; Ramsden et al., 2009), but as yet, it is unclear how this would change any neurostimulation of the chromaffin cells, or release of neuropeptides into the blood. Direct effects of nanometals on chromaffin cells are unknown, but one concern is that NPs accumulated in the vesicular trafficking system in the cells could interfere with catecholamine release.

The elevation of plasma cortisol, in the short term, is a beneficial response to stress resulting in the release of red blood cells into the circulation and stimulation of ion uptake at the gills (e.g., McDonald and Milligan, 1997). The release of cortisol from the interrenal tissue in the kidney is driven by stimulation of the hypothalamic-pituitary-interrenal (HPI) axis via the secretion of precursor, adrenocorticotrophic hormone (ACTH), from the pituitary. Speculation has been made on how metals may interfere with this process (Handy, 2003) including Cu-dependent stimulation of prostaglandin E_2 (PGE_2) which can modulate the anterior pituitary as well as neurological stimulation of the hypothalamus. It seems inevitable that this response will be influenced by brain pathology from NPs and nanometals are known to cause inflammation reactions, which could stimulate PGE_2 and therefore cortisol release.

10. Conclusions and a perspective on hazard assessment

Data on the physiological effects of nanometals on fish are now emerging, and although the data set is limited with much research to be done, there is an indication that nanometals may not be as acutely toxic as dissolved metals. The data on sublethal effects show that nanometals do have adverse effects on fish, and that some of these toxic effects are also well known for dissolved metals (e.g., respiratory distress, inhibition of ATPases, oxidative stress). A key question is whether or not there are additional hazards from the nanoform compared to the dissolved metal. This needs to be addressed on a case by case basis for each nanometal, but overall, the sublethal effects observed for NPs are (so far) comparable to existing metals. Unusual pathologies (e.g., in the brain of fish) from NPs suggest that there may be additional or novel hazard associated with the nanoscale. The neurological effects of NPs could be important in modulation of stress responses in fish, but this remains to be determined by experimentation. Critically, in order to advance our interpretation of physiological effects data, methods for measuring nanometals in tissues are urgently needed.

The mechanisms of uptake and bioavailability of nanometals are likely to be very different to dissolved metals, with the former not being able to utilise ion transporters for the uptake of NPs, but instead using other mechanisms such as endocytosis. It is also clear that NP chemistry is very different to that of dissolved metals, and the equilibrium models we currently use to calculate free metal ion concentrations have limited theoretical foundation for its use with nanometals. The chemistry of NPs is fundamentally different (dynamic instability, not an equilibrium process), with perhaps one very important exception; the dissolution of free metal ions from the surface of NPs. In the latter case, it may be possible to apply existing biotic ligand models to the dissolved fraction. The metals community has also been working on more sophisticated models for the behaviour of metals in natural water which account for metal binding to soft colloids in the water column (e.g., humic acids), and the compartmentalisation of metals in other colloidal fractions (Rotureau and van Leeuwen, 2008; Duval, 2009). Natural colloids operate at the nanoscale, and it may be possible to develop bioavailability models for nanometals by incorporating knowledge on natural colloids (e.g., Lead and Wilkinson, 2006) with aspects of metal speciation, and the behaviour of engineered NPs. This would be an important step in enabling regulatory agencies to link the hazard information from physiological effects studies with the environmental chemistry of nanometals. Ideally, the regulatory agencies would prefer to be able to apply corrections for water hardness, pH, ionic strength, etc., in a similar way to dissolved metals. Particle theory suggests that such abiotic factors will be important, and while this may lead to empirical corrections to water quality standards that could be applied by regulators, one must not forget that the reasons behind such corrections will be fundamentally different to that for dissolved metals.

The advent of increasingly complex hybrid versions of (nano) materials containing more than one NP or doped metal, suggests it may be important to elucidate whether these nanohybrids exert a mixtures effect that would be equivalent to the sum of the individual metal components (or cause synergistic effects), and whether the unique properties of the material would require some novel approach to the issue of composite materials. Clearly, the stability of such composite materials in both the water and tissues may be critical in any mixture effect. This and other aspects of metal toxicity will need revisiting as new nanomaterials emerge.

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