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# The effects of manufactured nanoparticles on fish physiology, reproduction and behaviour

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**THE EFFECTS OF MANUFACTURED NANOPARTICLES ON FISH  
PHYSIOLOGY, REPRODUCTION AND BEHAVIOUR**

by

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A thesis submitted to the University of Plymouth  
in partial fulfilment for the degree of

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# **The effects of manufactured nanoparticles on fish physiology, reproduction and behaviour**

**Christopher Simon Ramsden**

## **Abstract**

Over the last decade the development and use of nanomaterials (NMs) and nanoparticles (NPs) has increased at a great rate. As a result there is an ever increasing risk of exposing humans and wildlife to these potentially harmful materials. Titanium dioxide nanoparticles (TiO<sub>2</sub> NPs) and carbon nanotubes (CNTs) are two of the most widely used NMs at present. Their potentially harmful effects on organisms and physicochemical properties have been investigated in a growing number of scientific studies. However understanding the level of risk they may pose is far from satisfactory. The present body of work has addressed various aspects of this field. In order to better quantify the fate of TiO<sub>2</sub> NPs in the environment the methodology of measuring Ti from TiO<sub>2</sub> NPs was improved using ICP-OES and single particle ICP-MS was demonstrated to provide the first steps towards characterising the nature of TiO<sub>2</sub> NPs in liquid-phase media. The potential harm of TiO<sub>2</sub> NPs and single walled carbon nanotubes (SWCNTs) to zebrafish was investigated in two separate studies. Little evidence of physiological toxicity was found and the only nano-scale effect of note was an increase in total glutathione of zebrafish exposed to TiO<sub>2</sub> NPs. More subtle effects in reproductive studies were further investigated using the three-spined stickleback in a longer term investigation. Similarly to the zebrafish there was little evidence of any physiological disturbances and the well documented reproductive behaviour of the stickleback was not significantly altered as a result of TiO<sub>2</sub> NP exposure. This body of work has added to the understanding of the potential toxic effects caused by exposure to both TiO<sub>2</sub> NPs and SWCNTs. Improved methods for the detection and characterisation of TiO<sub>2</sub> NPs have been demonstrated and the most sensitive tools for ecotoxicological assessments of NP toxicity have been elucidated.

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**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.

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**Ramsden, C.S.**, Shaw, B.J., Turner, A., Handy, R.D., submitted April 2012. A simplified method for determining titanium form TiO<sub>2</sub> nanoparticles in fish tissue and preliminary findings from single particle ICP-MS.

**Ramsden, C.S.**, Henry, T.B., Handy, R.D., submitted May 2012. Sub-lethal effects of titanium dioxide nanoparticles on the physiology and reproduction of zebrafish.

### List of conferences attended:

Platform presentations:

**Ramsden, C.S.**, Boyle, D., Henry, T.B. and Handy, R.D. The effects of manufactured nanoparticles on zebrafish. Presented at a school seminar at the University of Plymouth, Plymouth, UK, February 2011.

**Ramsden, C.S.**, Boyle, D., Henry, T.B. and Handy, R.D. The effects of manufactured nanoparticles on zebrafish. Presented at the 6<sup>th</sup> International Conference on the Environmental Effects of Nanoparticles and Nanomaterials, The Royal Society, London, UK, September 2011.

Work presented by others:

Boyle, D, Al-Bairuty, G., **Ramsden, C. S.**, Sloman, K. A., Henry, T. B. and Handy, R. D. (2012) Effects of Nanomaterials on Animal Behaviour- Are the Defects all Neurological? "From theory to practice-development, training and enabling Nanosafety and health research." 4<sup>th</sup> NanoImpactNet Integrating Conference, 27<sup>th</sup> February-2<sup>nd</sup> March 2012, University College Dublin, Ireland. Abstract book p57.

Handy, R. D., Windeatt, K. M., Al-Bairuty, G., **Ramsden, C. S.**, Boyle, D. B., Sloman, K. A., Henry, T. B. (2012) Concerns for Nano Effect on Nerves, and Brain Pathologies From Different Nanomaterials (TiO<sub>2</sub>, SWCNT, C<sub>60</sub>, Cu-NPs). "From theory to practice-development, training and enabling Nanosafety and health research." 4<sup>th</sup> NanoImpactNet Integrating Conference, 27<sup>th</sup> February-2<sup>nd</sup> March 2012, University College Dublin, Ireland. Abstract book p56.

Boyle, D., Al-Bairuty, G., **Ramsden, C. S.**, Sloman, K. A., Henry, T. B. & Handy, R. D. (2010). Effects of TiO<sub>2</sub> nanoparticles on locomotor and social behaviour in rainbow trout. SETAC North America 31st Annual Meeting, 7-11<sup>th</sup> November, 2010, Portland, Oregon, USA, Abstract book number 522.

Boyle, D., Al-Bairuty, G., **Ramsden, C. S.**, Sloman, K. A., Henry, T. B. & Handy, R. D. (2010). Effects of TiO<sub>2</sub> nanoparticles on locomotor and social behaviour in rainbow trout. Third Annual Marine Institute Conference, Plymouth, Devon, UK.

Handy, R. D., Smith, T. J., **Ramsden, C. S.**, Shaw, B. J. Voskou, S. and Henry T. B. (2009) Target organ pathologies from nanomaterials: a histological review of trout and implications for human health. Society of Environmental Toxicology and Chemistry UK branch annual meeting, "Environment and Human Health", 14-15<sup>th</sup> September 2009, London, UK. Abstract No. 1.12.

Handy, R. D., Smith, T. J., **Ramsden, C. S.**, Shaw, B. J. Voskou, S. and Henry T. B. (2009) Target organ pathologies from nanomaterials: a histological review of dietary and aqueous exposure in rainbow trout. 4<sup>th</sup> International Conference on the Environmental Effects of Nanoparticles and Nanomaterials, University of Vienna, Austria, 6-9<sup>th</sup> September 2009, Abstract book.

Poster presentations:

**Ramsden, C.S.**, Shaw, B.J., Turner, A. and Handy, R.D. Determination of total titanium from titanium dioxide nanoparticles in fish and water samples. Presented at the 20<sup>th</sup> SETAC Europe Annual Meeting, Seville, Spain, May 2010.

**Ramsden, C.S.**, Shaw, B.J., Turner, A. and Handy, R.D. Determination of total titanium from titanium dioxide nanoparticles in fish and water samples. Presented at Spirit of Discovery, Marine Institute, University of Plymouth, Plymouth, UK, December 2010.

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Signed...C. S. Ramsden.....

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# 1. General introduction

## 1.1. Introduction

Nanotechnology and the use of nanoparticles (NPs) is a relatively new area of science and technology. The previous definition of nanoparticles was particles with at least one dimension  $< 100$  nm (Roco, 2003; SCENHIR, 2007), however this has been recently updated to define nanoparticles as natural, incidental or manufactured, where 50 % or more of the particles are 1-100 nm in the number size distribution (SETAC World, 2012). There are various other terms used in describing this group of particles, with the most common alternative being ultrafine particles. Particle sizes have been categorised in respiratory toxicity fields for many years with typical nomenclature being as follows: coarse particles ( $< 10$   $\mu\text{m}$ ); fine particles (0.01-2.5  $\mu\text{m}$ ); and ultrafine particles (0.01-0.1  $\mu\text{m}$ ) (von Klot et al., 2002). Nanomaterials (NMs) are not necessarily nano-scale themselves but are manufactured with NPs. The sorts of manufactured nanomaterials include carbon-based materials such as carbon nanotubes (CNTs), nanowires,  $\text{C}_{60}$  fullerenes and their derivatives (also called Buckminster fullerenes or Bucky Balls, (Fako and Furgeson, 2009)). Metals are also used to make NMs and metal NPs (e.g. Ag, Au, Cu, Zn), and there are also metal oxide NPs (e.g.  $\text{TiO}_2$ ,  $\text{CeO}_2$ , ZnO, (Hussain et al., 2005; Zhu et al., 2008)). There are also composite nanomaterials made of more than one chemical substance (e.g., quantum dots). These diverse types of manufactured nanoparticles can be found in a wide range of products including electronics, paints and dyes, sun creams, cosmetics, industrial coatings and even medical devices (Aitken et al., 2006; Boxhall et al., 2007; Klaine et al., 2008).

Information on the aquatic ecotoxicity of manufactured nanoparticles (NPs) is now emerging (reviews, Moore, 2006; Nowack and Bucheli, 2007; Handy et al., 2008c;

Klaine et al., 2008; Kahru and Dubourguier, 2010; Klaine et al., 2012), and the data generated so far generally shows that the lethal levels to aquatic species are at high mg l<sup>-1</sup> concentrations, and that sub-lethal effects can occur at around 1 mg l<sup>-1</sup> or less. However, information on sublethal effects is still being collected and the data sets, especially for fishes, remain limited (reviews, nanometals, Shaw and Handy, 2011; body systems-effects on fishes, Handy et al., 2011).

While manufactured NPs may present benefits to society, there is still very limited knowledge on the possible toxicological effects to human health and the environment (Handy and Shaw, 2007; Handy et al., 2008a). Recent research has focused on the toxicity of carbon nanotubes and C<sub>60</sub> (Fiorito et al., 2006; Zhu et al., 2006; Smith et al., 2007), as well as metal and metal oxide NPs (e.g. titanium dioxide, Gurr et al., 2005; Warheit et al., 2006; Federici et al., 2007; Ramsden et al., 2009). This research is important to provide data on hazard for risk assessments and legislation such as REACH (Owen and Handy, 2007; Crane et al., 2008), and to help establish ecotoxicity testing protocols in the UK and elsewhere. Notably, current legislation and test methods do not necessarily take into account the novel physicochemical properties and biological activities of these NPs and NMs, or account for particle size. This means that a novel NM version of an existing bulk material of greater size may not have been sufficiently tested. Furthermore, many NMs have the same chemical formula as existing bulk materials, and therefore the same CAS number, and may escape the triggers for testing a new substance. In effect, if the chemical has been tested and certified then any new version of the same chemical does not have to be tested again. This means that particle size and other novel properties may not be taken into account (Handy and Shaw, 2007). However, there is an ever increasing body of evidence to suggest that these new nanoparticle forms of existing materials may have different characteristics and may actually cause harm (Boxhall et al., 2007; Handy et al., 2008b).

The issue of dispersal of NPs is a common problem area in current research. Various dispersal techniques are used for the preparation of NP solutions ranging from sonication in ultrapure water (Oberdörster et al., 2006; Ramsden et al., 2009; Bilberg et al., 2010) to the use of a solvent such as tetrahydrofuran (THF) or a detergent such as sodium dodecyl sulphate (SDS) (Oberdorster 2004 and Smith et al., 2007 respectively). A debate has arisen about the best dispersion methods because the physicochemical properties of NPs are not totally understood. It is also unclear if these methods are environmentally relevant because the actual physical state of NPs in the natural environment is poorly described (Handy et al., 2008c). Indeed the level of aggregation of NPs may depend on several variables (water pH, calcium content, dissolved organic matter content etc.) all of which may change according to specific habitat. As a result there has been much discussion regarding these issues (see Handy et al. (2008a) for a review) and there are as yet no firmly agreed international protocols for toxicity testing of NMs. Currently it is very difficult to measure levels of manufactured NPs in the natural environment due to the presence of natural NPs and colloids (Boxhall et al., 2007; Handy et al., 2008c; Mueller and Nowack, 2008; von der Kammer et al., 2012). In the meantime the possible ecotoxicological effects of NPs are being examined in an attempt to determine to what extent controls for environmental protection need to be enforced. These studies will be reviewed and the issues of environmental relevance will be discussed.

This review aims to outline the current research in the field of nanoparticle toxicity, with particular reference to titanium dioxide nanoparticles and carbon nanotubes as these are currently some of the most widely used nanomaterials (Boxhall et al., 2007). The review will briefly examine the current knowledge on the effects of NPs in two model test species – the zebrafish (*Danio rerio*) and the three-spined stickleback (*Gasterosteus aculeatus*). The review will also outline the role of bioenergetics in the

life of the fish with reference to the possible impacts of environmental stressors such as nanomaterials.

## 1.2. Titanium dioxide

Titanium dioxide, also known as titanium (IV) oxide, titania and  $\text{TiO}_2$ , is the naturally occurring oxide of titanium. One of its primary uses is as a white pigment, in which case it is known as titanium white or Pigment White 6. There are three polymorphs of titania: anatase; rutile and brookite, with the most commonly occurring being rutile (Figure 1.1).

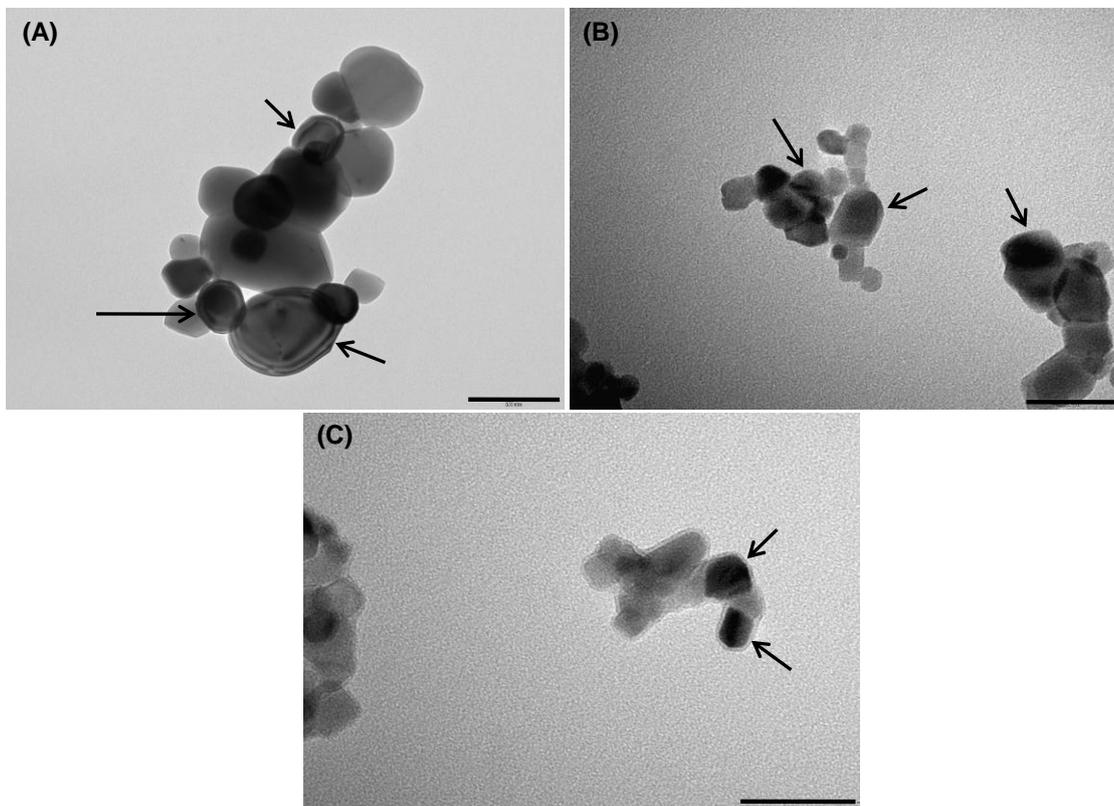


Figure 1.1. TEM images of  $\text{TiO}_2$  (A) bulk particles, scale bar = 200 nm, (B) and (C) NPs, scale bar = 50 nm. Arrows indicate rutile crystals – other crystals are anatase. Note the presence of nano-sized particles in the bulk media.

The main difference between these forms of titania lies in their crystal symmetries.

Titania is a good white pigment because of its brightness and very high refractive index.

It is used to provide whiteness and opacity in paints, coatings, plastics, inks, foods (food

dye E171) and toothpastes (Aitken et al., 2006; Jin et al., 2007). It is also found in sun creams due to its ability to absorb UV (Jaroenworarluck et al., 2006). Titanium dioxide is also used as a photocatalyst, being used in the presence of UV radiation (sunlight) to catalyse the oxidation of various compounds and therefore has uses in the treatment of waste water and in antifouling paints (Cho et al., 2004; Hirakawa et al., 2004; Gurr et al., 2005). The redox properties of titanium dioxide are of toxicological concern, with particular reference to oxidative stress in biota (Gurr et al., 2005), also outlined in Figure 1.2. In the presence of organic matter (a carbon source) and UV radiation  $\text{TiO}_2$  NPs act as catalysts in the generation of radicals of oxygen and water (Uchino et al., 2002; Reeves et al., 2008).

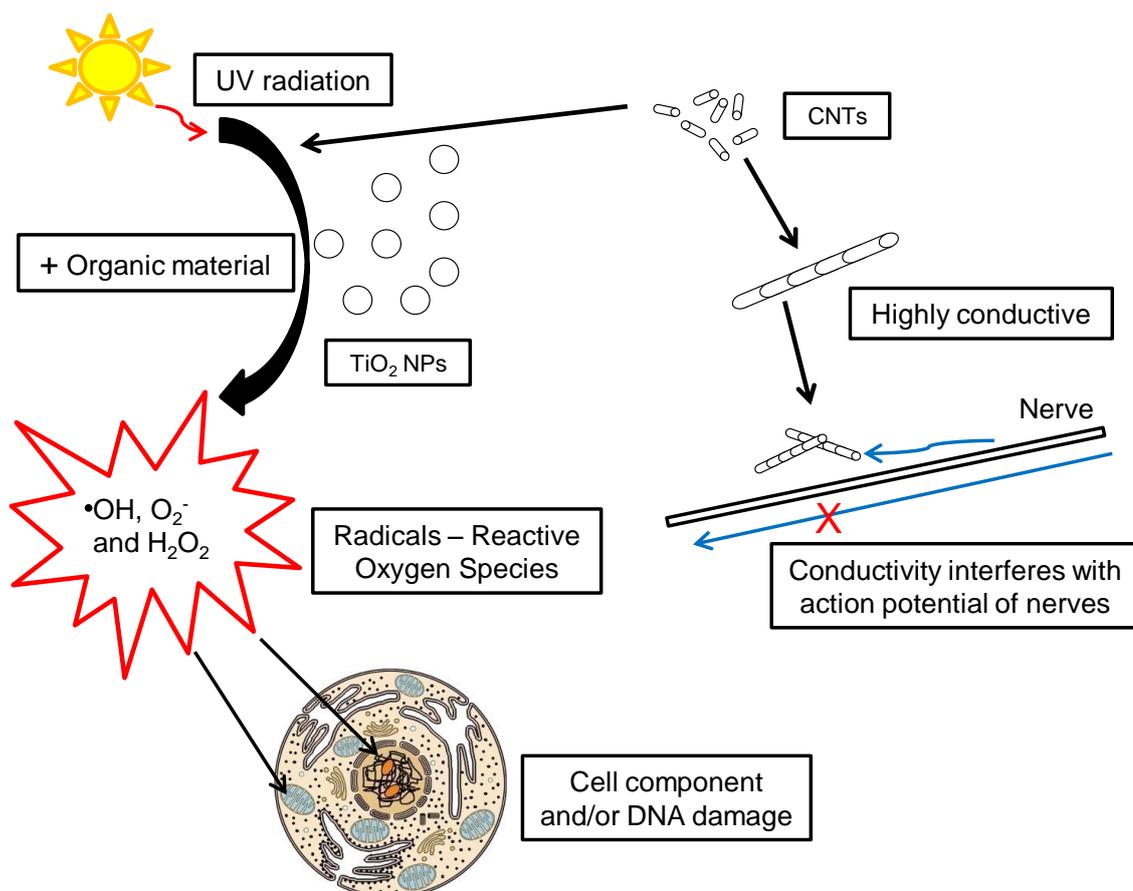


Figure 1.2. Possible mechanisms of toxicity of NPs. In the presence of UV radiation NPs may act as catalysts in the generation of reactive oxygen species. CNTs also display unique conductivity properties which may interfere with nervous system.

### 1.2.1. TiO<sub>2</sub> toxicity

Over the last decade a growing body of work on the effects of TiO<sub>2</sub> has been building up (Table 1-1). Early *in vivo* work was carried out with small mammals such as mice, rats and hamsters (Bermudez et al., 2004; Warheit et al., 2005; Warheit et al., 2006; Wang et al., 2007; Warheit et al., 2007; Park et al., 2009) and invertebrates (Lovern and Klaper, 2006), along with *in vitro* studies on human (Gurr et al., 2005; Hussain et al., 2009) and mouse (Xu et al., 2009) cell lines.

This early work focussed on the effect of particle size, surface area, crystal form and surface properties on toxicity. Some reviews had suggested that the smaller scale nanoparticles had a greater inflammogenic effect than larger particles (Donaldson et al., 2001; Oberdorster et al., 2005). Since then size effects on toxicity have been further examined both *in vivo* and *in vitro*. Lovern and Klaper (2006) tested the effects of TiO<sub>2</sub> NPs on *Daphnia magna*, testing the effect of aggregate size by filtering the stock solutions. They found that the filtered stock with smaller aggregates of around 30 nm was more toxic than unfiltered, sonicated stocks (Table 1-1). The same was observed by Wang et al. (2007) in mice and has also been demonstrated by Gurr et al. (2005) and Hussain et al. (2009) in *in vitro* studies using human bronchial epithelial cell lines. All of these studies report higher levels of toxicity caused by the smaller NPs – even when comparing two nano-scale particles Hussain et al. (2009) found that 15 nm particles produced a higher level of oxidative stress than larger 50 nm particles.

Whether this increased toxicity observed in tests using NPs compared to fine particles is a result of the increased surface area of the particle or due to other physicochemical property differences between particle sizes remains unclear. Warheit et al. have carried out several studies on particle size and surface area/surface treatment effects using rats (Warheit et al., 2005; Warheit et al., 2006; Warheit et al., 2007). They argue that any toxicity differences seen between NPs and fine particles are merely

transient (24 h) and that surface area is not correlated to toxicity. Warheit et al. also suggest that surface treatment effects such as the addition of  $\text{Al}_2\text{O}_3$  or  $\text{SiO}_2$  produces a higher level of toxicity than the  $\text{TiO}_2$  itself.

Also of interest is the issue of toxicity caused by oxidative stress and the generation of reactive oxygen species (ROS). Gurr et al. (2005) demonstrated that  $\text{TiO}_2$  NPs (10 & 20 nm anatase) were responsible for the generation of oxidative stress biomarkers in human bronchial epithelial cells as evidenced by increased lipid peroxidation and levels of measured ROS. This generation occurred in the absence of UV radiation. In contrast a study using a goldfish skin cell line reported that exposure to  $\text{TiO}_2$  NPs alone did not result in significant toxicity. Only when cells were exposed in conjunction with UVA was there a dose-dependent increase in toxicity evidenced by a decrease in cell viability (Reeves et al., 2008). A third study by Vevers and Jha (2008) used a rainbow trout cell line test the effect of UVA on  $\text{TiO}_2$  NP toxicity. Toxicity was observed in cells exposed to  $\text{TiO}_2$  NPs in the absence of UVA but an increase in DNA damage was evident when cells were exposed to NPs in combination with UVA. The generation of ROS may also be affected by the presence of organic matter. In their study on a human bronchial epithelial cell line Hussain et al. (2009) found that only produced significant levels of ROS when in conjunction with the cells. Cell-free trials resulted in no significant ROS production. The issue of organic matter being involved in the generation of ROS by  $\text{TiO}_2$  NPs was also suggested by Vevers and Jha (2008).

Recent work by Galloway et al. (2010) examined the effect of  $\text{TiO}_2$  NPs on the marine lugworm. The authors found that at the two higher doses (2 and 3  $\text{g kg}^{-1}$  sediment) evidence of cytotoxicity was seen with a significant reduction in neutral red retention time in coelomocytes and a significant increase in DNA damage. Whilst these concentrations were high compared to current estimated (computer modelled estimates which do not include the probability for aggregation and settling out of the water

column) environmental levels (Boxhall et al. (2007) predicted fresh water concentrations in the range of 24-245  $\mu\text{g l}^{-1}$  and Gottschalk et al. (2009) predicted even lower concentrations) it is worth noting that no real data has been collected for marine environmental concentrations of manufactured NPs. It is also thought that salinity may have an effect on NP aggregation (Handy et al., 2008c; Vevers and Jha, 2008), a result of which may be accumulation of aggregates of  $\text{TiO}_2$  NPs in the marine environment.

Table 1-1. Toxic effects of TiO<sub>2</sub> NP exposure (*in vivo* and *in vitro*).

Nanomaterial/chemical characteristics	Dose & exposure time	Exposure route	Species	Toxic effects	Reference
Ultrafine TiO <sub>2</sub> . Primary particle size 21 nm, directly dispersed into air.	0, 0.5, 2.0 or 10.0 mg m <sup>-3</sup> for 6 h per day, 5 d per week over 13 weeks. Recovery up to 52 weeks.	Aerosol inhalation	Mice, rats and hamsters	Pulmonary particle overload in mice and rats exposed to 10 mg m <sup>-3</sup> . These groups showed pulmonary inflammation evidenced by increased numbers of neutrophils and macrophages, as well as elevated levels of lactate dehydrogenase and proteins. Hamsters showed no real toxicity, clearing the uf-TiO <sub>2</sub> with no significant effects observed.	Bermudez et al. (2004)
Ultrafine TiO <sub>2</sub> . Primary particle sizes 10, 20, 200 or >200 nm anatase, 200 nm rutile, suspended in PBS.	10 µg ml <sup>-1</sup> for 1-72 h and with or without light depending on experiment.	<i>in vitro</i> exposure	Human bronchial epithelial cells BEAS-2B (ATCC CRL-9609)	Oxidative DNA damage caused by exposure to 10 and 20 nm anatase TiO <sub>2</sub> NPs in total darkness as evidenced by increased lipid peroxidation and levels of ROS. Larger anatase particles showed significantly lower/no oxidative stress.	Gurr et al. (2005)
TiO <sub>2</sub> NPs. Suspended in ultrapure water or THF. Filtered solutions with an average aggregate size of 30 nm, sonicated (un-filtered) aggregate range 100-500 nm.	0-10 mg l <sup>-1</sup> filtered, 0-500 mg l <sup>-1</sup> sonicated TiO <sub>2</sub> NPs for 48 h.	Aqueous exposure	Invertebrate <i>Daphnia magna</i>	Significantly higher toxicity caused by filtered TiO <sub>2</sub> NPs compared to un-filtered, sonicated. LC <sub>50</sub> value of 5.5 mg l <sup>-1</sup> for filtered TiO <sub>2</sub> whereas the sonicated solution did not produce sufficient mortality to generate an LC <sub>50</sub> value. No significant difference between TiO <sub>2</sub> NPs prepared with ultrapure water to those prepared with the THF vehicle.	Lovern & Klaper (2006)
TiO <sub>2</sub> NPs and fine particles. Primary particle sizes 6 nm (anatase dots), 92-233×20-35 nm (anatase rods) and 300 nm (rutile fine particles). Suspended in PBS and subjected to polytron dispersement.	1 or 5 mg kg <sup>-1</sup> body weight single dose. Sampled at 24 h, 1 and 3 months.	Intratracheal instillation	Rats	Short term lung inflammation and macrophage accumulations. No significant differences between fine and nano-scale particles. Surface area of particle also showed no difference in toxicity.	Warheit et al. (2006)

Nanomaterial/chemical characteristics	Dose & exposure time	Exposure route	Species	Toxic effects	Reference
TiO <sub>2</sub> NPs and fine particles. Primary particle sizes 25, 80 & 150 nm, suspended in HPMC K4M agent and sonicated.	5 g kg <sup>-1</sup> body weight single dose. Observed post-exposure for 14 d.	Oral exposure	Mice	Higher toxicity observed in NP exposed mice compared to fine sized TiO <sub>2</sub> . Females in NP groups showed inflamed livers with concurrent increase in liver pathology – namely lesions and necrosis. LDH significantly higher in NP exposed groups.	Wang et al. (2007)
TiO <sub>2</sub> NPs. Primary particle sizes 15 nm (anatase) and 50 nm (anatase rutile mix). Suspended in cell culture medium and sonicated.	24-760 mg l <sup>-1</sup> for 4-24 h depending on experiment.	<i>in vitro</i> exposure	Human bronchial epithelial cell line (16HBE14o)	Smaller NPs (15 nm) produced a higher level of oxidative stress than the larger ones (hydroethidine positive cells 18.6 and 13 % respectively). TiO <sub>2</sub> NPs induced oxidative stress through production of ROS only when in conjunction with cells – cell-free trials resulted in no significant ROS production.	Hussain et al. (2009)
TiO <sub>2</sub> NPs and fine particles. Primary particle sizes 5, 40 & 325 nm, suspended in distilled water and sonicated.	0-100 mg l <sup>-1</sup> for 24 h.	<i>in vitro</i> exposure	<i>gpt</i> delta transgenic mouse primary embryo fibroblasts (MEF)	Dose-dependent decrease in cell viability in cells exposed to the 40 nm TiO <sub>2</sub> , with an LD <sub>50</sub> value of 30 mg l <sup>-1</sup> . Similar dose-dependent increase in mutagenicity caused by exposure to the 40 nm particle. Significantly higher production of ROS in the TiO <sub>2</sub> NP-exposed cells compared to the 325 nm and control groups.	Xu et al. (2009)
TiO <sub>2</sub> NPs. Primary particle size 21 nm with mean aggregate size of 200 nm. Suspended in PBS and sonicated.	5, 20 or 50 mg kg <sup>-1</sup> single dose. Observed post-exposure for up to 14 d.	Intratracheal instillation	Mice	Levels of pro-inflammatory, Th1- type and Th2-type cytokines all significantly increased after exposure, with a 167 fold increase in pro-inflammatory cytokines after 24 h in the group exposed to 20 mg kg <sup>-1</sup> compared to the controls. Pathological toxicity observed in all exposed groups as evidenced by the presence of granulomatous lesions in the lungs.	Park et al. (2009)

Nanomaterial/chemical characteristics	Dose & exposure time	Exposure route	Species	Toxic effects	Reference
TiO <sub>2</sub> NPs. Primary particle size 23.2 nm, suspended in ultrapure water and sonicated.	1-3 g kg <sup>-1</sup> in sediment for 10 d.	Sediment exposure	Lugworm ( <i>Arenicola marina</i> )	Cytotoxicity evidenced by significantly reduced neutral red retention time in coelomocytes exposed to 2 and 3 g kg <sup>-1</sup> TiO <sub>2</sub> NPs. Significant increase in DNA damage at 2 and 3 g kg <sup>-1</sup> TiO <sub>2</sub> NPs.	Galloway et al. (2010)
TiO <sub>2</sub> NPs. Primary particle size 24 nm, suspended in ultrapure water and sonicated.	0, 0.1, 0.5 or 1.0 mg l <sup>-1</sup> for 14 d	Aqueous exposure	Rainbow trout ( <i>Oncorhynchus mykiss</i> )	Oxidative stress caused in all treatments of TiO <sub>2</sub> NP with changes in TBARS and total glutathione in a dose-dependent manner. Pathologies evident in gills and other tissues.	Federici et al. (2007)
TiO <sub>2</sub> NPs. Primary particle size 5 nm, suspended in phosphate buffered saline (PBS) and complete growth medium.	0.1-1000 mg l <sup>-1</sup> for 2 or 24 h and with or without UVA depending on the experiment	<i>in vitro</i> exposure	Goldfish ( <i>Carassius auratus</i> ) skin cell line GFSk-S1	Cell viability significantly decreased with co-exposure to TiO <sub>2</sub> and UVA in a dose-dependent manner. Exposure to TiO <sub>2</sub> alone had little effect. Some DNA damage caused by both TiO <sub>2</sub> and TiO <sub>2</sub> with UVA.	Reeves et al. (2008)
TiO <sub>2</sub> NPs. Primary particle size 24.4 nm, suspended in either ultrapure water or PBS.	0-50 mg l <sup>-1</sup> for 4-48 h and with or without UVA depending on the experiment	<i>in vitro</i> exposure	Rainbow trout ( <i>Oncorhynchus mykiss</i> ) cell line RTG-2	Toxicity without UVA was seen by a significant reduction in lysosomal integrity after 24 h exposure to 50 mg l <sup>-1</sup> TiO <sub>2</sub> NPs. Increase in DNA damage when exposed in combination with UVA.	Vevers and Jha (2008)
TiO <sub>2</sub> NPs. Primary particle size 50 nm, suspended in ultrapure water and sonicated.	0, 10, 50, 100 or 200 mg l <sup>-1</sup> for 20 d	Aqueous exposure	Common carp ( <i>Cyprinus carpio</i> )	Some respiratory distress and gill pathology increased in a dose-dependent manner. Oxidative stress evidenced by changes in SOD, CAT, POD and LPO levels in 100 and 200 mg l <sup>-1</sup> groups.	Hao et al. (2009)
TiO <sub>2</sub> NPs. Primary particle size 34.2 nm, suspended in trout perfusion ringer and sonicated.	100 µg per fish – single dose, sampled up to 90 d	Intravenous injection into caudal vein	Rainbow trout ( <i>Oncorhynchus mykiss</i> )	No toxicity observed but kidneys were shown to carry 10-19 % of the TiO <sub>2</sub> administered, with elevated levels observed up to 90 d post-injection.	Scown et al. (2009)

Nanomaterial/chemical characteristics	Dose & exposure time	Exposure route	Species	Toxic effects	Reference
TiO <sub>2</sub> NPs. Primary particle size 24.1 nm, suspended in ultrapure water and sonicated.	0, 10 or 100 mg kg <sup>-1</sup> TiO <sub>2</sub> NPs diets for 8 weeks followed by 2 weeks recovery on the control diet	Dietary exposure	Rainbow trout ( <i>Oncorhynchus mykiss</i> )	Ti accumulation occurred in several organs with the brain not clearing Ti after the exposure period. 50 % inhibition of brain Na <sup>+</sup> K <sup>+</sup> -ATPase activity in 100 mg kg <sup>-1</sup> group during exposure. Significant decreases in gill and intestine TBARS levels.	Ramsden et al. (2009)
TiO <sub>2</sub> NPs. Primary particle size 27.7 nm, suspended in 'fish' water containing 0.6 % Instant Ocean and ultrapure water, sonicated.	0, 0.1, 0.5, 1, 5 or 10 mg l <sup>-1</sup> from spawning to 5 days post fertilisation	Aqueous exposure	Zebrafish ( <i>Danio rerio</i> ) embryos	No significant effects on embryo hatchability or survival. Embryos exposed to 0.1, 0.5 and 1 mg l <sup>-1</sup> TiO <sub>2</sub> NPs showed decreased swimming activity compared to controls and 5 and 10 mg l <sup>-1</sup> TiO <sub>2</sub> NP groups.	Chen et al. (2011a)
TiO <sub>2</sub> NPs. Size range 80-110 nm, suspended in dechlorinated water and sonicated.	0, 1, 2, 4, 5 or 7 mg l <sup>-1</sup> for up to 6 months	Aqueous exposure	Zebrafish ( <i>Danio rerio</i> )	Increased mortality and decrease in growth in the fish exposed to 4, 5 and 7 mg l <sup>-1</sup> TiO <sub>2</sub> NP. Accumulation of Ti in gills, liver, heart and brain but pathology only evident in the gills of fish exposed to 5 and 7 mg l <sup>-1</sup> TiO <sub>2</sub> NP.	Chen et al. (2011b)
TiO <sub>2</sub> NPs. Primary particle size 21 nm, suspended in ultrapure water, sonicated and centrifuged.	0-14 mg l <sup>-1</sup> from spawning to 17 days post fertilisation	Aqueous exposure	Japanese medaka ( <i>Oryzias latipes</i> )	No effect on mortality of the embryos. Increased TiO <sub>2</sub> NP concentration resulted in decreased time to hatching. Some increase in incidence of moribund swimming and a resultant increase in fry mortality.	Paterson et al. (2011)
TiO <sub>2</sub> NPs. Aggregate size range 240-360 nm, suspended in ultrapure water and sonicated.	0, 0.1 or 1 mg l <sup>-1</sup> for up to 13 weeks	Aqueous exposure	Zebrafish ( <i>Danio rerio</i> )	Reduction in the cumulative number of eggs produced in TiO <sub>2</sub> exposed fish. Reduced survival of embryos produced by fish exposed to 0.1 mg l <sup>-1</sup> TiO <sub>2</sub> NP. Accumulation of Ti in the ovaries of females exposed to 0.1 and 1 mg l <sup>-1</sup> TiO <sub>2</sub> NP.	Wang et al. (2011)
TiO <sub>2</sub> NPs. Primary particle size range 20-70 nm, suspended in distilled water and sonicated.	0-300 mg l <sup>-1</sup> for lethality tests, 50 mg l <sup>-1</sup> for and oxidative stress tests	Aqueous exposure	Zebrafish ( <i>Danio rerio</i> )	96 h LC <sub>50</sub> of 124.5 mg l <sup>-1</sup> for TiO <sub>2</sub> NPs. High production of ·OH by TiO <sub>2</sub> NPs. Oxidative damage to gill tissue caused by TiO <sub>2</sub> NP exposure at 50 mg l <sup>-1</sup> .	Xiong et al. (2011)

### 1.2.2. TiO<sub>2</sub> toxicity in fish

The *in vivo* studies carried out on fish thus far have reported some common themes. Aqueous exposures of carp (Hao et al., 2009) and rainbow trout (Federici et al., 2007) to TiO<sub>2</sub> NPs and a dietary exposure to rainbow trout (Ramsden et al., 2009) have all reported toxicity to a greater or lesser extent (Table 1-1). The primary manifestation of toxicity in aqueous exposures can be expected to be gill injury. This was indeed the case for both carp and rainbow trout with gill pathologies and elevated ventilation rates observed in both aqueous studies (Federici et al., 2007; Hao et al., 2009). Oxidative stress was also recorded in all three studies with Federici et al. (2007) and Ramsden et al. (2009) both reporting significant decreases in Na<sup>+</sup>K<sup>+</sup>-ATPase activities in the gills and intestine, and the brain respectively. Changes in thiobarbituric acid reactive substances (TBARS) levels were also recorded in both studies. Hao et al. (2009) reported oxidative stress in exposed carp as evidenced by significant decreases in superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities in fish exposed to 100 and 200 mg l<sup>-1</sup> TiO<sub>2</sub> NPs.

All three of the above studies indicate that chronic rather than acute toxicity is the cause of concern in fish. Whilst individual toxicity may not be a primary concern it is possible that the physiological stresses induced by NP exposure as evidenced by the biochemical changes described above may result in compromised health over longer periods of time than those currently recorded in the literature. This message is further backed by the findings of Scown et al. (2009) who carried out an intravenous injection of TiO<sub>2</sub> NPs into rainbow trout. Whilst no significant toxicity was observed in the small number of assays carried out, it was noted that TiO<sub>2</sub> NPs were still present in the fish up to 90 d post-injection. This suggests that whilst the oxidative potential of these NPs may not be sufficient to produce acute tissue damage it is possible that over longer periods of time the health of the fish may be compromised by the presence of these particles.

### 1.2.3. Studies reporting little or no toxic effects

Whilst there have been several studies showing various levels of TiO<sub>2</sub> NP toxicity in a variety of different species and cell lines there is other data suggesting that TiO<sub>2</sub> NPs show little or no toxicity (Hussain et al., 2005; Warheit et al., 2006; Griffitt et al., 2008; Zhu et al., 2008; Griffitt et al., 2009; Scown et al., 2009). Hussain et al. (2005) used TiO<sub>2</sub> NPs as a control NP in their study on rat cells and Griffitt et al. (2008) found no toxicity caused by TiO<sub>2</sub> NPs in algae, *Daphnia*, or zebrafish. There is also evidence that whilst TiO<sub>2</sub> NPs may not themselves be directly toxic they may act as mediators of other potentially toxic chemicals such as cadmium and arsenic (Sun et al., 2007; Zhang et al., 2007; Sun et al., 2009). The lack of toxicity of TiO<sub>2</sub> NPs to zebrafish embryos and adults will be addressed later in this review; however it is worth noting that not all studies report toxicity. There are issues around the aggregation of TiO<sub>2</sub> NPs and the formation of colloids with organic matter in the natural environment (Lovern and Klaper, 2006; Handy et al., 2008c) which suggests that under realistic environmental conditions the level of toxicity caused by TiO<sub>2</sub> NPs may be negligible. Indeed TiO<sub>2</sub> fine particles have been used as an inert digestibility marker in fish nutrition for years (Weatherup and McCracken, 1998). While concern over the toxicity of NPs has arisen because of their nanoscale properties (Colvin 2003), there has been no consistent evidence of a relationship between any nanoscale properties of NPs and toxicological responses in fish (Petersen and Henry 2012). Whether or not TiO<sub>2</sub> NPs are absorbed also remains unclear and it is possible that the effects described above are secondary effects of NP exposure rather than direct toxicity caused by the presence of NPs in tissues. The conflicting evidence is not helped by the large diversity in particle type, preparation and exposure scenarios. As a result true comparisons cannot be made easily,

nor can they be considered in light of good bulk TiO<sub>2</sub> controls as these are not used often enough.

So it is clear that whilst there is a significant body of evidence growing indicating the toxic potential of TiO<sub>2</sub> NPs there are also a number of studies indicating otherwise. These studies all demonstrate the need for further research into the toxic effects of TiO<sub>2</sub> NPs. It is clear that whilst there may be some conflicting evidence concerning the level of toxicity of TiO<sub>2</sub> NPs there is still much work to be done to provide sufficient data for protocols on the regulation of these nanoparticles. Future studies should look at environmentally relevant levels, and explore the possibility that toxicity may be more long term than simply the test duration, with an emphasis on not only individual but also population health.

### **1.3. Carbon nanotubes**

Carbon nanotubes (CNTs) are an allotrope of carbon which take the form of a cylinder of carbon. The carbon molecules bond together creating a tube of carbon and are normally closed at one end with a hemisphere or semi-bucky ball shape. These nanotubes can take the form of either single-walled or multi-walled carbon nanotubes (SWCNT or MWCNT respectively, Figure 1.3). They have very high strength and unique electrical properties (high conductivity, low resistance) which means they have great potential in a variety of fields including electronics and computing, as well as possible uses in the medical field (Lam et al., 2003; Donaldson et al., 2006). The nanotubes (SWCNT) are typically 1-3 nm in diameter depending on the metal catalyst used in their manufacture, and can polymerise up to several micrometres in length (Donaldson et al., 2006; Shvedova et al., 2007; Smith et al., 2007). These individual tubes then show a tendency to aggregate into bundles or ropes as a result of attractive

van der Waals' forces between tubes (Donaldson et al., 2006), and then in turn into small clumps or ropes. These characteristics, together with their electrical properties are the main causes for concern in humans and other animals. A review by Handy and Shaw (2007) summarises known toxic effects of CNT and a recent review by Petersen and Henry (2012) discusses the potential problems associated with carbon-based NM testing. Factors such as surface coating, presence of trace material from the metal catalyst, UV radiation and dispersal vehicles can all influence the behaviour of the particles. Figure 1.2 outlines the potential toxicity pathways for CNTs.

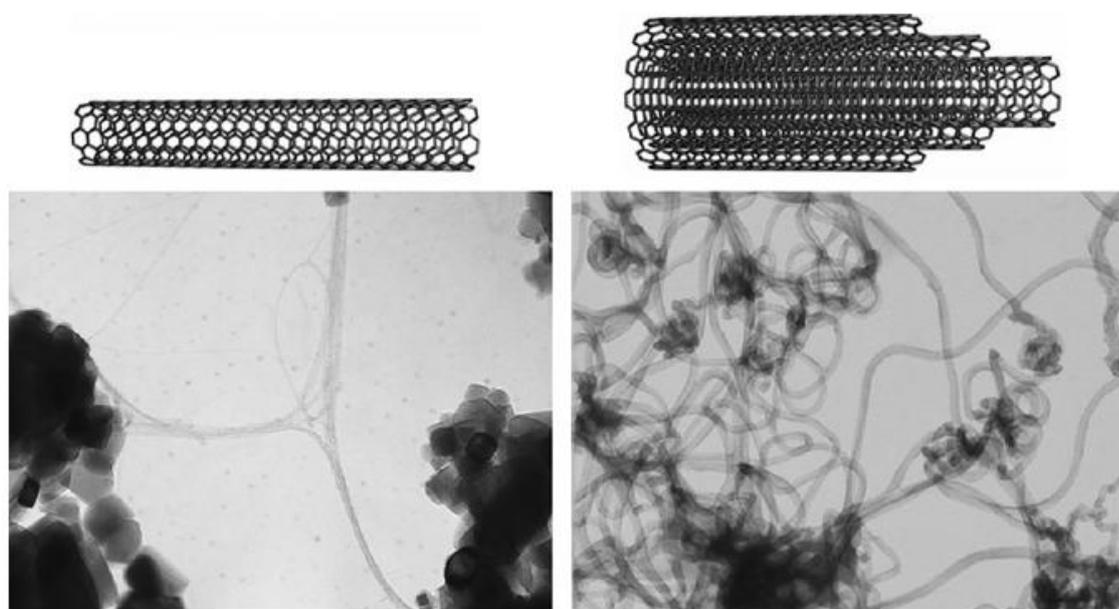


Figure 1.3. Molecular representations of SWCNT (left) and MWCNT (right) with typical TEMs below. From Donaldson et al. (2006).

### 1.3.1. Toxicity of CNTs

Of the body of work carried out investigating the toxic effects of carbon based NPs there is a fairly even split between  $C_{60}$  fullerenes and its derivatives (Oberdörster, 2004; Lovorn and Klaper, 2006; Oberdörster et al., 2006; Zhu et al., 2006; Xu et al., 2009), and CNTs (either single or multi walled) (Lam et al., 2003; Warheit et al., 2004; Shvedova et al., 2007; Smith et al., 2007; Mouchet et al., 2008; Scott-Fordsmand et al., 2008; Cheng et al., 2009) (Table 1-2). As was true for  $TiO_2$  NPs the earlier work carried

out on CNTs was biased towards respiratory toxicity studies on small mammals. While some studies were reporting high levels of toxicity caused by single walled CNT (SWCNTs) (e.g. Lam et al. (2003) reported 56 % mortality in mice exposed to 0.5 mg SWCNTs via intratracheal instillation within 7 days), others were suggesting that rather than oxidative stress or other forms of toxicity the primary cause of damage was through simple mechanical blockage of the airways (Warheit et al., 2004). This mechanical blocking was also deemed as the primary cause of mortality in a trial carried out by Mouchet et al. (2008) on frog larvae. The authors reported 85 % mortality in larvae exposed to DWCNTs at 500 mg l<sup>-1</sup> in static water. The authors suggested that toxicity was likely due to blocking of the gills and other organs.

Shvedova et al. (2007) examined the toxic effects of SWCNTs on mice fed either on diets with a vitamin E deficiency or the normal vitamin E sufficient diet. The authors found that while both sets of mice exhibited pulmonary inflammation responses, those mice fed on a vitamin E-deficient diet exhibited a greater decrease in pulmonary antioxidants. The result was a higher sensitivity to SWCNT-induced acute inflammation, as well as a greater accumulation of lipid peroxidation products. This study highlighted one potential mechanism of toxicity in that animals with weak redox defences can be more vulnerable to the oxidising properties of CNTs.

There has been very little research carried out on the effects of CNTs in fish. An aqueous exposure carried out by Smith et al. (2007) using SWCNTs reported respiratory distress caused by mechanical blockage of the gills, also evidenced by gill pathologies. This further supports the idea of mechanical blockage as a primary source of toxicity in CNTs. Smith et al. (2007) also reported some transient changes in TBARS and GSH levels but the authors suggested that these changes were not detrimental to the long term health of the fish. They did however suggest that subtle changes in tissue Zn and Cu levels, along with minor changes in brain histology, may indicate the possibility of more

chronic neurotoxic and cardiovascular effects (evidenced by possible behavioural changes) as a result of prolonged exposure to CNTs. In a dietary exposure to SWCNTs Fraser et al. (2010) found little evidence of toxicity in rainbow trout but there was a transient change in TBARS levels in SWCNT exposed fish.

A study on earthworms showed another aspect to nanoparticle toxicity. Scott-Fordsmand et al. (2008) exposed the earthworm *Eisenia veneta* to double-walled CNTs (DWNTs) and C<sub>60</sub>. The authors found no effect on mortality of the exposed individuals or hatchability of their offspring but did find that reproduction was affected with a 60 % decrease in cocoon production in those worms exposed to 495 mg kg<sup>-1</sup> DWNT. This highlights the importance of considering not only physiological effects on the exposed individuals, but also next generation and whole life cycle effects. A simple acute toxicity test will not suffice for complete ecotoxicological assessment – population level effects also need to be considered.

Table 1-2. Toxic effects of CNT exposure (*in vivo* and *in vitro*).

Nanomaterial/chemical characteristics	Dose & exposure time	Exposure route	Species	Toxic effects	Reference
Single walled CNTs (SWCNTs). No sizes given. Suspended in inactivated mouse serum and sonicated.	0.1 or 0.5 mg per mouse single dose. Observed post-exposure for 7 or 90 d.	Intratracheal instillation	Mice	56 % mortality in the high dose group within 7 days of exposure. Significant weight loss occurred in the high dose mice. Toxicity evidenced in by lung lesions and granulomas, inflammation and necrosis.	Lam et al. (2003)
Single walled CNTs (SWCNTs). 2 nm $\phi$ , 500 nm length, suspended in water and sonicated.	0-100 mg l <sup>-1</sup> for 1-5 d depending on experiment.	<i>in vitro</i> exposure	Human dermis fibroblast cells	Significant reduction in cell survival (58 % survival after 5 d at 25 mg l <sup>-1</sup> ). Refined SWCNTs more toxic than non-refined ones. Cell morphology altered by SWCNTs.	Tian et al. (2006)
Double walled CNTs (DWCNTs). 1-3 nm $\phi$ , aggregates of 10-20 nm suspended in ultrapure water.	0-500 mg l <sup>-1</sup> for 12 d in either static or aerated water.	Aqueous exposure	Amphibian <i>Xenopus laevis</i> larvae	85 % mortality in larvae exposed to 500 mg l <sup>-1</sup> in the static water compared to 5 % in aerated water. Dose dependent reduction in size of larvae for both static and aerated water. Toxicity is likely due to physical blockage of the gills and other organs.	Mouchet et al. (2008)
Single walled CNTs (SWCNTs). 1.1 nm $\phi$ , 5-30 $\mu$ m length, suspended in SDS and sonicated.	0.1-0.5 mg l <sup>-1</sup> for 10 d	Aqueous exposure	Rainbow trout ( <i>Oncorhynchus mykiss</i> )	Respiratory distress evidenced by increased ventilation rates and mucus production. Gill pathologies observed in exposed fish.	Smith et al. (2007)
Fluorescently labelled multi-walled CNTs (FITC-BSA-MWCNTs). 20 nm $\phi$ with a mean length of 800 nm, suspended in ultrapure water.	2 ng single dose at 1 or 72 hpf	Microinjection into embryo	Zebrafish ( <i>Danio rerio</i> ) embryos	No developmental abnormalities or toxic effects seen in the exposed embryos. Possible effect on reproductive success of exposed groups.	Cheng et al. (2009)

Nanomaterial/chemical characteristics	Dose & exposure time	Exposure route	Species	Toxic effects	Reference
Single walled CNTs (SWCNTs). 1-4 nm $\phi$ , suspended in PBS.	40 $\mu\text{g}$ suspended in 50 $\mu\text{l}$ PBS. Mice then fed basal or vitamin E-deficient diet for 28 d.	Pharyngeal aspiration	Mice	Pulmonary antioxidant levels decreased significantly in all SWCNT exposed mice with a greater decrease in those mice on the vitamin E deficient diet. Inflammation and lipid peroxidation products were highest in SWCNT exposed mice on the vitamin E deficient diet.	Shvedova et al. (2007)
Double walled CNTs (DWCNTs). 10-30 nm $\phi$ , 5-15 $\mu\text{m}$ length, suspended in ultrapure water and sonicated.	0-495 $\text{mg kg}^{-1}$ dry food weight every 7 d for 28 d.	Dietary exposure	Earthworm ( <i>Eisenia veneta</i> )	60 % reduction in cocoon production at 495 $\text{mg kg}^{-1}$ DWCNT. Growth 20 % lower in highest DWCNT group compared to control.	Scott-Fordsmand et al. (2008)
Single walled CNTs (SWCNTs). 1-4 nm $\phi$ , 5-30 $\mu\text{m}$ length, suspended in a range of natural and synthetic dispersing agents.	0-150 $\text{mg l}^{-1}$ for 3 or 24 h	<i>in vitro</i> exposure	<i>Escherichia coli</i> and rat liver epithelia cells	Cytotoxicity caused only in treatments containing Triton X-100 as the dispersant. SWCNTs not toxic.	Alpatova et al. (2010)

### **1.3.2. *In vitro* toxicity of CNTs**

*In vitro* studies have, in the majority, examined effects on particle size or shape. Work by Tian et al. (2006) looked at the difference in toxicity of SWCNT, MWCNT, carbon black, activated carbon and graphite using human fibroblast cells. The authors found that the highest level of toxicity was caused by the refined SWCNTs with only 58 % survival in cells exposed to 25 mg l<sup>-1</sup> for 5 d. The authors suggest that the surface area of the SWCNTs may be one of the important factors in the observed toxicity. The authors also highlight the importance of the surface properties and impurities in the level of toxicity induced by NPs. Contrary to the above study Fiorito et al. (2006) found very low levels of toxicity induced by refined SWCNTs in human macrophages at exposure concentrations up to 60 mg l<sup>-1</sup>. Fiorito et al. suggest that the lack of toxicity observed in purified SWCNTs was due to the absence of impurities such as metal catalyst particles. Clearly there is a level of debate still present in this field of research which will only be enlightened with further studies.

These studies all demonstrate the need for further research into the toxic effects of CNTs. It is clear that whilst there may be some conflicting evidence concerning the level of toxicity of CNTs there is still much work to be done to provide sufficient data for protocols on the regulation of these nanoparticles. Future studies should look at environmentally relevant levels, and explore the possibility that toxicity may be more long term than simply the test duration.

### **1.4. Main areas of controversy**

Whilst the two main NPs of interest here have been reviewed above, it is worth considering a few other NPs to highlight that many of the issues above also apply to

other types of NPs. Table 1-3 gives details of some of the main studies of interest which give evidence for toxicity caused by NPs.

There have been several studies on the toxic effects of C<sub>60</sub> fullerenes and its derivatives on cell lines (Xu et al., 2009), invertebrates (Lovern and Klaper, 2006; Oberdörster et al., 2006), embryonic zebrafish (Usenko et al., 2007; Zhu et al., 2007; Usenko et al., 2008) and fishes (Oberdörster, 2004; Oberdörster et al., 2006; Zhu et al., 2006). The common mechanism of toxicity attributed to C<sub>60</sub> in these studies is that of oxidative stress via the generation of ROS. The studies carried out by Zhu et al. (2006) and Lovern and Klaper (2006) both examined the difference between C<sub>60</sub> prepared with the use of the dispersal vehicle tetrahydrofuran (THF) to C<sub>60</sub> prepared by simple dispersion in ultrapure water. Both studies reported a significant level of toxicity caused by the THF-prepared C<sub>60</sub>. The water-prepared C<sub>60</sub> was significantly less toxic than THF-prepared solutions but there was still a significant level of toxicity caused by the water-prepared fullerenes. This possible vehicle effect has been further investigated by several groups and the general feeling amongst these studies is that the primary toxicity of C<sub>60</sub> is related to its method of preparation rather than the C<sub>60</sub> itself (Henry et al., 2007; Spohn et al., 2009). This has been an area of debate for some time now and it seems clear that care needs to be taken when attributing toxicity to C<sub>60</sub> – or any other NP – if the NP has been prepared with the use of a dispersal vehicle which itself is known to produce toxicity.

A second area in need of care and further research concerns the use of metal oxide NPs or surface coatings of NPs containing metals which are known to dissociate in solution (dissolution) with the production of metal ions (Handy et al., 2008c). This is not of concern in titanium NPs as TiO<sub>2</sub> is known to be stable in its oxidised form (Diebold, 2003) but studies on Ag and Cu NPs have identified the dissolution of metal ions as a potential source of toxicity (Griffitt et al., 2007; Griffitt et al., 2009; Bilberg et

al., 2010; Wu et al., 2010). Soluble metal ions used as controls in these studies are of some help but different methods such as the use of the filtrate of a NP solution as a control may be of more relevance (see Shaw and Handy 2011 for a review).

Table 1-3. Toxic effects of NP exposure (*in vivo* and *in vitro*).

Nanomaterial/chemical characteristics	Dose & exposure time	Exposure route	Species	Toxic effects	Reference
C <sub>60</sub> fullerenes (prepared with THF vehicle). 30-100 nm aggregates suspended in ultrapure water.	0.5 or 1 mg l <sup>-1</sup> for 48 h	Aqueous exposure	Juvenile largemouth bass ( <i>Micropterus salmoides</i> )	Significant increase in brain MDTA indicates lipid peroxidation caused by exposure to both levels of C <sub>60</sub> .	Oberdörster (2004)
C <sub>60</sub> fullerenes. 10-200 nm aggregates formed by stirring in ultrapure water for at least 2 months.	0.5 mg l <sup>-1</sup> for 96 h	Aqueous exposure	Fathead minnow ( <i>Pimephales promelas</i> )	Down-regulation in expression of the peroxisomal membrane protein PMP70.	Oberdörster et al. (2006)
C <sub>60</sub> fullerenes prepared either with or without use of THF vehicle, suspended in ultrapure water. No sizes given.	0.5 mg l <sup>-1</sup> of either water-stirred or THF prepared C <sub>60</sub> for 48 h	Aqueous exposure	Fathead minnow ( <i>Pimephales promelas</i> )	100 % mortality in fish exposed to THF-prepared C <sub>60</sub> within 18 h. Water-stirred C <sub>60</sub> was significantly less toxic but was still toxic as evidenced by significantly raised lipid peroxidation in the brain and gill compared to control fish.	Zhu et al. (2006)
C <sub>60</sub> fullerenes. Suspended in ultrapure water or THF. Filtered solutions with an aggregate range of 10-20 nm, sonicated (un-filtered) aggregate range of 20-100 nm.	0-880 µg l <sup>-1</sup> filtered, 0-9 mg l <sup>-1</sup> sonicated C <sub>60</sub> for 48 h.	Aqueous exposure	Invertebrate <i>Daphnia magna</i>	Significantly higher toxicity caused by filtered C <sub>60</sub> prepared by THF compared to sonicated C <sub>60</sub> prepared in ultrapure water. LC <sub>50</sub> value of 460 µg l <sup>-1</sup> for filtered THF C <sub>60</sub> compared to 7.9 mg l <sup>-1</sup> for sonicated ultrapure water C <sub>60</sub> .	Lovern & Klaper (2006)
C <sub>60</sub> fullerenes. 10-200 nm aggregates formed by stirring in ultrapure water for at least 2 months.	0-30 mg l <sup>-1</sup> for 48 h to 21 d depending on experiment.	Aqueous exposure	Invertebrate <i>Daphnia magna</i>	Toxicity evidenced by 40 % mortality in group exposed to 2.5 mg l <sup>-1</sup> . Chronic exposure caused delays in moulting and decreased production of offspring.	Oberdörster et al. (2006)
C <sub>60</sub> fullerenes. No sizes given. Suspended in distilled water and stirred for 60 d.	0-100 mg l <sup>-1</sup> for 24 h.	<i>in vitro</i> exposure	<i>gpt</i> delta transgenic mouse primary embryo fibroblasts (MEF)	Cell viability decreased in a dose-dependent manner from 0-10 mg l <sup>-1</sup> C <sub>60</sub> to around 50 % viability. The viability then remained relatively unchanged at higher doses. Dose-dependent increase in mutagenicity and significant increase in ROS production in cells exposed to C <sub>60</sub> .	Xu et al. (2009)

Nanomaterial/chemical characteristics	Dose & exposure time	Exposure route	Species	Toxic effects	Reference
Silver NPs. Primary particle size 81 nm, suspended in ultrapure water and sonicated.	63, 129 or 300 $\mu\text{g l}^{-1}$ overnight with 24 h pre & post exposure also monitored	Aqueous exposure	Eurasian perch ( <i>Perca fluviatilis</i> )	No effect on basal metabolic rate (BMR). Critical oxygen tension (Pcrit) increased significantly in 300 $\mu\text{g l}^{-1}$ treatment. Elevated gill ventilation rate and increase in mucus production.	Bilberg et al. (2010)
Silver NPs. Primary particle size 28.3 nm (median), suspended in deionised water.	0-8 $\text{mg l}^{-1}$ for 48 h - 60 d depending on experiment	Aqueous exposure	Japanese medaka ( <i>Oryzias latipes</i> ) embryos and adults	Acute toxicity tests showed an $\text{LC}_{50}$ value of 1.03 $\text{mg l}^{-1}$ for adult fish. Embryos suffered 100 % mortality at concentrations over 2 $\text{mg l}^{-1}$ . Chronic exposure test resulted in a dose-dependent developmental retardation along with developmental abnormalities.	Wu et al. (2010)
Tungsten carbide (WC) and tungsten carbide-cobalt (WC-Co) NPs. Primary particle size 56 and 62 nm respectively, suspended in ultrapure water (and 0.01 % (wt/vol) sodium polyphosphate solution for WC-Co) and sonicated.	7.5, 15 or 30 $\text{mg l}^{-1}$ WC, 8.25, 16.5 or 33 $\text{mg l}^{-1}$ WC-Co for 3-72 h	<i>in vitro</i> exposure	Rainbow trout ( <i>Oncorhynchus mykiss</i> ) cell line RTgill-W1	Agglomeration of NPs but cell uptake and toxicity still observed. Cell viability decreased with exposure to both WC and WC-Co NPs.	Kuhnel et al. (2009)

Another area of debate centres on the problem of whether or not NPs can be mobilised from the external environment across membranes and into cells rather than simply remaining in body cavities. Federici et al. (2007) reported some milky colouration in the lumen of fish exposed to TiO<sub>2</sub> NPs and Galloway et al. (2010) observed aggregates of TiO<sub>2</sub> NPs in the gut lumen of lugworms but there was no apparent movement of the NPs into the gut epithelial cells. There is however some evidence of movement of NPs into embryos as reported in zebrafish (Lee et al., 2007) and the see-through medaka (Kashiwada, 2006). These findings will be discussed in more detail in the zebrafish section. Johnston et al. (2010) demonstrated the possibility of nanoscale TiO<sub>2</sub> being able to cross gill epithelial membranes as evidenced by CARS imaging. The authors did however state that the level of internalisation of TiO<sub>2</sub> was likely under the detection limits of most commonly used analytical methods. There is clearly a need for further research into the movement of NPs from the exposure medium into tissues and cells. The use of coherent anti-Stokes Raman scattering (CARS) microscopy and other imaging methods such as those employed by Lee et al. (2007) will be vital in determining the movements of NPs in toxicity tests.

### **1.5. Zebrafish**

The zebrafish (*Danio rerio*) has become a widely recognised model species in toxicity testing over the last few decades for many reasons, including (i) the relative ease of maintaining large stocks of animals due to their small size; (ii) high fecundity; (iii) rapid development of the embryo and transparency of the chorion allows real time observations of development; (iv) similarity of the zebrafish genome and genetic pathways to man; (v) cost efficient biological testing due to quick generation turnover time (approximately 90 days from fertilisation to sexual maturity) and the suitability of the use of microwell plates for toxicity tests (Nusslein-Volhard and Dahm, 2002;

Berghmans et al., 2008). All of these advantages mean that the zebrafish is an ideal species to use in reproductive studies of NP toxicity.

The majority of studies using the zebrafish to examine toxicological effects of NPs thus far have used embryonic stages (Daroczi et al., 2006; Henry et al., 2007; Lee et al., 2007; Usenko et al., 2007; Zhu et al., 2007; Usenko et al., 2008; Zhu et al., 2008; Cheng et al., 2009; Fent et al., 2010; Nelson et al., 2010), with only a few studies on adult fish (Griffitt et al., 2007; Griffitt et al., 2009). The toxic effects of various NPs to zebrafish are outlined in Table 1-4.

Microinjection studies have been performed to determine the distribution of NPs inside the embryo. Cheng et al. (2009) injected multi-walled carbon nanotubes (MWCNTs) into zebrafish. The MWCNTs were conjugated to bovine serum albumin (BSA) and then labelled with fluorescein isothiocyanate (FITC) to produce functionalised MWCNTs (FITC-BSA-MWCNTs). These functionalised nanotubes were then injected into individual embryos at the 1-cell stage (2 ng/embryo), and in others at 72 hours post fertilisation (hpf). Embryos injected at the 1-cell stage showed fluorescence in the blastoderm throughout development, but not in the yolk cells. Embryos injected at 72 hpf showed an initial fluorescence throughout the body, but after 24 hours the fluorescence was mostly in the anterior region of the swim bladder. However, by 96 hours post-injection the signal was lost. This suggests some caution with fluorescent labels, it remains unclear whether the label was quenched, excreted, or metabolised, or even if the NPs remained attached to the label.

In a similar study, Kashiwada (2006) exposed see-through medaka to  $1 \text{ mg l}^{-1}$  of polystyrene microspheres (autofluorescent, 39.4 nm diameter). The author demonstrated deposition of the NPs on the chorion by 24 h. Once hatched, the larvae displayed most fluorescence in the gallbladder, oil droplets and yolk area, but not the liver. Particle size effects were also examined using particles ranging from 39-42,000 nm. Maximum

fluorescence was achieved with 474 nm particles. The author also examined the effect of salinity on the embryos. The embryo rearing medium (ERM, a buffered saline containing  $1 \text{ g l}^{-1}$  NaCl, equivalent to about 10 % seawater salinity) was used as a control against concentrates of up to x30 of the original medium. The fluorescence peaked using the 15 x concentration of the ERM. The author reported particle aggregation with increasing strength of the ERM. Details of how the ERM concentrates were made was not reported, it is therefore unclear if the effect is a salinity effect per se or an effect of the Ca, Mg, or bicarbonate buffer which was also in the medium.

Two separate studies using silver NPs have demonstrated toxicity in zebrafish embryos (Lee et al., 2007) and medaka (Wu et al., 2010). Lee et al. (2007) exposed zebrafish embryos to Ag NPs at a range of concentrations from 0-76.6  $\text{ng l}^{-1}$ . The diffusion of the NPs from the exposure medium (egg water with NPs) into the chorion via the chorion pore canals was imaged using dark-field single nanoparticle optical microscopy and spectroscopy (SNOMS). Toxic effects of the NPs were seen at concentrations over 8.6  $\text{ng l}^{-1}$  with various forms of developmental abnormalities, and mortality rates, increasing in a dose-dependent manner. A similar pattern of developmental abnormalities and mortality was observed by Wu et al. (2010) who exposed medaka embryos to AG NPs at concentrations of 0-1000  $\mu\text{g l}^{-1}$ . Mortality was 100 % in embryos exposed to AG NPs at 800  $\mu\text{g l}^{-1}$  and above.

The distribution of injected silica NPs and nanowires was examined by Nelson et al. (2010) using zebrafish. The materials were injected into the egg yolk of embryos at 6 hpf and 36 hpf. At both stages the nanowires and NPs were distributed around the embryo rather than remaining in the yolk. In a second trial Nelson et al. (2010) used transgenic zebrafish to explore the effects of the NPs on *sonic hedgehog (shh)* gene expression as a measure of gastrulation and neurulation. The authors found that 14 hpf control embryos displayed normal gene expression down the neural tube, whereas the

exposed embryos showed diffuse expression with 12 of 21 embryos showing a raised but flattened neural keel; indicating disruption of normal neurulation. The authors concluded that all the materials tested could enter the developing embryo, but only the high aspect ratio nanowires caused teratogenic effects.

Table 1-4. Effect of NPs on zebrafish.

Nanomaterial/chemical characteristics	Dose & exposure time	Exposure route	Species	Toxic effects	Reference
Fluorescently labelled multi-walled CNTs (FITC-BSA-MWCNTs). 20 nm $\phi$ with a mean length of 800 nm, suspended in ultrapure water.	2 ng single dose at 1 or 72 hpf	Microinjection into embryo	Zebrafish ( <i>Danio rerio</i> ) embryos	No developmental abnormalities or toxic effects seen in the exposed embryos. Possible effect on reproductive success of exposed groups.	Cheng et al. (2009)
C <sub>60</sub> , C <sub>70</sub> and C <sub>60</sub> (OH) <sub>24</sub> fullerenes suspended in DMSO. No sizes given.	100-500 $\mu\text{g l}^{-1}$ for C <sub>60</sub> & C <sub>70</sub> , 500-5000 $\mu\text{g l}^{-1}$ for C <sub>60</sub> (OH) <sub>24</sub> . Dechorionated and exposed at 24 hpf until 96 hpf	Aqueous exposure	Zebrafish ( <i>Danio rerio</i> ) embryos	Delayed development and abnormalities in embryos exposed to C <sub>60</sub> and C <sub>70</sub> . Functionalised C <sub>60</sub> (OH) <sub>24</sub> was significantly less toxic causing similar injuries at 2500 $\mu\text{g l}^{-1}$ compared to 200 $\mu\text{g l}^{-1}$ for the other fullerenes.	Usenko et al. (2007)
C <sub>60</sub> fullerenes (prepared with THF vehicle) and C <sub>60</sub> (OH) <sub>16-18</sub> fullerol suspended in ultrapure water. Mean particle size 100 nm.	1.5 mg l <sup>-1</sup> C <sub>60</sub> , 50 mg l <sup>-1</sup> C <sub>60</sub> (OH) <sub>16-18</sub> , with or without GSH depending on experiment. Dechorionated and exposed at 1 hpf for 5 d	Aqueous exposure	Zebrafish ( <i>Danio rerio</i> ) embryos	No significant toxicity caused by fullerol. Significant decrease in survival along with increase in development time for C <sub>60</sub> exposed embryos. Toxicity decreased and hatching rate increased with addition of GSH.	Zhu et al. (2007)
C <sub>60</sub> fullerenes suspended in DMSO. No sizes given.	0-500 $\mu\text{g l}^{-1}$ , with or without H <sub>2</sub> O <sub>2</sub> , DEM, BSO, NAC or reduced light depending on experiment. Dechorionated and exposed at 24 hpf for 5 d	Aqueous exposure	Zebrafish ( <i>Danio rerio</i> ) embryos	Decreased light showed significantly less mortality and malformations compared to same dose in normal light regime, except for highest 500 $\mu\text{g l}^{-1}$ dose which still produced mortalities. GSH inhibitors increased toxic effects in a dose-dependent manner.	Usenko et al. (2008)
Copper NPs. Primary particle size 50-60 nm, suspended in ultrapure water and sonicated.	0.25 or 1.5 mg l <sup>-1</sup> for 48 h	Aqueous exposure	Zebrafish ( <i>Danio rerio</i> )	Dose-dependent gill pathologies. Dose-dependent decrease in gill Na <sup>+</sup> K <sup>+</sup> -ATPase.	Griffitt et al. (2007)

Nanomaterial/chemical characteristics	Dose & exposure time	Exposure route	Species	Toxic effects	Reference
Copper NPs. Primary particle size 26.7 nm, suspended in ultrapure water and sonicated.	100 $\mu\text{g l}^{-1}$ for 48 h	Aqueous exposure	Zebrafish ( <i>Danio rerio</i> )	Significant increase in gill filament thickness in Cu NP exposed fish compared to controls and significantly higher than soluble Cu control. Gene expression significantly up-regulated in both Cu NPs and soluble Cu but differences between the two indicate different effects caused by the different forms.	Griffit et al. (2009)
ZnO NPs. Primary particle size 20 nm, suspended in ultrapure water and sonicated.	0-50 $\text{mg l}^{-1}$ for 96 h	Aqueous exposure	Zebrafish ( <i>Danio rerio</i> ) embryos	Dose-dependent decrease in survival of embryos/larvae. Increase in malformations in surviving embryos.	Zhu et al. (2008)
Fluorescently labelled silica nanowires (55 nm $\varnothing$ , mean length 2.1 $\mu\text{m}$ ) and silica NPs (primary particle size 50 or 200 nm) suspended in RNase-free water and sonicated.	10-1000 $\mu\text{g l}^{-1}$ single dose at 0-1, 6 or 36 hpf	Microinjection into embryo	Zebrafish ( <i>Danio rerio</i> ) embryos	Significant difference in level of toxicity between nanowires and NPs. High aspect ratio nanowires caused significant mortality when injected between 0 and 6 hpf. Gastrulation and neurulation stages most sensitive points in development as evidenced by <i>sonic hedgehog</i> ( <i>shh</i> ) gene expression.	Nelson et al. (2010)
Silver NPs. Primary particle size 11.6 nm, suspended in nanopure water.	0-76.6 $\text{ng l}^{-1}$ for 120 hpf	Aqueous exposure	Zebrafish ( <i>Danio rerio</i> ) embryos	Developmental toxicity observed in embryos exposed to concentrations over 8.6 $\text{ng l}^{-1}$ . Mortality increased in a dose-dependent manner.	Lee et al. (2007)

Several authors have reported NP toxicity to zebrafish embryos or larvae. Zhu et al. (2008) exposed zebrafish embryos to three different metal oxide NPs (0-50 mg l<sup>-1</sup> ZnO, 0-100 mg l<sup>-1</sup> Al<sub>2</sub>O<sub>3</sub>, and 0-500 mg l<sup>-1</sup> TiO<sub>2</sub>), compared to bulk powder controls, for 96 h. Only the nano and bulk forms of ZnO produced a dose-dependent increase in mortality, with LC<sub>50</sub> values of 1.793 and 1.550 mg l<sup>-1</sup> for nano and bulk ZnO respectively. Subsequent tests on filtered ZnO solutions revealed that toxicity was still evident, albeit at a reduced level, suggesting dissolution of dissolved Zn<sup>2+</sup> ions from the surface of the NP may have caused some of the toxicity. In the study above, Nelson et al. (2010) also reported lethal toxicity for silica nanowires, the LD<sub>50</sub> was 110 pg g<sup>-1</sup> embryo. The LD<sub>50</sub> for the silica nanoparticles was nearly three orders of magnitude higher at 20 ng g<sup>-1</sup> embryo; suggesting an important effect of shape on embryo toxicity.

In two separate studies Usenko et al. examined the effect of and C<sub>60</sub>, C<sub>70</sub> and C<sub>60</sub>(OH)<sub>24</sub> (Usenko et al., 2007) and C<sub>60</sub> (Usenko et al., 2008) on zebrafish embryos. In their first study embryos were dechorionated and the waterborne exposure began at 24 hpf, proceeding until 96 hpf; at which point the remaining embryos were cleaned and raised in clean fresh water. The authors reported LC<sub>50</sub> values of around 200 µg l<sup>-1</sup> for C<sub>60</sub> and C<sub>70</sub>, but 4000 µg l<sup>-1</sup> for C<sub>60</sub>(OH)<sub>24</sub>. At 200 µg l<sup>-1</sup> the authors observed morphological abnormalities in the embryos exposed to both C<sub>60</sub> and C<sub>70</sub> fullerenes with caudal fin malformations. Embryo development was also delayed by 12 – 20 hours. C<sub>60</sub> exposure also resulted in pericardial and yolk sac oedema. Embryos exposed to C<sub>60</sub>(OH)<sub>24</sub> displayed similar toxicological effects but only at concentrations over 2500 µg l<sup>-1</sup>, suggesting that hydroxylated fullerenes are much less toxic than underivatized fullerenes. Figure 1.4 outlines the possible mechanisms of toxicity of NPs to zebrafish including possible developmental effects as described here.

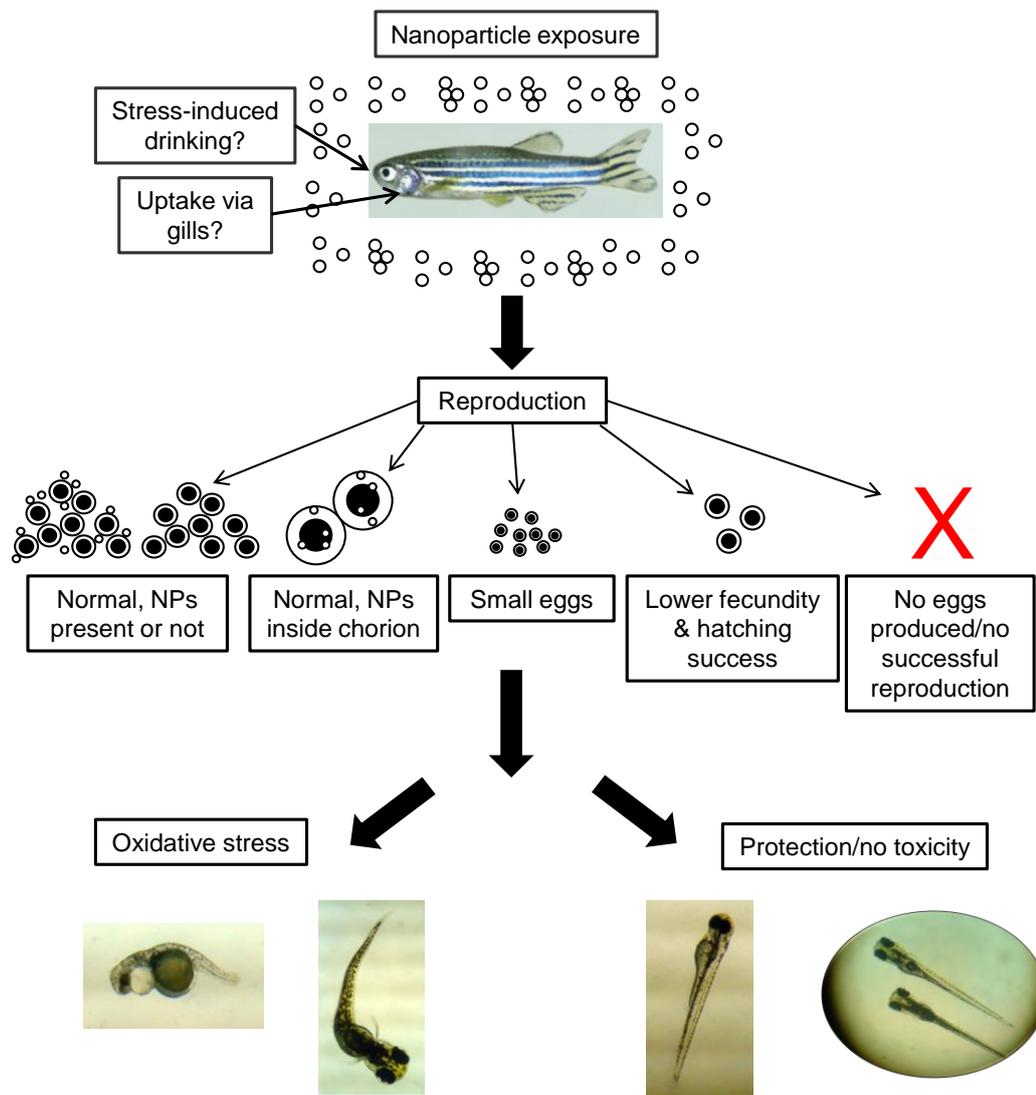


Figure 1.4. An overview of the possible causes of toxicity of NPs to fish – focusing on zebrafish and reproduction.

Usenko et al. (2008) examined the potential for oxidative stress from  $C_{60}$ , including the effects of light (normal aquarium lighting present or absent) and oxidising/reducing agents. Exposure of zebrafish embryos to 200 and 300  $\mu\text{g l}^{-1}$   $C_{60}$  in reduced light led to a significant reduction in fin malformations, pericardial oedema and mortality compared to controls. The authors used buthionine sulfoximide (BSO) and diethyl maleate (DEM) to slow the production of glutathione, and found a concomitant increase in toxicity. Additions of *N*-acetylcysteine (NAC, an antioxidant) reduced toxicity, while additions

of H<sub>2</sub>O<sub>2</sub> in the presence of C<sub>60</sub> caused a significant increase in toxicity compared to C<sub>60</sub> alone.

Zhu et al. (2007) also attempted to explore the effects of C<sub>60</sub> or fullerol (C<sub>60</sub>(OH)<sub>16-18</sub>) on zebrafish embryos. The experimental design included a vehicle control (solvent control) of THF with a series of THF concentrations up to 144 mg l<sup>-1</sup>, and a treatment containing 60 mg l<sup>-1</sup> of reduced glutathione (GSH). Embryos were exposed from spawning (under 1.5 hpf) for 5 days with toxicity end points studied throughout (survival, hatching success, pericardial oedema and heartbeat). Neither C<sub>60</sub>(OH)<sub>16-18</sub> nor the solvent controls resulted in any significant toxicity. C<sub>60</sub>-exposed embryos suffered a significant decrease in survival (45 % survival at 96 hpf) compared to the controls (over 95 % survival); and the C<sub>60</sub> plus GSH treatment showed statistically significant improvement on this (80 % survival at 96 hpf). Hatching rate was delayed in embryos exposed to C<sub>60</sub> with only 15 % hatched at 96 hpf, compared to 100 % for the controls, and 70 % for the C<sub>60</sub> plus GSH group. The authors concluded that the THF vehicle was not the cause of toxicity and that GSH was able to mitigate a significant portion of the toxicity caused by C<sub>60</sub>.

The latter finding might suggest that oxidative stress is a key mechanism for C<sub>60</sub> toxicity in fish embryos. Usenko et al. (2008) supports this by demonstration of up-regulation of the genes involved in oxidative defences including, glutathione S-transferase pi (GST-pi), and a glutamate-cysteine ligase catalytic subunit (GCLc). Usenko et al. (2008) used dimethyl sulfoxide (DMSO) as the vehicle control. Henry et al. (2007) demonstrated that the up-regulation of GST-pi was attributed to the THF vehicle (and its oxidation products  $\gamma$ -butyrolactone and tetrahydro-2-furanol), not the C<sub>60</sub> itself. As discussed earlier, the choice of solvent is a critical issue in studies on carbon-based NPs, and vehicle controls should always be included in the experimental design.

Evidence against NP toxicity has already been alluded to in the study on C<sub>60</sub> carried out by Henry et al. (2007) and is supported by Oberdörster et al. (2006) and Spohn et al. (2009). There are also a few other studies on other NPs which have also reported little or no toxicity. Studies using TiO<sub>2</sub> NPs have reported no significant toxicity to adult zebrafish (Griffitt et al., 2008; Griffitt et al., 2009) and embryonic zebrafish (Zhu et al., 2008). Al<sub>2</sub>O<sub>3</sub> and silica NPs have also been reported to have induced no significant toxicity to zebrafish embryos by Zhu et al. (2008) and Fent et al. (2010) respectively.

There is clearly still a level of debate over NP toxicity to zebrafish and early life stages of fish but the studies so far on fish embryos highlight some key concepts for NPs. Firstly, the chorion may offer some protection from waterborne exposure, but once the fish has hatched the internal organs including the central nervous system (CNS) can become contaminated. An overview of the possible causes of toxicity of NPs, with particular reference to zebrafish and reproduction, is detailed in Figure 1.4. There are also toxic effects, albeit at high mg levels rather than more environmentally relevant µg levels, and these adverse effects can include delays in development, as well as abnormal development. In this regard, NPs are not that different from a myriad of other traditional chemicals, and in terms of exposure concentrations, might be orders of magnitude less toxic than some dissolved trace metals which normally have effects at microgram rather than milligram levels. However, the studies of Kashiwada (2006) and Nelson et al. (2010) raise new concerns about the effect of particle shape and size on the toxicity of materials to fish embryos.

## **1.6. The three-spined stickleback**

The three-spined stickleback (*Gasterosteus aculeatus*) is widely distributed throughout boreal and temperate regions of the northern hemisphere and inhabits a variety of aquatic systems ranging from marine coastal areas to freshwater streams and

ponds (Bell and Foster, 1994). The species complex includes marine, anadromous and resident freshwater populations. As a result of this wide distribution and varying habitats the species complex has become rather diverse and there are various different morphological adaptations suited for each population. Nevertheless the species as a whole is still best defined by its unique behavioural traits which it displays throughout its life history. It is these behavioural traits that make the three-spined stickleback an ideal species for reproductive behaviour and success testing.

A variety of work has been done on the three spine stickleback including studies on endocrine disruption (Bell, 2004; Hahlbeck et al., 2004); trace metal toxicity (Wibe et al., 2001; Roussel et al., 2007), and the effects of stress and/or contaminants on behaviour (Bell, 2004; Craig and Laming, 2004). Studies on the toxic effects of NPs on the three-spined stickleback are scarce – indeed the recent work by Sanders et al. (2008) seems to be the only study on NPs in sticklebacks currently available for review.

Sanders et al. (2008) exposed groups of sticklebacks to 5, 50 or 500  $\mu\text{g l}^{-1}$  cadmium sulphide NPs of 4.2 nm diameter for 21 days. There were no mortalities but the authors observed some hepatocellular nuclear pleomorphism pathologies in the livers of the highest exposure fish (500  $\mu\text{g l}^{-1}$ ). There was also some elevation of oxidised glutathione in the gills of the exposed fish at both 50 and 500  $\mu\text{g l}^{-1}$ . The authors also aimed to examine the impact of CdS NPs on the reproductive fitness of the sticklebacks. Unfortunately a large proportion of the fish did not show physiological maturation as confirmed by the absence of any nest building behaviour in all groups of male fish at all treatment levels in one of the replicates. As vitellogenin induction was absent in the males the authors suggested that the level of intracellular  $\text{Cd}^{2+}$  was not sufficient to stimulate oestrogen receptors.

Clearly, with only one published report of NP toxicity to the three-spined stickleback it is rather too early to make any generalisations about toxic effects. It is however

becoming apparent that NPs may display their toxicity in more subtle ways, namely neurological changes leading to behavioural alteration and other physiological toxicity caused by chronic exposure. In both cases the result of NP exposure may be susceptibility of the species at the population level, rather than in 100 % toxicity at the individual level. As a result it may be more appropriate to examine the possible long term effects on NP exposure and the effects they have on the behaviour of fish. In this case the three-spined stickleback is a good candidate. With the potential discharges into the natural waterways of the local environment detailed in the reports by Boxhall et al. (2007) and Mueller and Nowack (2008) and the abundance of the stickleback in the local waters there are few others species which present themselves as such an ideal ecotoxicological testing candidate.

### **1.7. Bioenergetics**

In the presence of an external stressor a fish has various options available to mitigate against the potential negative impact of the exposure. One of the main ways in which a fish can re-allocate supplies to deal with stressors is through the manipulation of its energy budget (a field known as bioenergetics). Bioenergetics concerns the rates of energy expenditure, the losses and gains, and the efficiencies of energy transformation, as functional relations of the whole organism (Brett and Groves, 1979). The bioenergetics of a fish involves energy gains, losses, and transfers and can simply be represented as:

$$E(\text{In}) = E(\text{Out}) + E(\text{P})$$

where  $E(\text{In})$  is the energy ingested as food,  $E(\text{Out})$  is energy losses and  $E(\text{P})$  is energy retained as production or growth (Jobling, 1994). To understand this better we need to consider the details of energy division and loss. Energy intake ( $R$ ) can be lost as faeces ( $F$ ); some energy is used to provide energy for bodily functions through metabolism

(*M*); metabolic energy losses (lactate, various other compounds and heat) (*U*); and excess energy intake can then be used for growth or energy storage (*P*) (Jobling, 1994).

This results in a new equation:

$$R = F + U + M + P$$

We can also divide metabolic energy use into three subdivisions: energy used in basic bodily functions, energy used in activity, and energy used in digestion, absorption and processing of food. The final division is made for excess energy input (*P*) by isolating energy used in body (somatic) growth ( $P_S$ ), and reproductive energy ( $P_R$ ) used for the production of gametes (Jobling, 1994).

It is useful to think of bioenergetics in terms of an energy budget. Energy coming into the system is used for body maintenance, metabolism and locomotor activity, and any excess energy is saved and invested (e.g., in growth and maturation of the reproductive system). If the energy coming in is insufficient to allow savings then the vital bodily functions must be dealt with at the sacrifice of body or reproductive investment. It is possible that at certain times a fish has insufficient energy to fulfil the basic bodily requirements and must therefore supplement the energy budget with energy stores already in reserve. The possible effects of NPs on the bioenergetics of fish are outlined in Figure 1.5.

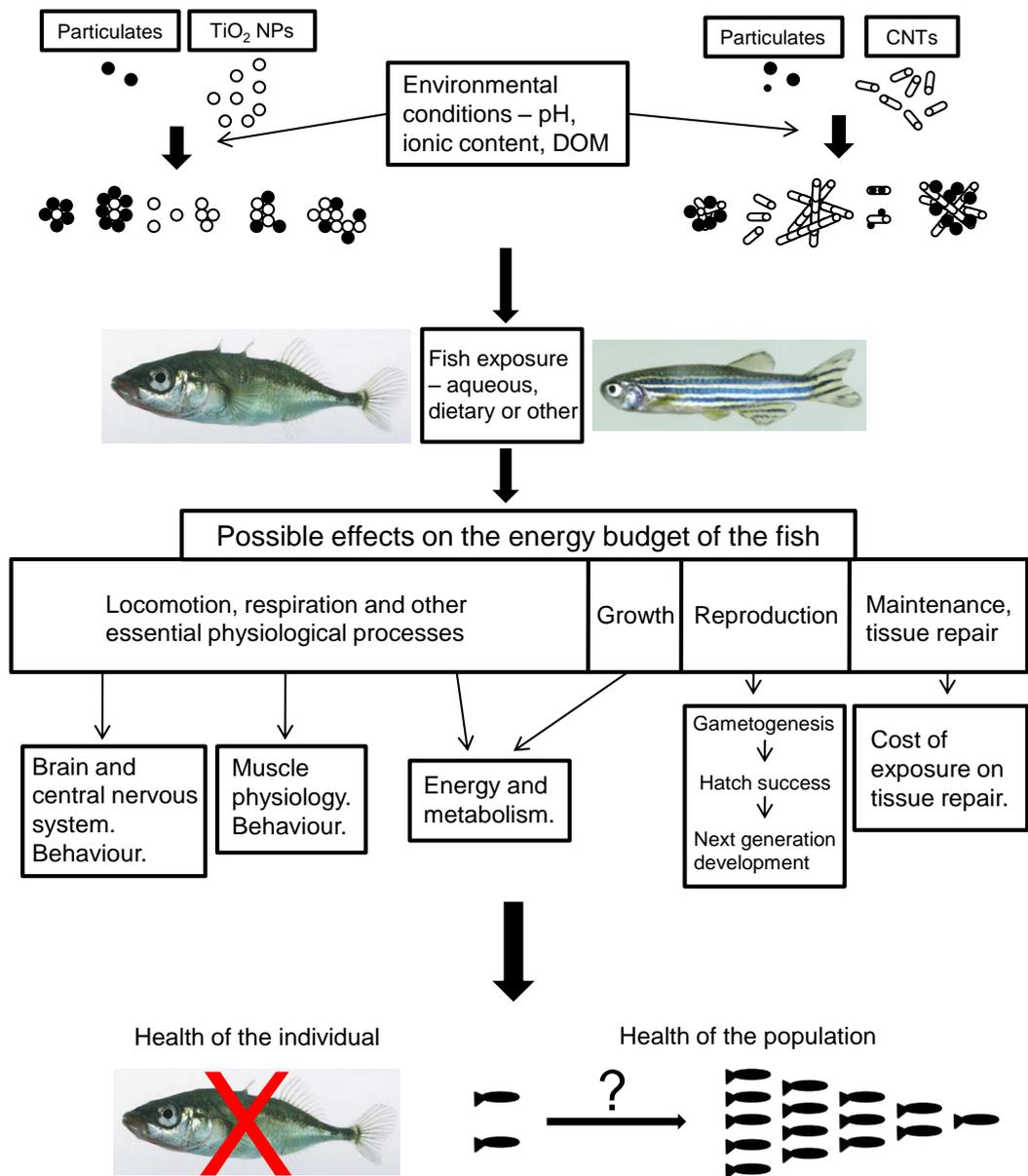


Figure 1.5. An overview of the possible effects of manufactured NP exposure on fish. Fish exposed to NPs in the environment may suffer various impacts on their growth, behaviour and reproduction.

### 1.7.1. Metabolic Scope and environmental stressors

The term metabolic scope is defined as the difference between basal metabolism and the maximum metabolic rate of an animal (Priede, 1985). Clearly, an animal at rest will have a larger metabolic scope than one that is not. In fish, a large proportion of the daily metabolic scope is used up by locomotion (Priede, 1985) and experiments on swimming speed have therefore traditionally been used to investigate metabolic scope. The term “scope” is often used loosely by ecotoxicologists as an indicator of energy reserves for

specific functions (e.g., scope for growth, (Priede, 1985)). However, the correct term is metabolic scope and this capacity might be used on any aspect of the energy expenditure budget in fish – for example increasing the budget spent on body maintenance/tissue repair at the expense of locomotion or growth (Handy et al., 1999). Theory predicts that fish with a large metabolic scope will have a greater chance of survival in the long term (Priede, 1977), i.e. the animal has flexibility to respond to demands on energy utilisation, such as pollutant stress.

There are a variety of biotic and abiotic factors which can put a fish under stress. These stressors cause a compensatory physiological response (Jobling, 1994). If a fish is able to restore normal physiological function it has acclimated to the stress and overcome it. Alternatively the stressor may be of sufficient strength that the fish cannot adapt and overcome it, thereby succumbing to the stressor and losing its life. There is also a third division of stressors – sublethal stressors. These can cause a cascade of changes and in the long term may lead to cessation of growth, little or no reproductive activity, and increased susceptibility to disease. These chronic exposures can therefore lead to poor reproductive success and a decrease in survival of individuals. The net result of this chronic stressor may be the decline in population numbers, or even entire population risk (Jobling, 1994). The manner in which an individual is able to cope with environmental stressors will have a direct impact upon the entire population.

### **1.7.2. A special case: bioenergetics of the three-spined stickleback**

Energy expenditure in the adult three-spined stickleback is divided up into the main areas outlined above. There is however a difference in the proportion of energy allocated to each area, with reproductive energy expenditure using the greatest proportion of the total energy budget in mature fish. In females this energy is used in the production of the eggs – energy can be used from the total sum entering the fish in food, but can also be obtained from energy stored in the body (Wootton, 1994). In males the

greatest proportion of reproductive energy expenditure is given to the energy used in reproductive behaviours and to the production of spiggin for nest building (Wootton, 1994). The fact that so much of the energy budget of the three-spined stickleback is allocated to reproductive success means that, bioenergetically, the animal can be very vulnerable to any external factor which may increase energy demands in other areas of the budget. The metabolic scope of the fish may be compromised by its commitment to reproductive success.

#### **1.7.2.1. Osmoregulation vs nest building in males**

Organs involved in osmoregulation include the gills, kidney, gastrointestinal tract, skin, opercular membrane and urinary bladder. In the three-spined stickleback the kidney is not only used for osmoregulation but for the production of spiggin – a glycoprotein glue used to stick together the nest. This glue is produced by the male during the breeding season as a response to endogenous androgens. These hormones cause the epithelial cells of the kidney to increase in size and synthesise the protein (Jakobsson et al., 1999).

As a result of the need for nest building and the production of spiggin the kidney has to serve a dual purpose of osmoregulation and glue production. The urine production in the kidney is reduced along with a concomitant increase in spiggin and mucus production. The male stickleback must ensure that not only is osmoregulatory control maintained but that nest building, and therefore reproductive success, is also successful. As this mucus is secreted it is possible that some osmoregulatory function is retained in saltwater fish as the mucus may bind to some divalent ions, thereby acting as a form of ion excretion (Guderley, 1994). In freshwater fish this osmoregulatory function is partly assumed by the intestine through renal excretion of hypotonic fluid (Guderley, 1994).

The behavioural trait of nest building in the male stickleback necessitates adaptation of the kidney and other organs in order to maintain osmoregulatory control. The need to balance osmoregulation against spiggin production is potentially a key indicator of physiological fitness. In the event of a stressor such as a pollution event it is possible that osmoregulatory control must take priority over spiggin production. The result may be the sacrifice of nest building and therefore a decline in reproductive fitness in order to stay alive. In terms of chronic exposure it is possible that the fish may not be able to bioenergetically afford this courtship behaviour and as a result the population survival would be put at risk. This is only one of the ways in which a shift in energy utilisation is required as a result of a behavioural trait.

#### **1.7.2.2. Reproductive energy expenditure**

The energy requirements for reproductive success in the stickleback are great. The male has several tasks: territory acquisition and defence, nest site competition, nest building and spiggin production, courtship behaviours, fertilisation, and paternal care of the offspring. The female must devote a large portion of her energy budget to the production of eggs.

Females can spawn several times during the breeding season, being able to have consecutive spawning events only a few days apart (Wootton, 1994). The energy required to meet such high demands is provided by one of two main sources: the food ingested, or by the depletion of the energy stores already in the body – the soma. The female can vary the energy income invested in egg production and in times of poor food supply the fecundity of the female stickleback is likely to fall. Should the supply be very poor the female may not spawn at all (Wootton, 1994).

Male sticklebacks also require a large energy allowance for reproductive success. The male energy budget is dominated by behavioural costs and a relatively small

gamete production cost compared to the female. The energetic costs for the male are: territoriality, nest building, and parental care (Wootton, 1994). Once successful courtship and fertilisation has taken place the female's role in reproduction is complete. The male will then drive the female away and care for the fertilised eggs. This paternal role requires two main tasks – the oxygenation of the eggs and aggressive defence of the brood throughout the early life stages. The oxygenation of the eggs is carried out by the male through fanning of the brood with the pectoral fins and is a key role throughout the parental cycle from fertilisation to hatching (Stanley, 1983). The defence of the nest is also a key role and can be the cause of major energy expenditure. Males may deplete lipid and glycogen stores in order to defend their territory, with longer fights using larger quantities of the supplies (Chellappa and Huntingford, 1989). The male may guard the fry for up to 2 weeks post-hatching. The male will try to keep the fry together by collecting strays and spitting them back into the centre of the brood (Whoriskey and FitzGerald, 1994). These parental roles are costly both in terms of time and energy, whilst also exposing the male to predation attack (Whoriskey and FitzGerald, 1994).

The data available currently on the effects of NP exposure in fish indicates that whilst acute toxicity might not be of primary importance there is a possible risk of chronic toxicity effects. The life style of the three-spined stickleback is such that it has a vulnerable window in its energy budget which may be subject to stress through NP exposure. For this reason the testing of NP toxicity on reproductive success in the three-spined stickleback should provide good indications as to the extent that NPs pose risks if their release into the natural environment remains unchecked.

## **1.8. Aims and objectives**

The aim of this thesis was to examine the potential toxicological effects of exposure to manufactured nanoparticles using the fish as a model test organism. The thesis also

examined the current problems with nanoparticles characterisation and aim to improve certain areas of NP detection and characterisation. The thesis also aimed to investigate the effects of NP exposure on general fish physiology, as well as effects on reproduction and behaviour. Experiments were conducted with two example nanoparticles where effects on the brain, behaviour or reproduction were already known or suspected. These materials were TiO<sub>2</sub> NPs and CNTs and the fish species they were tested on were the zebrafish and the three-spined stickleback.

### **1.8.1. Specific objectives and hypotheses**

The specific objectives tested the main hypothesis that exposure to manufactured NPs would result in disturbances to general physiology, reproduction and the behaviours of fish. The specific objectives were to:-

1. Improve the detection and characterisation methods currently available for the analysis of NPs in stock suspensions and more complex matrices.
2. Use the zebrafish as a model organism to test the null hypothesis that there would be no effects of NP exposure on the general physiology and reproductive output of exposed fish.
3. Test the null hypothesis that there would be no effects of NPs on reproductive behaviours of fish using a nest building and reproductive behaviour assay with three-spined sticklebacks primarily, but also the zebrafish reproduction model.
4. Link the above to the bioenergetics of the fish and relate this to the probability of survival at not only an individual level but also in the wider scope of population survival.

## **2. General methodology & method development**

### **2.1. Risk assessment**

Risk assessment forms and relevant Control of Substances Hazardous to Health (COSHH) forms were completed prior to any work being carried out. The risks included in these assessments ranged from working in an aquarium (e.g. risks of slipping and electricity in an aqueous environment) to the unique risks associated with certain chemicals (e.g. inhalation of NPs).

### **2.2. Fish husbandry and water quality**

Where fish were sourced from outside the University of Plymouth (either a wholesale supplier or wild caught) it was necessary to keep the fish for a quarantine period to ensure good health and to acclimate the fish to the new environment. For the zebrafish and stickleback trials this was also important ensure successful reproduction.

In order to ensure that the water quality and fish husbandry standards were maintained a fish husbandry plan was designed for each experiment. Water quality parameters were tested at regular intervals including temperature, pH and dissolved oxygen, as well as water ammonium, nitrate and nitrite levels (see individual experimental chapters for details). Photoperiods suited to the test species were kept using automated lighting. The feed type and feeding frequency were appropriate for the species and the health status requirements of the experiments (details given in experimental chapters). The aquarium systems were built specifically for the

investigation of the effects of NPs on fish in an aqueous exposure regime and with the specific challenges of maintaining NP exposures in mind.

### **2.3. Method development**

As experiments were planned it became apparent that some testing of existing protocols was needed to ensure their efficacy for the specific test species and NPs to be used in the experiments. There were three main areas that needed to be addressed as a result of some preliminary trials and previously published work (Ramsden et al., 2009): a method for the separation of zebrafish adults from freshly spawned embryos, the development of an optimised protocol for the histological analysis of whole zebrafish samples, and the development of analytical techniques for the characterisation of TiO<sub>2</sub> NPs. The first two of these issues are detailed in the following sections. The latter issue has been addressed in both this chapter (the use of NanoSight for particle size distribution analysis) and in Chapter 3 (measuring TiO<sub>2</sub> NPs in complex matrices).

#### **2.3.1. The separation of adult zebrafish from freshly spawned embryos – a novel solution**

For breeding trials with zebrafish it was necessary to separate the fish from the freshly spawned eggs to stop predation. There were various techniques considered for this but none were deemed effective for the specific needs of the experiment. The exposure of fish to NPs was planned to be in larger experimental tanks with breeding taking place in separate smaller tanks without exposure. It was known that glass was a suitable material for carrying out aqueous NP exposures with little material adhering to the glass (preliminary studies). As a result it was necessary to design a mesh which was made of only the materials present in the tank – namely glass and silicone sealant. Using only glass and silicone meant that there were no additional material types

introduced which may have had unknown effects (e.g. particle adhesion). Marbles are a common separation material but the workload involved with separating marbles from eggs in a tank where 6 females had potentially spawned hundreds of eggs was not feasible. The new design involves the use of a glass frame fitted with glass rods (Figure 2.1). The rods were placed close enough together to ensure the fish could not swim through the gaps, yet far enough apart that the eggs could fall through the gaps into the safe water below the mesh. With the use of a specially designed removable handle the mesh could be lowered into position for breeding and then later removed once the fish had been removed from the breeding tank.

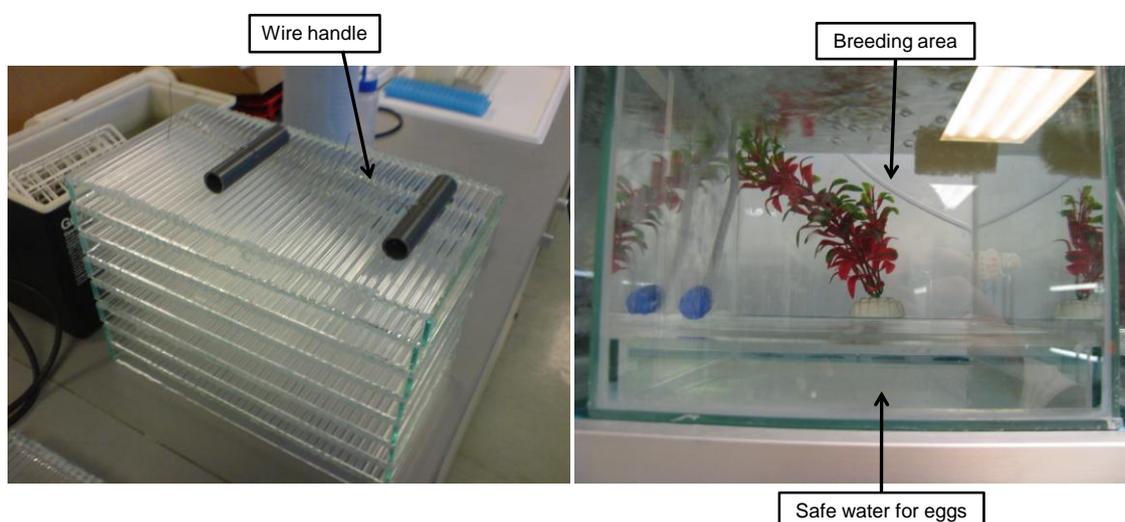


Figure 2.1. Glass rod meshes for breeding tanks.

### 2.3.2. Histological analysis of whole zebrafish samples

Histological examination of fish tissues and whole fish is necessary to assess potential body system pathology related to NP exposure.

#### 2.3.2.1. Original protocol

In order to learn the techniques involved in fixing and processing fish samples for sectioning a trial run was carried out. This trial involved the use of an accepted histological processing routine used for fish tissue samples. This routine is detailed in Table 2-1. Once tissues had been carefully harvested, or whole zebrafish terminally

anaesthetised followed by an incision to open the body cavity to allow good penetration of the fixative, they were placed directly into 10 % formal saline as a fixative. When ready for histological processing the samples were placed into labelled cassettes and put into a cradle for the automatic tissue processor (Leica TP1020). The desired programme was selected and the procedure was left to run. The tissue processor has an agitation function which was used throughout the method development. This function raises and lowers (pulses) the carousel every 10 s to ensure good mixing of the reagents between samples.

Table 2-1. Original tissue processor routine (programme 2 for large tissue samples).

<b>Station</b>	<b>Reagent</b>	<b>Duration (h)</b>
1	10 % Formal Saline	2
2	50 % alcohol	2
3	70 % alcohol	12
4	90 % alcohol	8.5
5	Industrial methylated spirits (IMS)	5
6	Absolute alcohol	5
7	Absolute alcohol	5
8	Histolene	2
9	Histolene	2
10	Histolene	2
11	Paraffin	3
12	Paraffin	3
<b>Total duration (h)</b>		<b>51.5</b>

Once the tissues or zebrafish had finished the processing stage they were loaded into the embedding centre (Leica EG150H) for embedding in paraffin wax. This was carried out by placing the sample into a metal mould and filling the mould with molten wax via the dispenser tap. The sample was then orientated and one half of the cassette was placed on the top of the mould. The cassette became the base of the block which was mounted in the specially sized chock on the microtome for sectioning. This mould and cassette assembly was then cooled rapidly with the adjacent cold plate. Rapid cooling stops the formation of layers within the paraffin block and allows for better sectioning.

Once embedded the sample was ready for sectioning. This was done with a microtome (Leica RM2235) loaded with a cassette chuck and a blade. The original protocol had the section thickness set at 8  $\mu\text{m}$ . Sections were cut using the original protocol but there were a few problems with the outcome. The block tended to section poorly with the wax slicing well but the sample crumbling out of the section. The result was a section with a few small bits of tissue but mostly blank air space. Some gentle use of the cold plate was employed in an attempt to stiffen the block and the sample within it. This method allowed a few sections to be collected before the block warmed and the sections deteriorated again. The useable sections were then processed further by mounting onto slides and staining. Sections were moved from the microtome to a waterbath set at 50°C to allow creases to settle out. Slides were prepared to receive sections by applying a very small amount of egg albumen to allow the section to adhere to the slide. Mounted slides were then allowed to air dry whilst all slides were prepared. Slides were then loaded into a staining rig for staining.

The staining was carried out using an auto-stainer (Leica Autostainer XL) and with a well-practised staining schedule (Table 2-2). For general staining the Mayer's Haematoxylin and Eosin (H&E) staining protocol is very effective. Specialised staining protocols were not used for zebrafish sections.

Table 2-2. Original haematoxylin & eosin (H&E) staining protocol (programme 3, sections 7-8  $\mu\text{m}$ ).

<b>Step</b>	<b>Station</b>	<b>Reagent</b>	<b>Time (min:sec)</b>
1	Oven		05:00
2	1	Histolene	02:00
3	2	Histolene	02:00
4	3	Absolute alcohol	02:00
5	4	Absolute alcohol	02:00
6	5	90 % alcohol	02:00
7	6	70 % alcohol	02:00
8	7	50 % alcohol	02:00
9	8	Mayer's Haematoxylin	40:00
10	Wash 1	Tap water	05:00
11	9	LiCO <sub>3</sub>	00:02
12	Wash 2	Tap water	02:00
13	10	Acid alcohol	00:02
14	Wash 3	Tap water	02:00
15	11	Eosin	01:00
16	Wash 4	Tap water	05:00
17	12	90 % alcohol	00:30
18	13	Absolute alcohol	02:00
19	14	Absolute alcohol	02:00
20	15	Histolene	02:00
21	16	Histolene	02:00
22	17	Histolene	02:00
23	Exit	Histolene	

Once finished in the auto-stainer the slides were left in histolene in preparation for cover-slipping. Cover slips were mounted onto each slide using the mounting medium DPX (mixture of distyrene, a plasticiser, and xylene) and allowed to air dry in a vented fume hood.

Slides were then examined under a microscope to observe the success of the test run. The first trial run using whole zebrafish was not very successful (Figure 2.2). Processing artefacts were seen in the sections with missing tissue and juddering as a result of poor cutting by the blade (most likely a result of poor wax infiltration).

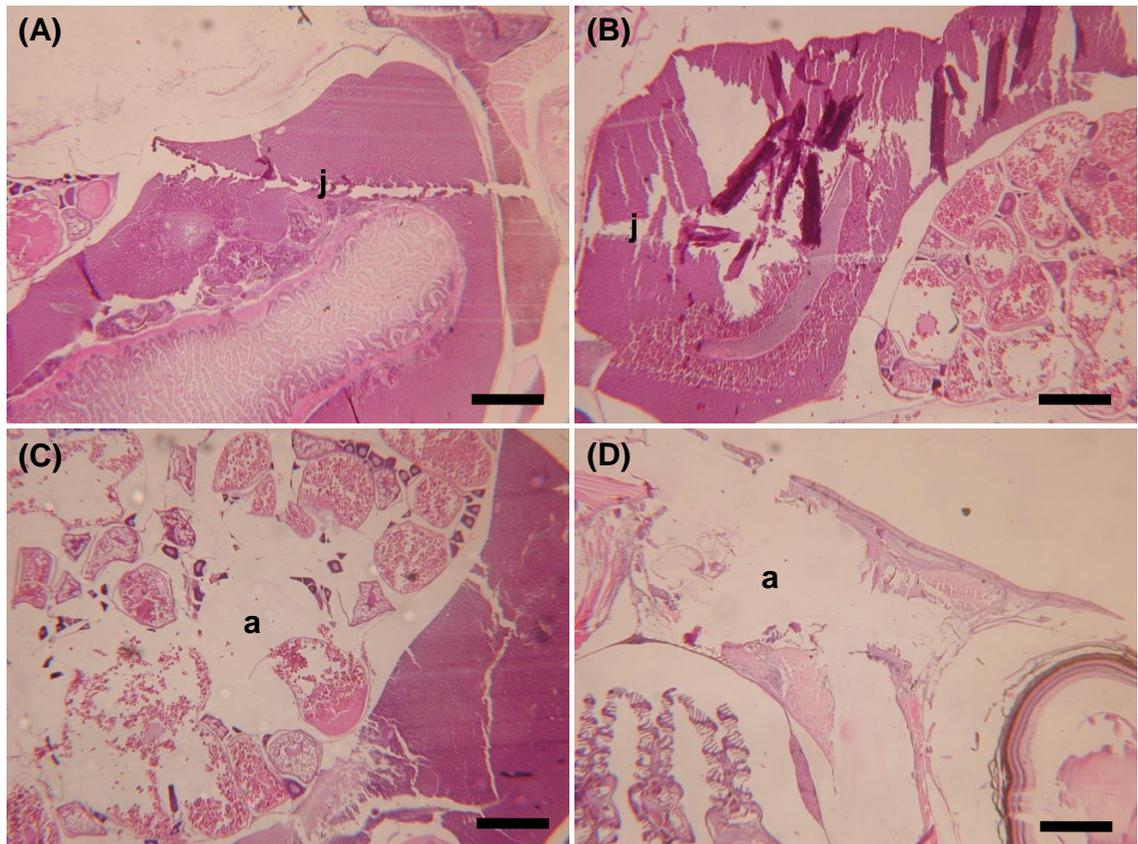


Figure 2.2. Histology slides from zebrafish sections processed by the original method showing (A) liver and intestine, (B) liver and ovary, (C) ovary and (D) absent brain tissue at 4 × magnification. Scale bar = 0.5 mm. a = absent tissue, j = judder artefact from the blade.

With such poor results from the original method it was necessary to amend the protocols. A series of optimisation trials followed with various aspects of the protocol altered and new runs carried out. Changes to the original protocol included: (i) changing tissue processing times for various parts of the process (decreasing or increasing time in each solution and the paraffin wax), (ii) changing staining protocol times, (iii) changing sectioning methodology (type of blade, section thickness, use of cold plate, breathing techniques). After several poor runs and a trip to the CEFAS laboratory in Weymouth the final optimised protocol was developed.

### 2.3.2.2. Optimised protocol

The main theme of the optimised protocol was the same as the original – that is the main steps were still fixation, processing to wax, embedding, sectioning, staining and cover-slipping. There were some major changes and some more subtle changes associated with each of these steps. These are reported in bullet points.

#### *Fixation*

- 10 % Formal saline as outlined above.
- Zebrafish may be truncated by cutting the tail section off.

#### *Processing to wax*

- The same apparatus was used – an automated tissue processor.
- Samples were loaded into cassettes with labels and loaded into the processor.
- A new programme was employed (Table 2-3).

Table 2-3. The optimised protocol for zebrafish (programme 7).

<b>Station</b>	<b>Reagent</b>	<b>Duration (h)</b>
1	10 % Formal Saline	20 min
2	50 % alcohol	12
3	70 % alcohol	12
4	90 % alcohol	12
5	Industrial methylated spirits (IMS)	8
6	Absolute alcohol	8
7	Absolute alcohol	8
8	Histolene	8
9	Histolene	8
10	Histolene	8
11	Paraffin	7.5
12	Paraffin	7.5
<b>Total duration (h)</b>		<b>99 h 20 min</b>

#### *Embedding*

- Samples were moved directly from station 12 paraffin of the tissue processor to the reservoir of the embedding centre.

- Samples and cassettes were orientated as described above and moved directly onto the cold plate (which had been turned on for at least 20 min to cool).
- Samples were left in the metal moulds on the cold plate for approximately 30 min – this allowed the block to cool completely and allowed for easy removal of the block from the mould.

### *Sectioning*

- The cold plate was used throughout the sectioning stage – it needed to be at its operational temperature to cool the blocks sufficiently.
- Blocks were ‘faced up’ by cutting sections of 10  $\mu\text{m}$  to get down to the desired section of the sample. N.B. if sections cut whilst facing up were too thick the sample was pulled and torn, causing damage deeper into the block.
- Blocks were put onto the cold plate after facing up for at least 15 min to allow them to cool properly.
- When sectioning proper blocks were allowed to cool after the facing up and a fresh blade was used for sectioning. One blade was used in 3 positions (left, centre and right) to cut, with each position being good for 3-5 blocks (when facing up has been done with an old blade).
- Sections were cut at 5  $\mu\text{m}$  which enabled a cleaner, more intact, section to be cut.
- When sectioning turning the wheel smoothly and with enough speed was important to keep a smooth section coming off the block. N.B. breathing on the ribbon as it comes off the blade helped to keep it free of too many wrinkles and formed a better section.
- Good sections were placed onto the chopping board to select the best sections for mounting.

- Using a wetted, fine paint brush or a fine set of forceps, sections were picked up and placed directly into the waterbath.
- It was important not to leave the sections in the waterbath for too long – longer than about 5-10 min resulted in the section breaking apart.
- Sections were carefully applied to a labelled slide with a fine layer of egg albumen already applied to the mounting surface of the slide (a small drop of raw egg albumen rubbed onto the surface of the slide) and slides were allowed to air dry on a flat surface.

### *Staining*

The original staining protocol proved to be insufficient for the thinner sections of the optimised protocol. The main improvement was a longer period of time in each of the two stains (Table 2-4).

Table 2-4. Optimised haematoxylin & eosin (H&E) staining protocol (programme 6, sections 5  $\mu$ m).

<b>Step</b>	<b>Station</b>	<b>Reagent</b>	<b>Time (min:sec)</b>
1	Oven		05:00
2	1	Histolene	02:00
3	2	Histolene	02:00
4	3	Absolute alcohol	02:00
5	4	Absolute alcohol	02:00
6	5	90 % alcohol	02:00
7	6	70 % alcohol	02:00
8	7	50 % alcohol	02:00
9	8	Mayer's Haematoxylin	60:00
10	Wash 1	Tap water	05:00
11	9	LiCO <sub>3</sub>	00:02
12	Wash 2	Tap water	02:00
13	10	Acid alcohol	00:02
14	Wash 3	Tap water	02:00
15	11	Eosin	02:00
16	Wash 4	Tap water	05:00
17	12	90 % alcohol	00:30
18	13	Absolute alcohol	02:00
19	14	Absolute alcohol	02:00
20	15	Histolene	02:00
21	16	Histolene	02:00
22	17	Histolene	02:00
23	Exit	Histolene	

### *Cover-slipping*

- Cover slips were placed onto a flat surface with blue roll.
- Slides were removed from the histolene trough (without the slides drying out) and DPX was applied over the section – this was done with care to avoid disturbing the section and with sufficient volume of DPX to allow complete coverage of the cover slip.
- Slides were turned over (section facing down) and gently lowered towards the cover slip. The cover slip was sucked onto the slide by capillary action.
- The slide was turned back over and pressure was gently applied to the centre of the cover slip with a metal spatula to encourage any air bubbles out from under the slip.
- The DPX was allowed to dry under the fume hood for at least 2 h.

With the optimised protocol new images were taken of the resultant slides (Figure 2.3). The sections showed very little incidence of blade judder and all samples appeared to be very well infiltrated with wax.

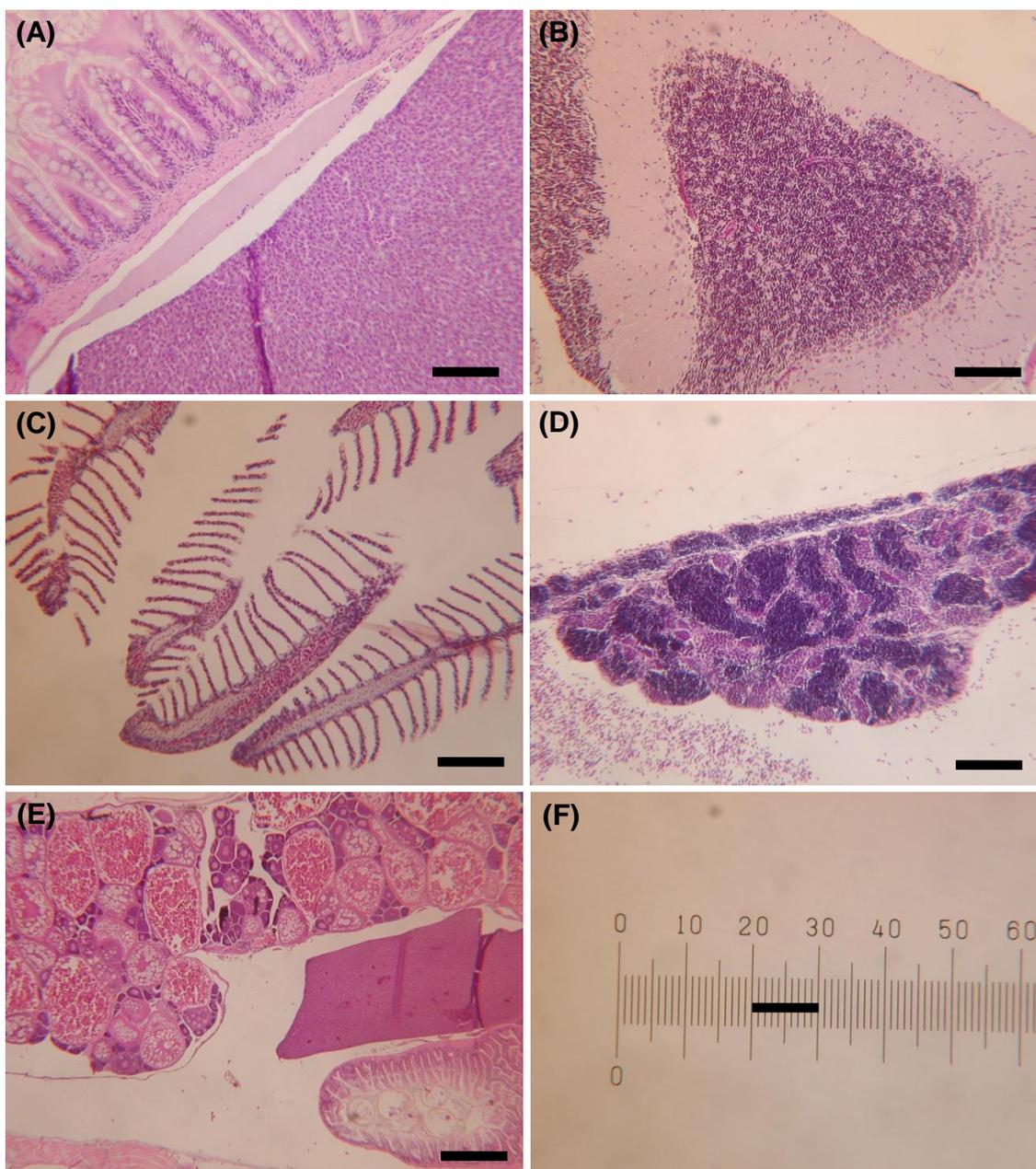


Figure 2.3. Histology slides from zebrafish sagittal sections processed by the optimised method showing (A) liver and intestine, (B) brain, (C) gills, (D) testis and (F) graticule at 20 × magnification; and (E) ovary, liver and intestine (with *Artemia* cysts) at 4 × magnification. Scale bar = 100 μm for (A, B, C, D, F), and 0.5 mm for (E).

### 2.3.3. The use of NanoSight for particle size distribution analysis

Particle characterisation of NPs is very important to ensure manufacturer's information is correct and to confirm nominal exposure parameters. Information about the stock solution of a NP can be broken down into five sections: chemical concentration of stock solution, particle concentration, particle size distribution, primary particle size, and manufacturer's information including batch details and impurities.

Concentration measurements were performed using inductively coupled plasma optical emission spectrometry (ICP-OES, Varian 725 ES), whilst primary particle size measurements were made using transmission electron microscopy (TEM, JEOL 1200EXII).

To measure particle size distribution a relatively new instrument was employed – the NanoSight LM10 (NanoSight Ltd, Amesbury, UK). This instrument uses a laser unit (LM10) which fires a laser beam through the sample chamber. When the laser hits a particle its beam is scattered. The forward scattered light is captured by the camera which shows the particle as a point light scatter. By making a video recording of these points of light and tracking the distance and speed of the particles moving under Brownian motion with the Nanoparticle Tracking Analysis software (NTA version 2.0) an estimate of their hydrodynamic diameter is made (Figure 2.4 shows a screen grab of a recording being analysed). Estimates of particle concentration can also be calculated using the software.

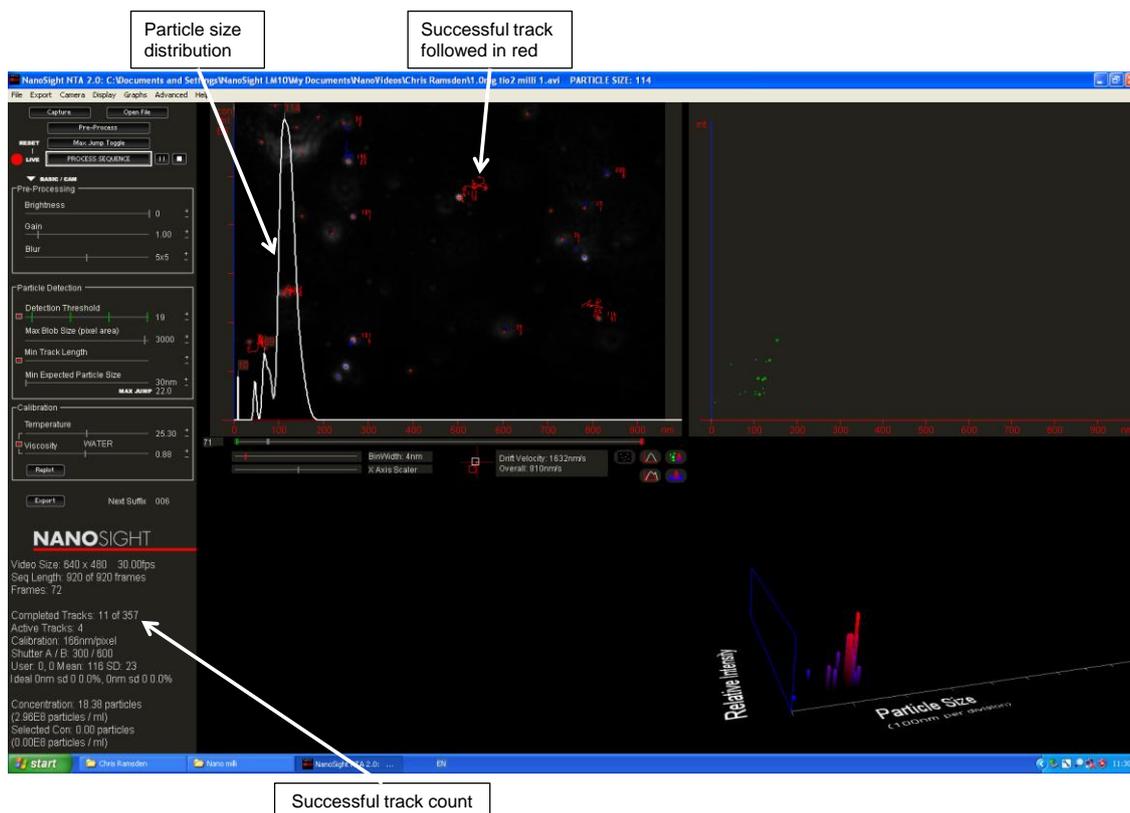


Figure 2.4. A screen grab from the NTA 2.0 software as a video sequence is analysed. The video is replayed and tracks are labelled on the screen as they are formed. Particle size distribution graphs are also drawn ‘live’ as the video is analysed. The settings on the left hand side of the screen are optimised for TiO<sub>2</sub> NPs.

As this instrument is very much similar to the TEM in that its primary role is to make size measurements there is no calibration required. The instrument can be tested using the certified reference NPs to ensure accuracy. There are various controls both on the camera and analysis software which can be altered to optimise video footage and reliability of the results depending on which NP is being analysed. For example certain metal NPs will have higher reflective indices than certain plastic NPs, therefore demanding different settings for optimal analysis. The instrument was therefore optimised for the primary NP of concern in this study – TiO<sub>2</sub>. Various runs were performed and individual settings changed each time (optimised settings in Figure 2.5) in an effort to produce a video with sufficient successful tracks of as many particles as possible. The higher the number of successful tracks, and the higher the number of

visible particles on the video screen, the more accurate the analysis software will be in estimating particle sizes and concentration of particles.

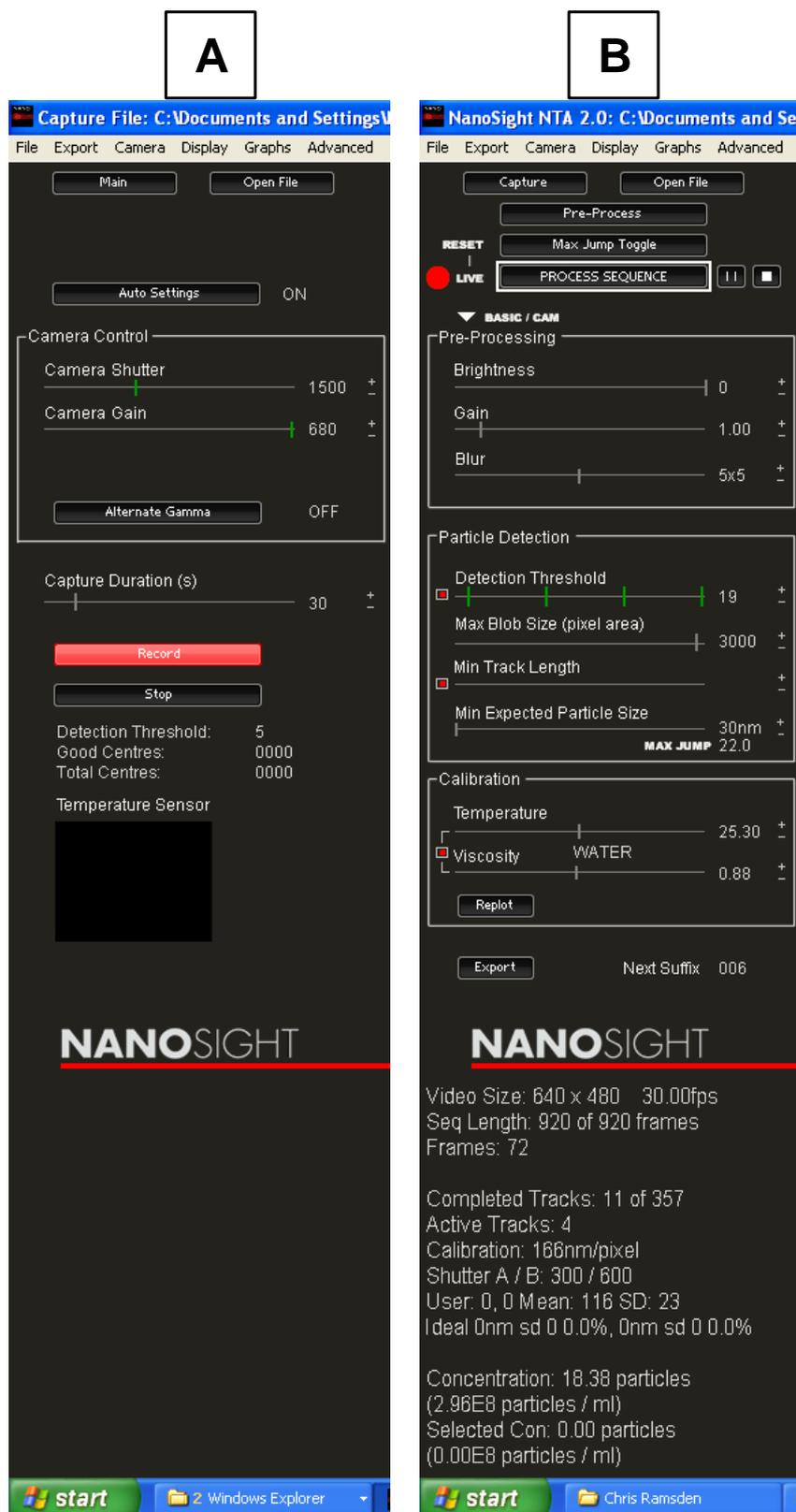


Figure 2.5. Optimised settings for the NanoSight LM10. (A) Camera settings – automatic settings turned on, (B) all other settings.

During these trials a few key points were identified to ensure good tracking: (i) samples must be of a sufficient concentration to give a good number of particles visible on screen at any one point (or video frame) – this number needs to be in the range of 100-300 particles. For TiO<sub>2</sub> NPs this optimal concentration is in the range of 0.1-1.0 mg l<sup>-1</sup>, (ii) samples must be in a suitable medium to ensure sufficient dispersion of particles – in a medium where particles aggregate there may only be 5 particles (or more likely particle clusters) analysed thereby making the estimate of size distribution and concentration poor, (iii) video recordings must be of a sufficient duration that a sufficient number of successful tracks can be attained – this number should be in the range of 100-300, achieved for TiO<sub>2</sub> NPs at a minimum time of 30 s per video at 1.0 mg l<sup>-1</sup>, (iv) samples should be run in triplicate to account for variation between runs of the same sample.

When these settings had been optimised for TiO<sub>2</sub> NPs a series of standards were analysed. Standards were made up with either TiO<sub>2</sub> NPs or bulk TiO<sub>2</sub> particles and were made in either ultrapure (Millipore) water or water taken from the conditioned tap water for the aquarium tanks in the zebrafish room. The LM10 unit was taken apart and cleaned with ethanol and ultrapure water. The unit was then reassembled and ultrapure water run as a blank. To introduce the sample to the unit a disposable syringe was used (1 ml, BD Plastipak) and the blank was reintroduced and checked until there were no visible particles in the unit. Standard samples were then analysed and a major difference between standards made with ultrapure and those made with tank water became apparent. Figure 2.6 shows the mean data taken from three analyses of each standard from the TiO<sub>2</sub> NP in ultrapure water. The data showed fairly uniform distributions with a low level of variation between analyses.

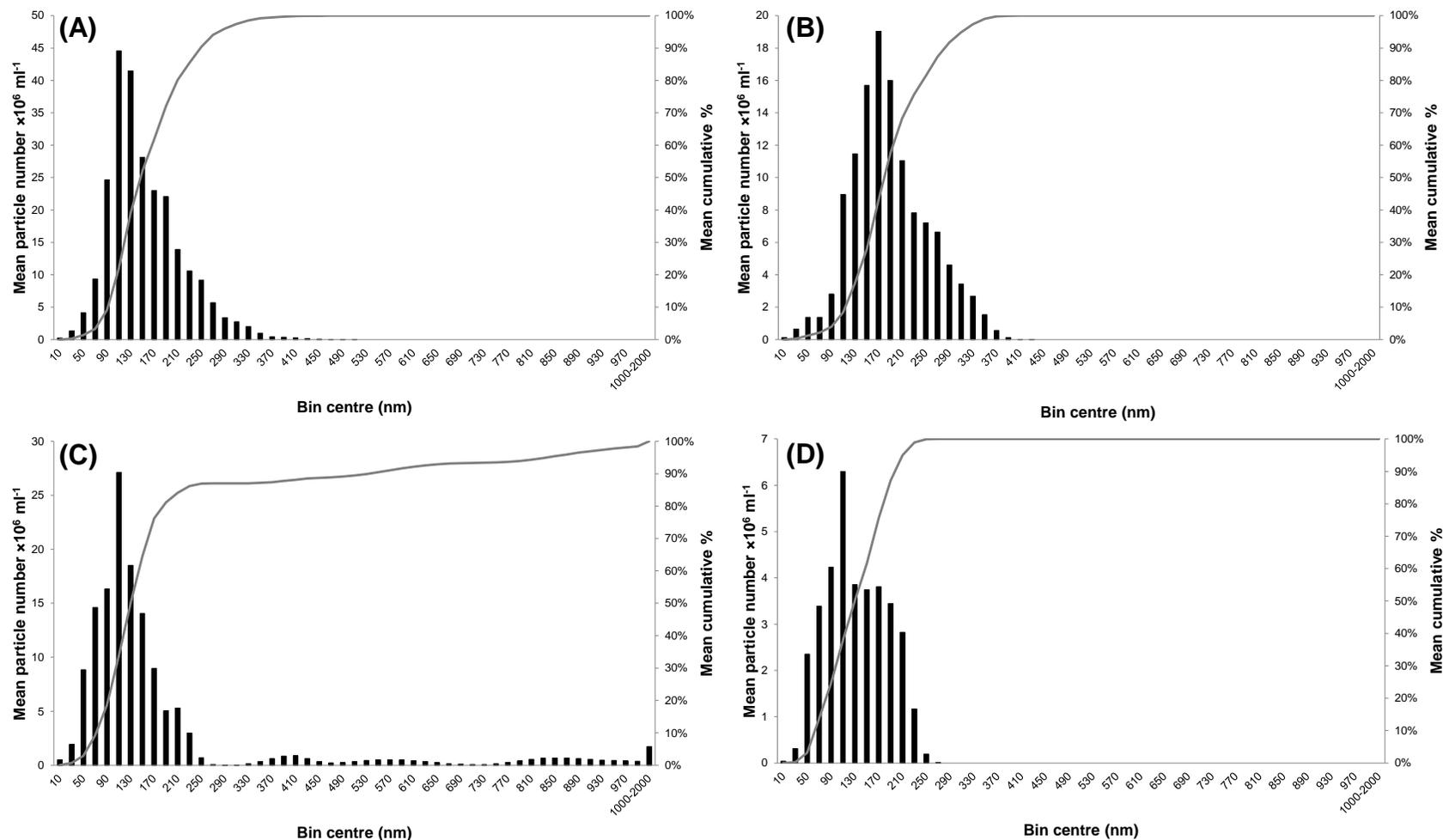


Figure 2.6. Standards made with TiO<sub>2</sub> NPs in ultrapure water at concentrations of: (A) 1.0 mg l<sup>-1</sup>, (B) 0.5 mg l<sup>-1</sup>, (C) 0.1 mg l<sup>-1</sup> and (D) 10 µg l<sup>-1</sup>. Black bars are mean ( $n = 3$ ) particle concentration, grey lines are mean ( $n = 3$ ) cumulative % of total sample.

When the standards were made using water taken from the experimental systems the results were somewhat different (Figure 2.7). It became apparent that larger aggregates of NPs had formed and as a result the number of successful tracks dropped significantly. This meant that only standards made using ultrapure water were reliable to use as particle size distribution measures for the stock suspensions.

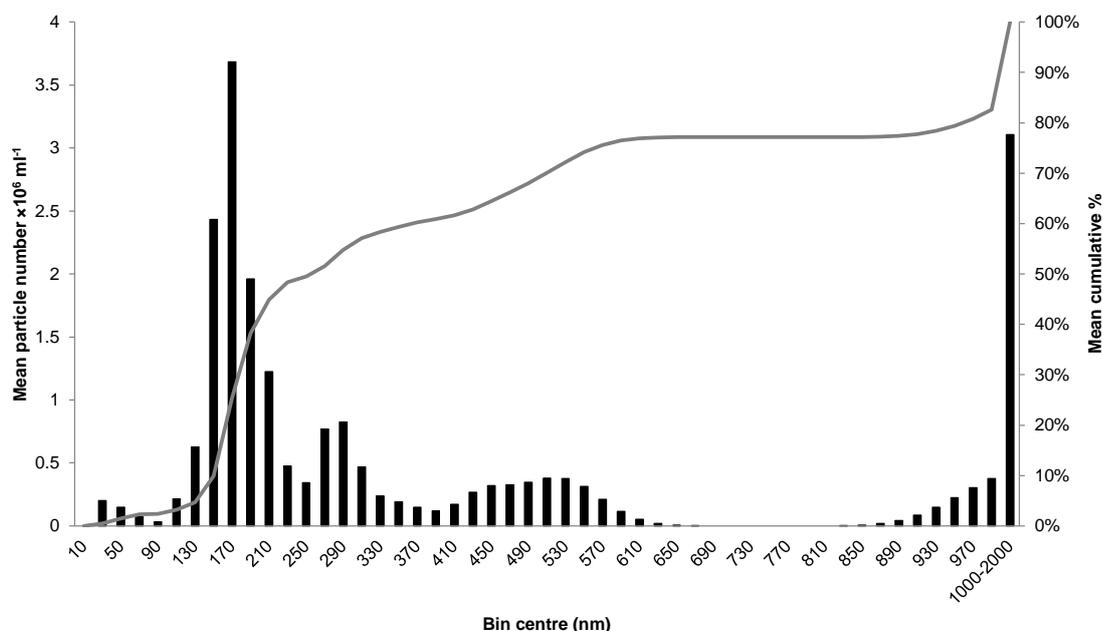


Figure 2.7. Standard made with  $\text{TiO}_2$  NPs in tank water at  $1.0 \text{ mg l}^{-1}$ . Black bars are mean ( $n = 3$ ) particle concentration, grey lines are mean ( $n = 3$ ) cumulative % of total sample.

The effect of the vehicle (water type) on reliability of results was clear. The possible explanation for this difference may be to do with the zeta-potential of the NPs – in ultrapure water it is likely that the zeta-potential is higher (more positive or negative, see von der Kammer et al. (2010) for a more comprehensive study) than in tank water. The effect of this difference in zeta-potential is that particles will aggregate more strongly when they have minimal charge – if they have a charge (positive or negative) there will be repulsion between particles meaning that the particles aggregate less, resulting in a lower range in the particle size distribution at the smaller particle size of the spectrum.

The NanoSight is a useful tool in estimating particle size distributions of NPs however there are some limitations. For measuring stock solutions of TiO<sub>2</sub> NPs in ultrapure water it is a reasonably quick and reliable method to employ. However, for TiO<sub>2</sub> NPs at least, it is really quite variable and does not produce reliable data. When the number of particles identified on a video sequence is under  $n = 10$  the NTA 2.0 software is flawed. The main problem is caused by the LM10 unit. The sample chamber is 3-dimensional and as such it allows particles to move under Brownian motion (the whole basis of the measurement algorithms behind the software). The problem with this is that particles can move in and out of the field of view and they can move in and out of focus. The camera is set up and the microscope focussed at the start of an analysis to give the highest number of particles visible to be measured. However during the run the particles move out of focus and the software loses the track. The same particle may then move back into view and be re-tracked. Unfortunately when this happens the software may measure the same particle 3 or 4 times and give 3 or 4 completely different size estimates, and in turn a rather poor estimate of particle concentration is attained.

Despite this flaw the fact that the TiO<sub>2</sub> NPs aggregate in tank water cannot be ignored. The homogeneity of the sample will play a major role in the use of this instrument with evenly sized and distributed particles giving far more reliable measurements than heterogeneous mixes. The NanoSight does have an 'extended dynamic range' mode which enables the user to have two video recordings of the same sample recorded at the same time. This allows the camera to be tuned for very large bright particles, and also for smaller particles which would otherwise be lost from view if the gain is set for brighter particles. Despite this the NanoSight was still not able to give reliable data for the TiO<sub>2</sub> NPs in tank water – the range of particle sizes was simply too great for the instrument to read them all and there was not a sufficient number of visible particles to obtain a sufficient number of successful tracks. It is also worth

noting that the SWCNTs used in Chapter 5 were also analysed with the NanoSight instrument with similar problems to those described for TiO<sub>2</sub> NPs.

#### **2.4. Titanium dioxide NP stock dispersions and dosing**

The titanium dioxide NP powder used here was from the same batch previously characterised by our laboratory (Federici et al., 2007; Ramsden et al., 2009), and the stock dispersion preparation followed our published procedure. Briefly, dry powder of TiO<sub>2</sub> NPs (“Aeroxide” P25 TiO<sub>2</sub>, DeGussa AG), was obtained via Lawrence Industries, Tamworth, UK (revised manufacturer’s information; crystal structure approximately 25 % rutile and 75 % anatase; purity 99 % TiO<sub>2</sub>, maximum impurity 1 % Si, average primary particle size of 21 nm, specific surface area of  $50 \pm 15 \text{ m}^2 \text{ g}^{-1}$ ). Chemical analysis of stock suspensions revealed no metal impurities and the batch purity was high (data not shown), with a measured mean primary particle size of  $22.8 \pm 0.6 \text{ nm}$  (mean  $\pm$  S.E.M.,  $n = 169$  particle measurements, see electron micrograph in Figure 2.8). A  $10 \text{ g l}^{-1}$  stock suspension of TiO<sub>2</sub> NPs was made (no solvents) by dispersing the NPs in ultrapure (Millipore) water by sonication (bath type sonicator, 35 kHz frequency, Fisherbrand FB 11010, Germany) for 6 h. Dry powder of TiO<sub>2</sub> (Titanium (IV) oxide, Acros Organics, supplied via Fisher Scientific, Loughborough, UK, purity 99.7 % TiO<sub>2</sub>) was used as the bulk material in the experiments. A  $10 \text{ g l}^{-1}$  stock suspension of the bulk TiO<sub>2</sub> powder was made (no solvents) by dispersing the powder in ultrapure (Millipore) water. Chemical analysis of the bulk powder dispersion revealed no metal impurities (data not shown), with a measured mean primary particle size of  $153.7 \pm 13.3 \text{ nm}$  (mean  $\pm$  S.E.M.,  $n = 149$  particle measurements, see electron micrograph in Figure 2.8). The size distribution of bulk TiO<sub>2</sub> was larger than that of the NPs with a large number of particles in the <100 nm range. The crystalline structure ratio was similar to that of the NP stock (approximately 25 % rutile, 75 % anatase).

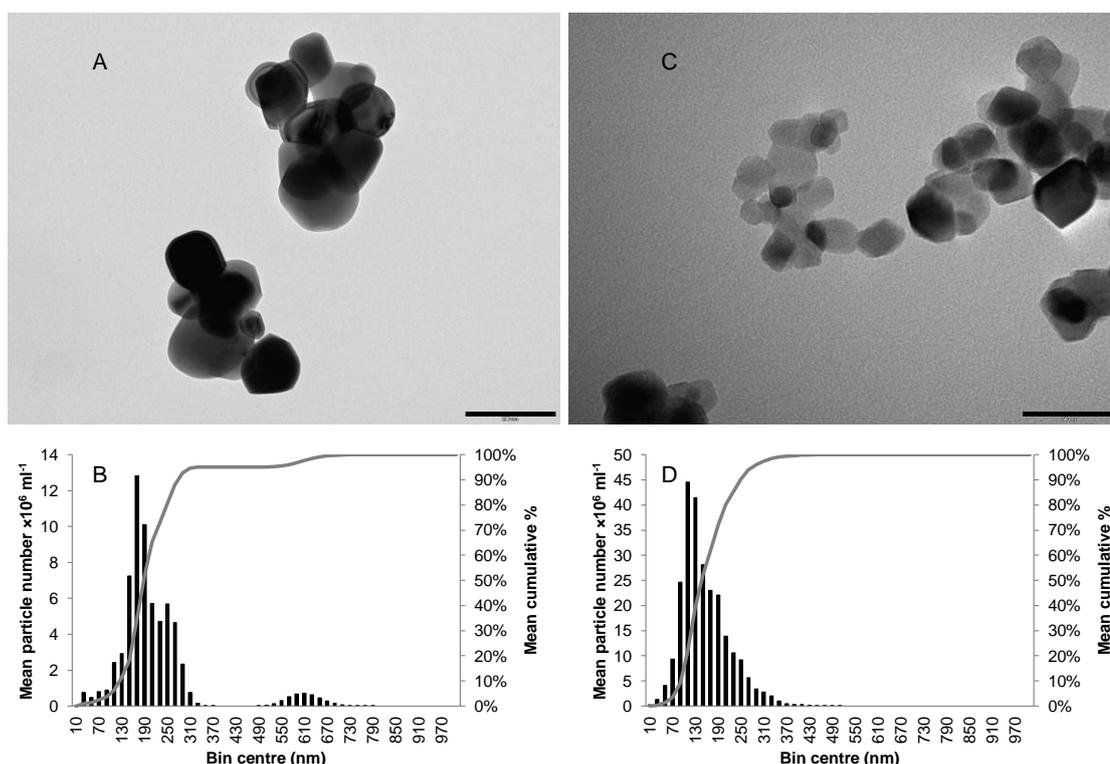


Figure 2.8. Transmission electron micrographs and particle size distribution plots for (A & B) TiO<sub>2</sub> bulk and (C & D) TiO<sub>2</sub> NP stock suspensions. Scale bars for the micrographs are 200 (A) and 50 (C) nm. Example particle size distribution plots are for 1 mg l<sup>-1</sup> stock dispersions of TiO<sub>2</sub> in Millipore water using nanoparticle tracking analysis (NTA, Nanosight LM10). The example plots are a mean plot of three measurements.

Stock suspensions of both TiO<sub>2</sub> bulk and NPs were characterised by transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA) using a NanoSight LM10 (NanoSight Ltd, Amesbury, UK) (Figure 2.8). For primary particle size measurements by TEM, the initial stock suspensions of 10 g l<sup>-1</sup> were diluted to 1 mg l<sup>-1</sup> and then mounted onto plastic coated copper grids for TEM (JEOL 1200EXII). Particle size distributions by NTA were determined in the 1 mg l<sup>-1</sup> stock suspension in triplicate to generate a mean particle size distribution plot (Figure 2.8, B & D). Attempts were made to perform NTA on samples of exposure water from the experimental tanks. However, the background noise from natural colloids and aggregation behaviour of the materials resulted in an insufficient number of completed tracks to give a reliable size distribution plot for Plymouth freshwater.

## 2.5. Anaesthesia and dissection

All procedures on fish were ethically approved and carried out under a Home Office project licence (PPL 30/2772 'Environment and Health: Hazards of Metals and Nanomaterials'). For anaesthesia fish were immersed in water containing MS222 (tricane methane sulphonate, MS222/1-V1, PHARMAQ) buffered to neutral pH with  $\text{NaHCO}_3$ . After fish were euthanised and, where necessary, blood had been collected fish were weighed, total length recorded and any observations on gross morphology noted. When relevant, dissection was carried out using clean instruments with regular cleaning and blade replacement between treatment groups to avoid cross contamination.

## 2.6. Gross condition indices

Gross condition indices such as condition factor and organo-somatic indices are useful as first level screening biomarkers in studies on the ecotoxicology of pollutants in fish (Schmitt and Dethloff, 2000; van der Oost et al., 2003). The indices used in the present studies were condition factor ( $K$ ), gonadosomatic index (GSI) and splenosomatic index (SSI). Condition factor is based upon the ratio between body weight and length and gives an idea of the health of an individual. Condition factor is calculated as,  $K (\%) = (\text{weight (g)}/\text{total length}^3 (\text{cm})) \times 100$ . The power value used in the condition factor equation is based on the relationship between the log of weight versus the log of total length for a fish population (Craig et al. 2005). The power value varies between populations of fish but in general the power of 3 adequately describes the typical relationship between weight and length in most fish body forms when simple proportional comparisons are being made. Gonadosomatic index is based on the ratio of gonad tissue relative to the total body weight of an individual and gives an idea of the

reproductive maturity and health of an individual. GSI is calculated as,  $GSI (\%) = (\text{gonad weight (g)}/\text{wet body weight without gonad (g)}) \times 100$ . Splenosomatic index is based on the ratio of spleen tissue relative to the total body weight of an individual and gives an idea of the condition of the haematological status and immune system health of an individual. SSI is calculated as,  $SSI (\%) = (\text{spleen weight (g)}/\text{wet body weight without spleen (g)}) \times 100$ .

## **2.7. Haematology**

Fish were carefully anaesthetised with buffered MS222, weight and total length were recorded, and the fish were rinsed in ultrapure water to remove any test media from the body surface. The tail was cut off with a sharp scalpel (under deep anaesthesia), and whole blood was collected from the caudal vein into heparinised capillary tubes and then carefully expelled onto a clean glass slide. Samples of approximately 3  $\mu\text{l}$  of the blood were mixed with 147  $\mu\text{l}$  of Dacie's fluid, stored and later counted for red and white blood cells according to Handy and Depledge (1999).

## **2.8. Whole body ion and trace metal analysis**

The protocol was modified from Federici et al. (2007) to include a dispersion step in the final dilution of the acid digest. The following is a shortened version of the results of the method development detailed in Chapter 3. Instrument settings and standard calibration details are also detailed in Chapter 3. Briefly, following blood sampling, whole zebrafish (including digestive tract) or dissected stickleback organs were placed onto clean glass slides and oven dried to a constant weight, then digested in 1 or 4 ml of concentrated nitric acid at 70 °C for 2 hours, and allowed to cool. Then, a 10 % solution of Triton X-100 was slowly added to each of the digested fish samples to achieve a final

concentration of 2 % Triton X-100 in each tube (0.8 or 3.2 ml of the 10 % Triton X-100 solution for small or large digests respectively). Each sample was then diluted with ultrapure water to achieve final volumes of 4 or 16 ml (adding 2.2 or 8.8 ml ultrapure water to small or large digests respectively) 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 were used throughout, and the acidity and matrix of the standards was matched to the samples. Spiked samples showed good recoveries for trace metals (over 95 %) and Ti metal (approximately 70 % from TiO<sub>2</sub> NP-spiked digests, data shown in Chapter 3). Ti concentrations in fish samples were reported as total Ti concentrations not TiO<sub>2</sub>.

## **2.9. Biochemistry**

Samples of either whole fish or dissected tissues were analysed for indicators of osmoregulatory dysfunction, oxidative stress and energy status. Exact details of the samples collected and the method of tissue homogenisation vary slightly between experiments and as such details for each methodology are given in each chapter. There are however some assays which have been used in all chapters, the details of which are given below.

### **2.9.1. Na<sup>+</sup>K<sup>+</sup>-ATPase activity**

The Na<sup>+</sup>K<sup>+</sup>-ATPase assay was performed according to Bouskill et al. (2006) with minor modifications. Briefly, samples (15 µl crude homogenate) were dispensed in triplicate into 1.5 ml microcentrifuge tubes containing 400 µl of either a K<sup>+</sup>-containing buffer (100 mmol l<sup>-1</sup> NaCl, 10 mmol l<sup>-1</sup> KCl, 5 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 1.25 mmol l<sup>-1</sup> Na<sub>2</sub>ATP, 30 mmol l<sup>-1</sup> HEPES, pH 7.4) or the equivalent K<sup>+</sup>-free buffer (plus 1.0 mmol l<sup>-1</sup> ouabain), then incubated at 37 °C for 20 min. The reaction was stopped by adding 1ml

of ice cold trichloroacetic acid (8.6 % w/v) and then the tubes were centrifuged 12,300 g (Heraeus Biofuge Pico) for 2 minutes to remove cell membrane debris. The supernatants (140  $\mu$ l) were mixed with 100  $\mu$ l of colour reagent (9.6 % w/v  $\text{FeSO}_4 \cdot 6\text{H}_2\text{O}$ , 1.15 % w/v ammonium heptamolybdate dissolved in 0.66M  $\text{H}_2\text{SO}_4$ ), and colour allowed to develop for 30 minutes at room temperature. Absorbances were measured at 660 nm (Molecular Devices Versa Max microplate reader) against 0-2.0  $\text{mmol l}^{-1}$  phosphate standards.

### **2.9.2. Total glutathione (GSH)**

Total glutathione (GSH) was determined according to Owens and Belcher, (1965). Briefly, 20  $\mu$ l of diluted (final dilution of 1:100) tissue homogenate, blank or standard (0-20  $\mu\text{mol l}^{-1}$  reduced glutathione), was added in triplicate to wells of a 96-well plate containing 20  $\mu$ l of 10  $\text{mmol l}^{-1}$  DTNB (5,5'-dithiobis- (2-nitrobenzoic acid)), 260  $\mu$ l of assay buffer (100  $\text{mmol l}^{-1}$  potassium phosphate buffer containing 5  $\text{mmol l}^{-1}$  EDTA, pH 7.5), and 20  $\mu$ l of 2 U  $\text{ml}^{-1}$  glutathione reductase (Sigma-Aldrich, UK). The reaction was commenced by the addition of 20  $\mu$ l of 3.63  $\text{mmol l}^{-1}$  NADPH, with changes in absorbance at 412 nm (microplate reader as above) recorded over 15 minutes, and total GSH ( $\mu\text{mol g}^{-1}$  wet weight tissue) determined using the standard calibration curve.

### **2.9.3. Protein concentration**

When necessary biochemistry data were normalised against homogenate protein content. Briefly for the protein assay, 5  $\mu$ l of diluted 1:100 homogenate was dispensed into triplicate wells of a 96 well plate along with 250  $\mu$ l of Bradford Reagent, the colour was allowed to develop for 30 minutes at room temperature, then absorbance values were measured at 595 nm (using the microplate reader above) against 0-1.4  $\text{mg ml}^{-1}$  bovine serum albumin standards.

## **2.10. Histology**

Histology followed the protocol of Handy et al. (2002), with some amendments as detailed in section 2.3.2.2. Briefly, fish were terminally anaesthetised with an overdose of buffered MS222, weight and total length were recorded, then the abdomen was cut open (to allow rapid penetration of the fixative) and the whole fish submerged in an excess volume of buffered formal saline. Fish were processed for routine wax histology (Leica TP1020 tissue processor), longitudinal whole body sections were then cut (5 µm sections, Leica RM2235 microtome), mounted, and then stained with Mayer's Haematoxylin and Eosin (using a Leica Autostainer XL). All fish were prepared simultaneously in batches containing specimens from all treatments in order to eliminate temporal fixation or staining artefacts. Sections were reviewed by light microscopy and photographed (Olympus Vanox AHBT paired to Olympus E620). Quantitative analysis of tissues was performed as described in Handy et al. (2002).

Briefly, for gill tissue analysis a minimum of 100 secondary lamellae were counted for each fish where possible and scored for damage including the incidence of clubbed tips and the presence of any oedema or lesions. Club tip damage was reported as the percentage of the total number of secondary lamellae exhibiting clubbed tips. For liver tissue one slide per fish was scored for the number of points on a grid where the tissue underneath was sinusoid space rather than somatic tissue (the parenchyma); this was reported as a percentage of sinusoid space relative to the parenchyma. Incidence of other markers of tissue damage such as foci of lipidosis was also noted. For gonad tissue female tissue samples were scored for the mean diameter of follicles by measuring 10 follicles per slide (to give an idea of any potential changes which may warrant further investigation) at random in the horizontal plane. This data was reported as mean follicular diameter. Other features which were noted in the gonad tissue

included the relative proportions of follicular developmental stages in females and the gross morphology of the male gonad tissue. Incidence of ova-testis was also recorded.

### **2.11. Statistical analysis**

All data were analysed using StatGraphics Plus version 5.1 and graphs were drawn using Microsoft Excel. Data were checked for inter-tank variability and where no tank effects were observed throughout the experiment data was pooled by treatment for statistical analysis. Data were checked for normality by evaluating kurtosis (the heaviness of tails relative to a normal distribution), skewness (the symmetry of a distribution relative to the normal distribution) and unequal variance (using the Bartlett's test).

Data were then tested for treatment and/or time effects by one-way or multifactor-ANOVA. For data sets where there were several treatments at more than one time point data were analysed using a two-way ANOVA to test for treatment, time and time  $\times$  treatment interactions. Where these tests showed statistically significant differences ( $P < 0.05$ ), one-way ANOVA tests were used to look for differences either between time points within a treatment group or, more usually, differences between treatment groups within time points. Where one-way ANOVA analysis showed a significant difference between means ( $F$ -test,  $P < 0.05$ ), a post hoc Fisher's 95 % least significant difference test was used to look for significant differences between mean values of data sets.

Where data were non-parametric (failed Bartlett's test,  $P < 0.05$ ), data were log-transformed and re-tested. If the data were still non-parametric the Kruskal-Wallis test was used to test for differences between the medians of data sets. If this test rejected the null hypothesis that there were no differences between median values, the differences between data sets were located by notched box and whisker plots. Where necessary, in data sets where there were only two data sets to compare, pair-wise comparisons were

analysed using the student's *t*-test. All results are presented as mean  $\pm$  SEM unless otherwise specified, and all statistics use a rejection level of 5 %.

# **3. An improved method for the determination of total titanium from titanium dioxide nanoparticles in fish, including the use of single particle ICP-MS for sample characterisation**

## **3.1. Abstract**

The reliable characterisation and detection of Ti from TiO<sub>2</sub> nanoparticles (NPs) is required to support ecotoxicological research on NP uptake, and the food safety industry also requires a method for measuring edible fillets (muscle) of fish. Existing metal analytical techniques are inadequate yielding low recoveries in fish intestine spiked with TiO<sub>2</sub> NPs compared to Ti metal spikes and were (all data mean  $\pm$  SEM,  $n = 6$ ),  $18.1 \pm 9.1$  and  $97.5 \pm 2.9$  % respectively. Investigations of analyte loss showed no effect of 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<sub>2</sub> NPs by 3-10 fold. Spikes of  $100 \mu\text{g l}^{-1}$  TiO<sub>2</sub> NPs produced the highest recoveries and were  $69.7 \pm 8.6$ ;  $65.0 \pm 1.7$ ;  $55.0 \pm 5.8$ ;  $44.7 \pm 5.8$ ; and  $41.3 \pm 4.0$  % for whole zebrafish, and trout gills, muscle, intestine, and liver respectively. Precision and accuracy were also within the limits generally accepted for dissolved metals. The new method represents a marked improvement in determining Ti metal from TiO<sub>2</sub> NPs in tissues with potential uses in

both ecotoxicology and food safety. Single particle ICP-MS characterisation of TiO<sub>2</sub> NPs was tested in both water and tissue samples. Good calibration was achieved with TiO<sub>2</sub> NPs and combined with the improved ICP-OES methodology a more complete characterisation of complex matrix samples can now be achieved.

### **3.2. Introduction**

Engineered nanoparticles (NPs) can be defined as novel materials with at least one dimension < 100 nm (SCENHIR, 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<sub>60</sub> fullerenes), as well as a range of nano-ceramics, quantum dots and nano-composites that are each made from several chemical substances (Boxhall et al., 2007; Stone et al., 2010). The interest in NPs partly arises from their unique physical and chemical properties at the nanoscale (Stengl et al., 2007; Behnajady et al., 2008; Handy et al., 2008c; Ju-Nam and Lead, 2008). However, some of these properties such as poor solubility in water, the colloidal behaviour of nanoparticles in aqueous media (aggregation) and adsorption to surfaces are creating new challenges for analytical chemists (see (Handy et al., 2008c; Hasselov et al., 2008) for reviews of measurement methods for NPs).

Titanium dioxide (TiO<sub>2</sub>) 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<sub>2</sub> 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 per cent in foods. However, the potential benefits of TiO<sub>2</sub> 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., 2008b) and, more generally, to humans (Handy and Shaw, 2007) with oral exposure to TiO<sub>2</sub> NPs causing lesions in the liver and kidneys of mice (Wang et al., 2007). A reliable method of detecting titanium from TiO<sub>2</sub> 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 U where very low detection limits are required, (e.g., Bourrachot et al., 2008). However, for TiO<sub>2</sub> NPs, these traditional methods give variable results and very poor spike recovery tests (e.g., Scown, et al., 2009). There are some techniques which will slowly dissolve TiO<sub>2</sub> but these involve the use of either hot concentrated sulphuric acid (Lomer et al., 2000; Sun et al., 2007) or hydrofluoric acid (Lomer et al., 2000). Both of these methods require specialist equipment and, particularly in the case of HF acid, are extremely dangerous and do not lend themselves for cost and time effective analysis of large numbers of samples. Researchers have therefore tried various approaches to improve digestion and quantification. 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<sub>2</sub>, and in the absence of certified standards for TiO<sub>2</sub> NPs in fish tissues, dual calibrations, where titanium metal (Ti-Me) standards are run against carefully prepared TiO<sub>2</sub> NP dispersions have been used to

correct for the calibration responses of instruments (Federici et al., 2007; Zhang et al., 2007).

As well as determining mass concentration it would also be useful to assess particle number in tissue digests to allow alternative dose metric plots of the data (Handy et al. 2012). There has been an increase in interest in the use of ICP-MS for single particle characterisation over the past few years with groups looking at microparticles and colloids (Degueldre and Favarger, 2003; Degueldre et al., 2004; Degueldre et al., 2006a; Degueldre et al., 2006b) and nanoparticles (Garcia et al., 2010; Suzuki et al., 2010; Laborda et al., 2011; Poda et al., 2011). However, so far there is no data available where this technique was used to assess NPs in digested fish tissue. Full details of the analytical method development for measuring Ti in tissues from TiO<sub>2</sub> NPs have not been documented. For practical use in food safety the accuracy and precision of techniques, 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 aims of this study were to describe, test, and validate a method of quantifying total tissue Ti levels from fish tissues exposed to TiO<sub>2</sub> NPs; 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, and to carry out a preliminary study to examine the possibility of using single particle ICP-MS as a tool in characterising TiO<sub>2</sub> NPs in complex digest samples. This study investigated the hypothesis that standard analytical procedures for the analysis of metal concentrations in fish tissues were not sufficient for the novel problems associated with NPs. A reliable method of Ti analysis is also a pre-requisite for the

development of certified reference fish tissues for TiO<sub>2</sub> NPs, which are currently not available.

### **3.3. Methods**

#### **3.3.1. Titanium dioxide NP stock solution**

The details of the titanium dioxide NP powder used here are given in Chapter 2.

#### **3.3.2. 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<sup>-1</sup> of Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup> 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.

#### **3.3.3. Established metal detection method**

The starting point for the experiments was an existing method for trace element analysis in fish tissues used many times by our laboratory (e.g., Handy et al., 1999; Handy et al., 2000; Shaw and Handy, 2006) where spike recovery tests performed on tissue samples typically gave recoveries of 100 ± 5 % of the target value (e.g., Handy et

al., 2000). Briefly, fish tissues were oven dried to constant weight over 48 h (100°C, Gallenkamp Oven BS Model OV- 160), then transferred to 20 ml plastic polypropylene (with polyethylene cap) scintillation vials (VWR International Ltd, Poole, UK) and approximately 0.3-0.1 g of dried tissue was digested in 4 ml of concentrated nitric acid (69 % analytical grade, Fisher Scientific) for 2 h at 70°C in a water bath, for at least 10 minutes out of the water bath, and then diluted to 20 ml using ultrapure water (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 to a minimum of 1 ml of nitric acid, and diluted to a final volume of 4 ml. Samples were analysed for Ti, Cu, Zn, Mn, Ca, Na and K by inductively coupled plasma optical emission spectrometry (ICP-OES, Varian 725-ES, Melbourne, Australia).

#### **3.3.4. Instrumentation and quality control**

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<sup>-1</sup>, respectively, and instrument stabilisation, time uptake delay, and replicate read time, 10, 15, and 4 s, respectively, with a wavelength of 336.122 nm for Ti. Calibration was achieved with mixed, matrix-matched standards between 0-1000 mg l<sup>-1</sup> (depending upon analyte), prepared from Aristar<sup>®</sup> 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 Ti-Me or TiO<sub>2</sub> NPs and for practical reasons (see discussion) the former was used throughout. The detection limit of the instrument for Ti-Me (3 x standard deviation of the 25 % nitric acid blank) was 7.04 µg l<sup>-1</sup> (*n* = 18). The procedural detection limit of Ti-Me for tissue digests going through the entire protocol (3 x standard deviation of the digestion protocol blank) was 4.58 µg l<sup>-1</sup> (*n* = 6). Titanium dioxide values were calculated from the Ti-Me 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<sub>2</sub> NP content of tissues, spike recovery tests using both Ti-Me and TiO<sub>2</sub> NPs were conducted using rainbow trout intestine. Briefly, samples were oven dried as described above and known concentrations of Ti-Me or TiO<sub>2</sub> NPs were added to the vial prior to the addition of concentrated nitric acid and the subsequent digestion. Samples were then diluted with ultrapure (Millipore) water as described above. However, the existing method of Ti analysis, as expected, gave poor results for TiO<sub>2</sub> NP spiked tissue and it was evident that the established method of analysis needed improving and optimising for TiO<sub>2</sub> NPs.

### **3.3.5. Method development**

A series of experimental tests were conducted to improve the methodology for detection of TiO<sub>2</sub> 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 the ICP-MS gave poor results (low compared to OES) with a high level of variability between samples (i.e., more than  $\pm$  20%, data not shown). The ICP-OES showed far greater sensitivity to Ti in the form of TiO<sub>2</sub> 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<sub>2</sub> NP standard solutions compared to Ti-Me standards and optimising the instrument settings so that both responses were linear; (ii) trials to determine where any losses of TiO<sub>2</sub> NPs could be occurring during the digestion protocol. This involved spiking samples with known concentrations of TiO<sub>2</sub> 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 finally 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; (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<sub>2</sub> NPs or Ti-Me), and in some of the trials tissue samples that were spiked with known amounts of Ti-Me (positive controls) were used to compare with TiO<sub>2</sub> 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 variation over time.

### **3.3.6. Final optimised method for determining Ti from TiO<sub>2</sub> 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 were cool, Triton X-100 was slowly added to each digested sample prior to dilution with ultrapure 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  $145 \text{ r min}^{-1}$  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, Mn, Ca, Na and K (ICP-OES parameters set precisely as described in the 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  $\text{TiO}_2$  NP spikes ( $100\text{-}1000 \mu\text{g l}^{-1}$ ). Data for precision are presented per g tissue.

### **3.3.7. Single particle ICP-MS characterisation of $\text{TiO}_2$ NPs**

Standards and samples were prepared exactly as described above in the final optimised method section. Standards were prepared using Ti metal ICP standards,  $\text{TiO}_2$  bulk and  $\text{TiO}_2$  NPs at concentrations of 0, 25, 50, 100 and  $200 \mu\text{g l}^{-1}$ . All standards were matrix matched to the samples with 25 % nitric acid and 2 % Triton X-100. To test the ability of single particle ICP-MS to detect  $\text{TiO}_2$  NPs in tissue digests gill samples from rainbow trout were digested and spiked in the same standard range as given above. Gill digest samples were also prepared to test spike recovery by spiking three samples with  $200 \mu\text{g l}^{-1}$   $\text{TiO}_2$  NPs and leaving three samples without a spike. A Thermo Fisher Scientific X Series 2 ICP mass spectrometer (Hemel Hemstead, UK) was used for the

single particle characterisation. The sample introduction system consisted of a concentric glass nebuliser and a PC<sup>3</sup> spray chamber assembly cooled to 5 °C.

Instrument and data acquisition parameters were as follows: RF Power = 1400 W, Plasma = 13 l min<sup>-1</sup>, Auxiliary = 0.7 l min<sup>-1</sup>, Nebulizer = 0.82 l min<sup>-1</sup>, Sample uptake = 1 ml min<sup>-1</sup>, Points per spectral peak = 1, Sweeps = 1, Dwell time = 0.1 ms, Readings per replicate = 4,000 and Integration time = 0.4 s. Each standard and sample were run as a new experiment to standardise time delays in instrument wash time and introduction delays, and for ease of data output.

### **3.3.8. Statistical analysis**

All data were analysed using StatGraphics version 5.1. Details of the statistical analysis protocol followed are given in Chapter 2.

## **3.4. Results**

### **3.4.1. Quality control and assurance**

Calibration with both Ti-Me and TiO<sub>2</sub> 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 Ti-Me and TiO<sub>2</sub> NP standards with both producing correlation coefficients of > 0.9995, with  $P$  values of  $2.50 \times 10^{-8}$  and  $1.14 \times 10^{-6}$  for Ti-Me and TiO<sub>2</sub> NP respectively (Pearson Product Moment Correlation).

Standards were run 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 carryover. Recovery of Ti-Me spikes was complete using the standard metal

method in fish intestine samples (data were, mean  $\pm$  SEM,  $n = 6$ ;  $97.5 \pm 2.9$  %), but this original method gave very poor results for intestine samples spiked with TiO<sub>2</sub> NPs (recovery of  $18.1 \pm 9.1$  %,  $n = 6$ ).

### 3.4.2. 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,  $P > 0.05$ ; Figure 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 Ti-Me and TiO<sub>2</sub> NP spiked samples (students *t*-test,  $P > 0.05$ ; data not shown).

Samples were 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 (Figure 3.1B), nor did the use of the anionic surfactant, SDS, in spiked samples (recovery appeared reduced in these samples, though not significantly; ANOVA,  $P > 0.05$ ; Figure 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) and SDS (ANOVA,  $P < 0.05$ ; Figure 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).

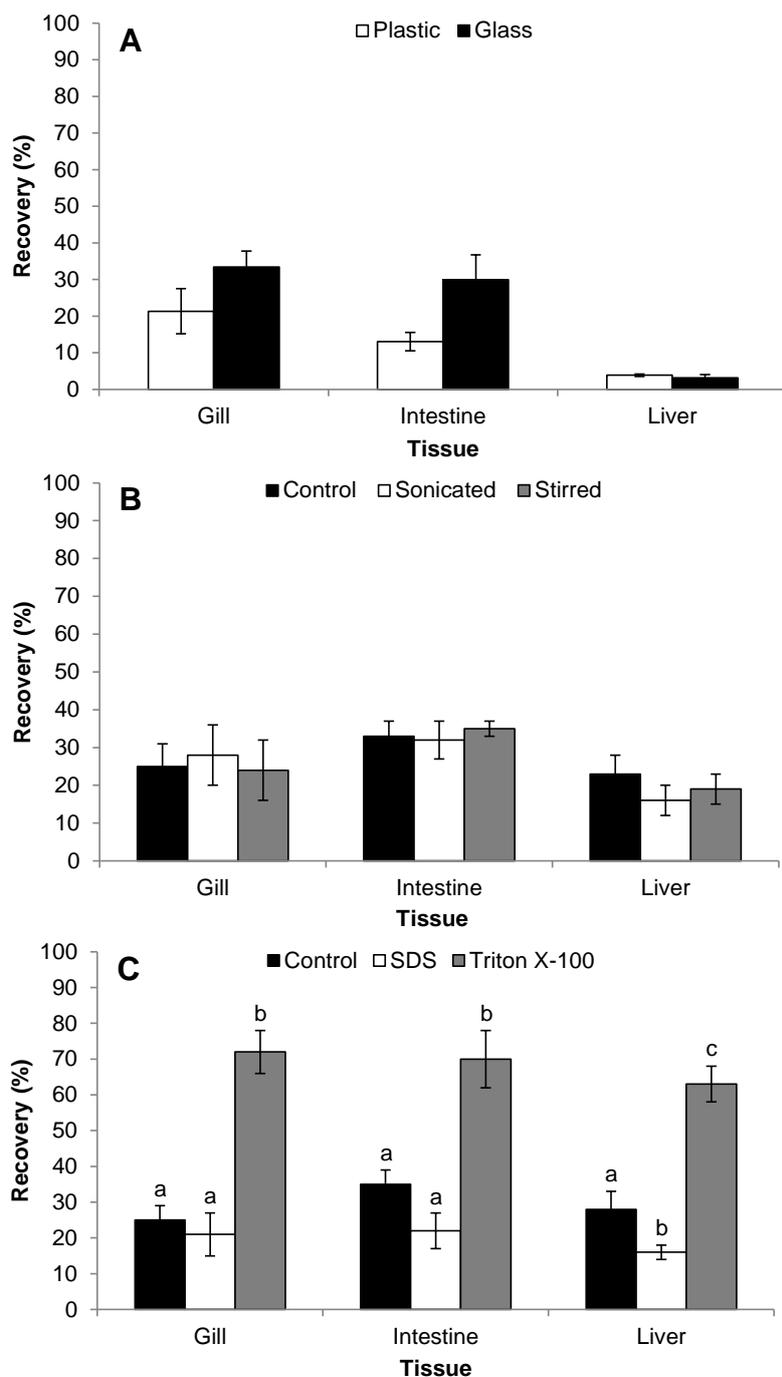


Figure 3.1. Recovery of Ti in rainbow trout tissue (gill, intestine, liver) spiked with  $100 \mu\text{g l}^{-1}$   $\text{TiO}_2$  NPs, effects of protocol variables: (A) glass versus plastic polypropylene digestion vials, and (B) 2 h stirring or sonication of samples, and (C) the effects of SDS and 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  $\pm$  SEM, % recovery of  $\text{TiO}_2$  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.

### **3.4.3. Optimisation of Triton X-100 method for Ti determination from TiO<sub>2</sub> NPs in fish tissue**

A series of spike recovery experiments (with 100 µg l<sup>-1</sup> TiO<sub>2</sub> 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) 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 ultrapure water dilution of the acid digest (students *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., Figure 3.2A).

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<sup>-1</sup> 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, data not shown).

### **3.4.4. Validation of optimised protocol**

Following optimisation of the protocol a series of spike recovery tests were conducted using a serial dilution of TiO<sub>2</sub> NPs (0-1000 µg l<sup>-1</sup>) in order to validate the efficacy and precision of the method. Recovery was tissue and spike concentration dependent with the highest recovery achieved using whole zebrafish and the lowest using liver, whilst 100 µg l<sup>-1</sup> TiO<sub>2</sub> NP spikes provided the highest recovery in each

tissue type compared with more concentrated spikes (see Figure 3.2B for % recovery data of  $100 \mu\text{g l}^{-1}$   $\text{TiO}_2$  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 ranged from (data; % mean  $\pm$  SEM,  $n = 6$  samples for each tissue at each spike concentration);  $36.2 \pm 4.5$  to  $53.3 \pm 4.4$  for liver;  $45.9 \pm 1.7$  to  $55.0 \pm 5.8$  for muscle; and  $32.5 \pm 5.6$  to  $52.1 \pm 1.1$  for intestine. Spike recovery was significantly higher in zebrafish samples spiked with  $100 \mu\text{g l}^{-1}$   $\text{TiO}_2$  NPs than was achieved at higher spike concentrations (Kruskal-Wallis;  $P < 0.05$ ) with recoveries of  $69.7 \pm 8.5$ ,  $53.8 \pm 2.5$ ,  $54.4 \pm 2.3$ ,  $42.2 \pm 5.0$ ,  $45.5 \pm 0.6$ , and  $44.9 \pm 2.8$  from 100, 200, 300, 400, 500, and 1000  $\mu\text{g l}^{-1}$   $\text{TiO}_2$  NP spikes respectively (data mean  $\pm$  SEM % recovery,  $n = 6$ ).

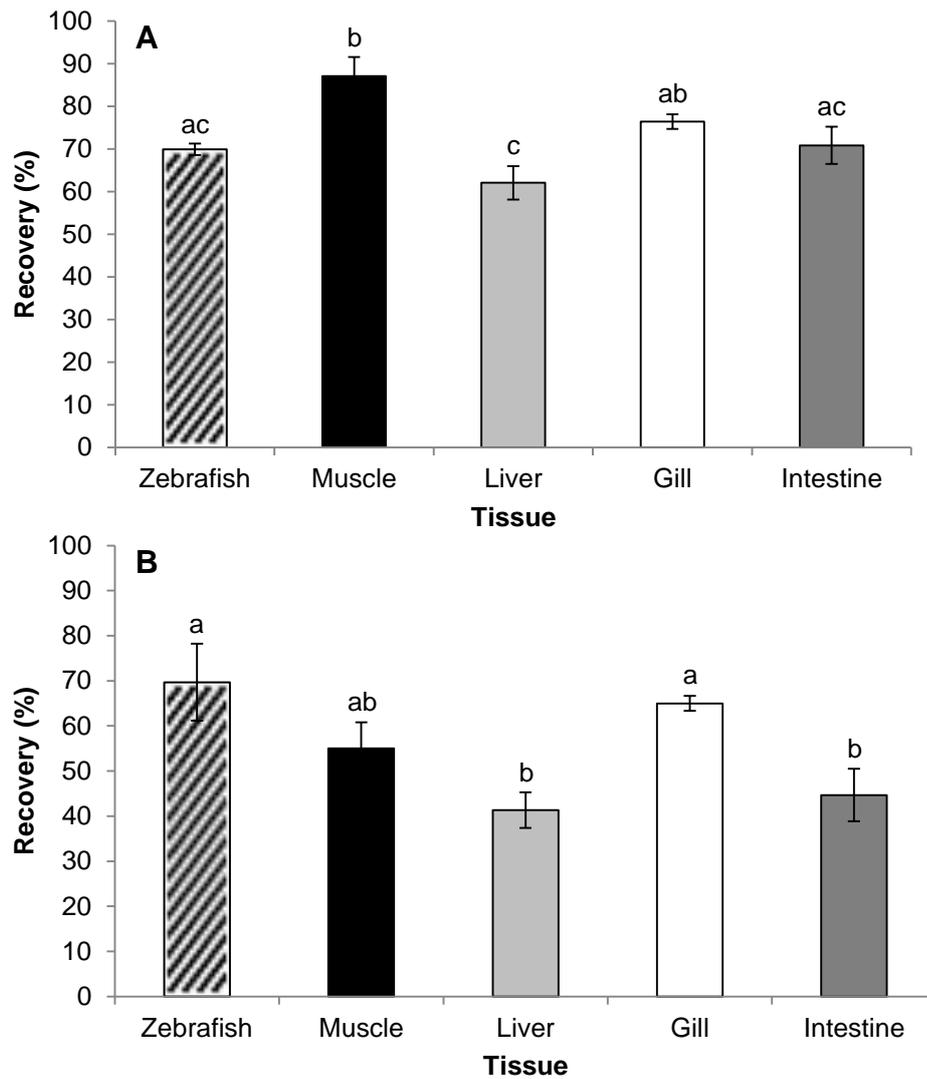


Figure 3.2. Recovery of Ti in whole zebrafish and rainbow trout tissue (muscle, liver, gill, intestine) samples spiked with  $100 \mu\text{g l}^{-1}$   $\text{TiO}_2$  NPs with 2 % Triton X-100 added following acid digestion and measured on ICP-OES calibrated using (A) standards with no added Triton 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  $\pm$  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  $\mu\text{g l}^{-1}$   $\text{TiO}_2$  NP spiked samples and the 400, 500, and 1000  $\mu\text{g l}^{-1}$  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  $\mu\text{g l}^{-1}$   $\text{TiO}_2$  NPs compared with all other spikes (ANOVA,  $P < 0.05$ ; data mean  $\pm$  SEM % recovery (nominal  $\text{TiO}_2$  NP spike concentration in  $\mu\text{g l}^{-1}$ ); 65.0  $\pm$  1.7 (100) 59.8  $\pm$  1.8 (200); 57.8  $\pm$  1.8 (300); 58.7  $\pm$  1.3 (400); 56.2  $\pm$  2.2 (500); and 54.4  $\pm$  2.7 (1000),  $n = 6$ ).

The effective shelf life of tissue digest samples containing 2 % Triton X-100 was investigated by measuring the samples immediately after 2 hours, 3 and 14 days after the addition of Triton X-100. Notably, 14 days after adding the Triton X-100, Ti recovery from the  $\text{TiO}_2$  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 days compared to 2 h. In order to ascertain whether samples containing Triton X-100 could also be used to accurately measure other analytes, samples were tested for differences in tissue Ca, Na, Cu, Zn, Mn, 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 (data not shown; ANOVA,  $P > 0.05$ ) indicating that interferences were not a concern.

The results of precision testing of the optimised protocol are presented in Table 3-1 and Table 3-2. Within-sample precision using rainbow trout muscle tissue (triplicate readings from the same sample; Table 3-2) 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

Table 3-1. Instrument and procedure precision in rainbow trout muscle tissue at different TiO<sub>2</sub> NP spike concentrations.

TiO <sub>2</sub> NP spike (µg l <sup>-1</sup> )	Fish and sample number	Ti metal (µg g <sup>-1</sup> )			Mean ± SD	SEM	Within-sample CV (%)
		Triplicate reading					
		1	2	3			
100	Fish 1 sample 1	2.88	2.67	3.09	2.88 ± 0.21	0.12	7.41
100	Fish 1 sample 2	2.13	1.92	2.45	2.17 ± 0.27	0.15	12.38
100	Fish 1 sample 3	2.03	2.13	2.45	2.20 ± 0.22	0.13	10.07
100	Fish 1 sample 4	2.03	2.03	2.45	2.17 ± 0.24	0.14	11.36
	Within-fish Ti metal mean ± SD (SEM)	2.36 ± 0.38 (0.11)					
	Within-fish CV (%)	15.98					
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 ± 2.94 (0.98)					
	Within-fish CV (%)	6.84					

Within sample coefficient of variation (CV (%)) at end of each row is the variation from repeat (×3) measurements of the same tissue sample; CV (%) at the bottom of a column is the total procedural variation from acid digestion of pieces of muscle from the same fish.

Table 3-2. Procedural precision from triplicate digestions of rainbow trout gill tissue at different TiO<sub>2</sub> NP spike concentrations.

TiO <sub>2</sub> NP spike (µg l <sup>-1</sup> )	Fish number	Ti metal (µg g <sup>-1</sup> )					Between-sample CV (%)
		Tissue replicate			Mean ± SD	SEM	
		1	2	3			
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<sub>2</sub> spike concentrations. Between sample coefficient of variation (CV (%)) is the total procedural variation from acid digestion of pieces of gill from the same fish.

higher (15.98 %, Table 3-1) than the within-sample variation indicating a minor 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 3-2). Gills from six different trout were digested following spiking with a serial dilution of TiO<sub>2</sub> NP ( $n = 3$  gill samples per fish, Table 3-2). The variation in the gill tissue was lower than muscle with all coefficients being under 10 %.

#### **3.4.5. Single particle ICP-MS characterisation of TiO<sub>2</sub> NPs**

An investigation into the viability of the use of single particle ICP-MS as a tool in NP characterisation in acid digestion samples of fish tissues produced some interesting results. The raw data from the ICP-MS <sup>47</sup>Ti time scans (Figure 3.3, (A-D)) shows the differences between the top standard concentration (200 µg l<sup>-1</sup>) Ti metal, TiO<sub>2</sub> bulk and TiO<sub>2</sub> NP standards and gill tissue digests spiked with 200 µg l<sup>-1</sup> TiO<sub>2</sub> NPs. Individual peak height gives a proxy of particle size whilst the number of peaks in a given time period gives a proxy of particle concentration. The Ti metal time scan shows a maximum <sup>47</sup>Ti count of 30,030, whereas the bulk and NP TiO<sub>2</sub> and gill scans display much greater peaks (up to 170,170 counts for TiO<sub>2</sub> NPs in gill tissue digests (Figure 3.3 (D))). Calibration graphs for the three series of standards and the gill tissue digest standard series were all generated successfully (Figure 3.3, (E-H)).

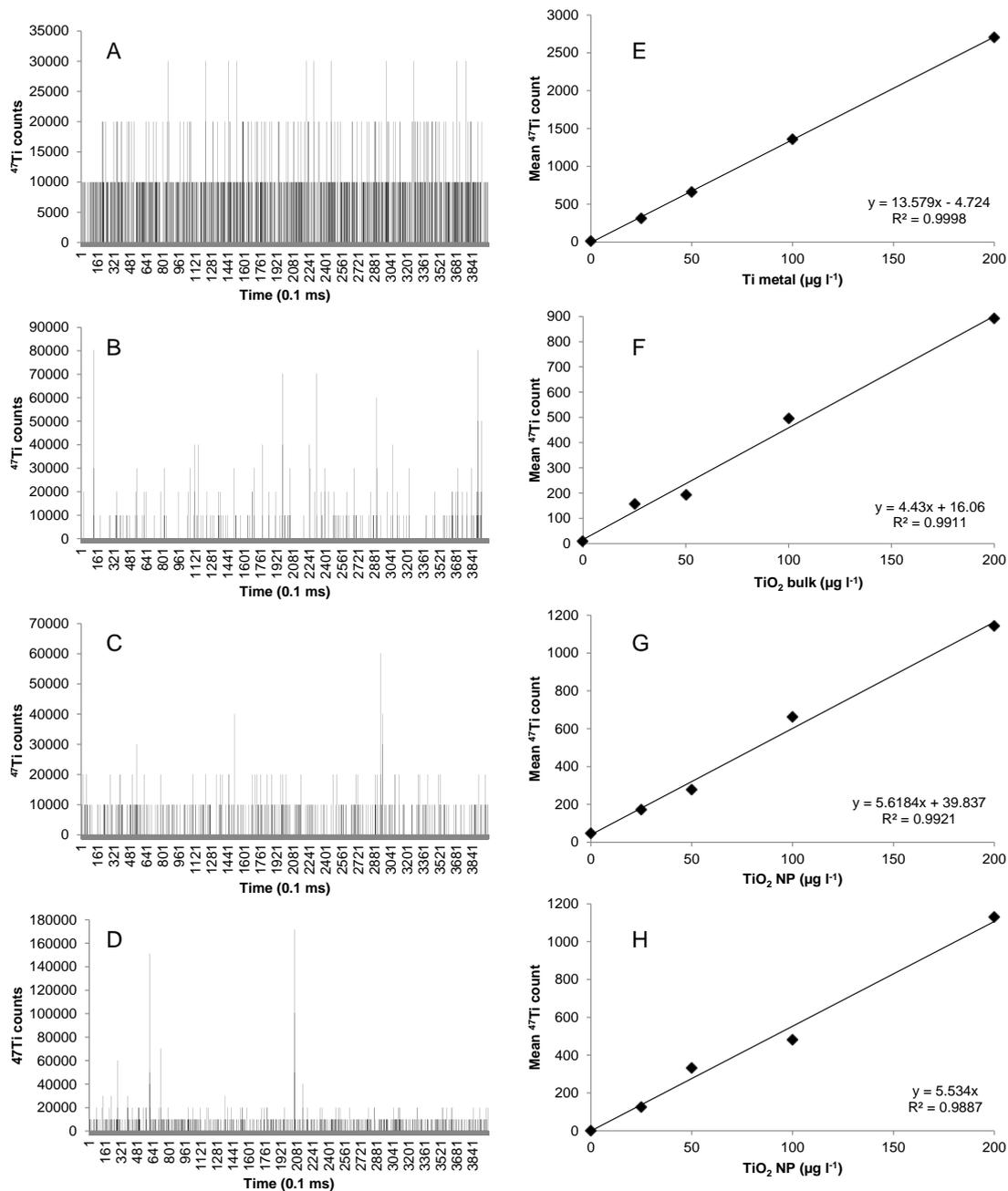


Figure 3.3.  $^{47}\text{Ti}$  time scans of (A) Ti metal, (B)  $\text{TiO}_2$  bulk, (C)  $\text{TiO}_2$  NP standards and (D) gill tissue digests, all at  $200 \mu\text{g l}^{-1}$ .  $^{47}\text{Ti}$  calibration graphs of standards ranging from  $0$ - $200 \mu\text{g l}^{-1}$  for (E) Ti metal, (F)  $\text{TiO}_2$  bulk, (G)  $\text{TiO}_2$  NP and (H)  $\text{TiO}_2$  NP in gill tissue digests. Linear equations and  $R^2$  values are included for each calibration.

Raw data from the time scans were plotted as signal distribution histograms with cumulative percentage plots (examples shown in Figure 3.4). The vast majority of readings in the raw time scans gave  $^{47}\text{Ti}$  counts of  $0$  so these have been excluded from the plots. Histograms vary depending on the source of Ti; Ti metals histograms have a higher percentage of count readings in the lower count bins (up to  $30,030$ ),  $\text{TiO}_2$  bulk

histograms have a higher number and greater spread of count readings further up the count bin scale which extends the tail further right (up to 90,090), the TiO<sub>2</sub> NP standard histogram (Figure 3.4 (A)) has a greater number of counts at higher levels than the Ti metal but fewer than the bulk particle (up to 70, 070) and the TiO<sub>2</sub> NP histogram for gill tissue digests has the highest spread of all with counts up to 170,170.

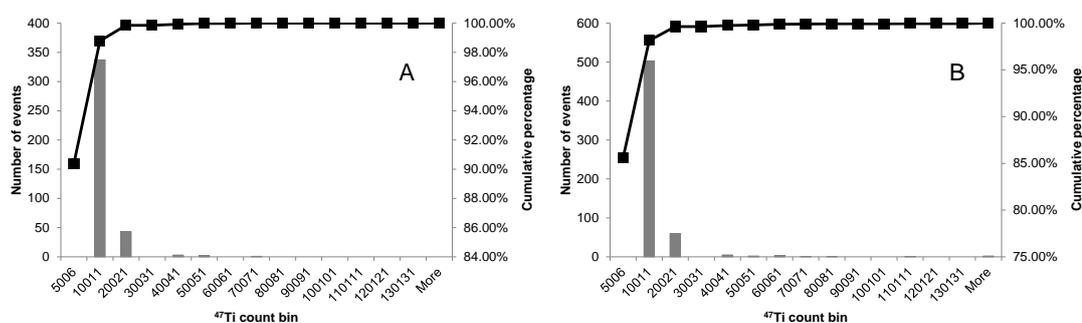


Figure 3.4. <sup>47</sup>Ti signal distribution histograms and cumulative percentage plots excluding the zero count bin from time scans of (A) TiO<sub>2</sub> NP standard at 200 µg l<sup>-1</sup> and (B) gill tissue digest with 200 µg l<sup>-1</sup> TiO<sub>2</sub> NPs.

### 3.5. Discussion

With the expected future growth in the nanotechnology sector and predicted increasing environmental releases of engineered NPs (Boxhall et al., 2007; Owen and Handy, 2007; Ju-Nam and Lead, 2008) 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 needs to be monitored for public safety. Here we have established a method to measure Ti from TiO<sub>2</sub> NPs in the tissues of fish 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 Ti-Me and TiO<sub>2</sub> NPs, with each showing good linear correlation (correlation coefficient *P* values of < 0.00001). In the 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<sub>2</sub> NPs in tissues remains unknown, but total metal in the tissues has been detected in previous studies (Handy et al., 2000; Federici et al., 2007).

Determining analyte recovery (spike recovery) from the protocol is an important part of method validation, and for TiO<sub>2</sub> NPs, the traditional trace element methods for fish tissues gave a poor recovery (e.g., < 35 %, Figure 3.1A). Experiments were therefore conducted using TiO<sub>2</sub> 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<sub>2</sub> NPs) was seen when for acid digestions performed in either plastic or glass vials (Figure 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 that the use of NPs was 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<sub>2</sub> NPs, Federici et al., 2007), stirring (e.g., C<sub>60</sub> fullerenes, Henry et al., 2007) and the use of surfactants (SWCNT, Smith et al., 2007). However, this appeared to have no effect on spike recovery and neither stirring nor sonication of the sample 2 h prior to measuring on ICP-OES improved spike recoveries (Figure 3.1B). Similarly, the use of the surfactant SDS to disperse the NPs in solution did not improve recovery, and sometimes decreased it (liver in Figure 3.1C).

However, the use of another surfactant, 2 % Triton X-100, successfully improved Ti recovery from TiO<sub>2</sub> NP spikes (Figure 3.1C). The concentration of Triton X-100 used was much higher than the critical micelle concentration (information from the

manufacturer's data sheet: CMC; 0.22 to 0.24 mM for Triton X-100 in water) and this would most likely have aided the dispersion of any NPs in the sample. 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., Figure 3.2). Although micelle formation with Triton X-100 can be affected by the viscosity of the solution or presence of organic matter (Mukerjee and Mysels, 1970), 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  $75 \pm 8$ ,  $64 \pm 9$ ,  $25 \pm 8$ ,  $12 \pm 4$ , and  $0.5 \pm 0.02$  % (mean  $\pm$  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, Fuguet et al., 2005). 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.

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., Figure 3.1C and Figure 3.2A). 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 days 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  $100 \mu\text{g l}^{-1}$   $\text{TiO}_2$  NPs (Figure 3.2B). 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  $\text{TiO}_2$  NPs, and our method is a major improvement. Scown et al. (2009) reported a  $\text{TiO}_2$  NP digestion efficiency of 28.82 %, and the new method here more than doubles this recovery. However, in that study 200  $\mu\text{l}$  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 recovery values of 90-105 % in fish tissues. However, the latter study used spiked samples containing unrealistically high Ti concentrations ( $0.80 \text{ g Ti ml}^{-1}$  of final analysis solution). This method and data were published in further studies by the same group, (Zhang et al., 2007; Sun et al., 2009) but our 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. 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  $100 \mu\text{g l}^{-1}$   $\text{TiO}_2$  NPs and produced coefficients of variation (CV) between 7.29 and 12.32 % for rainbow trout muscle, whilst a higher spike concentration ( $1000 \mu\text{g l}^{-1}$   $\text{TiO}_2$  NPs) produced lower CV values of 5.45-7.92 % (Table 1). Between sample variation again showed a concentration effect in muscle tissue with CV of 14.88 and 3.93 % for 100 and  $1000 \mu\text{g l}^{-1}$   $\text{TiO}_2$  NP

spikes respectively. However, no such relationship was seen in trout gills exposed to a serial dilution of spikes (100-1000  $\mu\text{g l}^{-1}$   $\text{TiO}_2$  NPs) with CV values ranging from 2.65-8.37 % (Table 3-2). 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.

In addition to the improved methods for determination of Ti levels in tissues containing  $\text{TiO}_2$  NPs this study has also demonstrated the possibility to use single particle ICP-MS as a method for characterisation of particles in digestion samples. The method and results detailed above are by no means a complete analysis or a finalised protocol for characterisation of  $\text{TiO}_2$  NPs in fish tissues but they are at least a sound foundation for further investigation. Work done on the use of single particle ICP-MS by Laborda et al. (2011) gives insights into how this technique can be applied and the results we obtained echo some of the patterns they observe. Whilst the results shown here did not show clear evidence of particle groups of one, two or three NPs the trend for greater  $^{47}\text{Ti}$  count peaks in digests containing  $\text{TiO}_2$  bulk and NP particles suggests that with some adjustment of dwell time, total run time and particle concentration a good representation of particle characteristics could be achieved. The methods for digestion and characterisation could become a valuable tool for ecotoxicological tests enabling a more complete analysis of the fate and distribution of NPs in fish toxicological studies.

In conclusion, an improved method is established for determining Ti metal from  $\text{TiO}_2$  NPs in whole zebrafish and rainbow trout tissues with potential uses in ecotoxicology and food safety. The new method is not only one of the simplest and cost and time effective methods we are aware of but it also 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. The potential for the use of single particle ICP-MS in the characterisation of NPs and a more complete analysis of tissue digests is also presented.

### **3.6. Acknowledgments**

Dr Andy Fisher is thanked for technical assistance on all ICP work and Ben Eynon is thanked for fish supply and husbandry.

# 4. Sub-lethal effects of titanium dioxide nanoparticles on the physiology and reproduction of zebrafish

## 4.1. Abstract

There are limited data on the sub-lethal physiological effects of titanium dioxide nanoparticles (TiO<sub>2</sub> NPs) in adult fishes, and the consequences for reproduction are also unclear. This study aimed to examine the sub-lethal effects of TiO<sub>2</sub> NPs on the physiology and reproductive health of zebrafish. Fish were exposed to either 0.1 or 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NPs compared to a no added TiO<sub>2</sub> control and a 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub> powder for 14 days. Fish were examined for haematology, electrolyte and trace metal profiles, biochemistry and histology. Then during a recovery period of 21 days a reproduction trial was conducted to investigate the subsequent effects of TiO<sub>2</sub> NP exposure on embryo production and survival. Ti exposure was confirmed in water samples. Whole body acid digests showed a 40-fold increase in Ti concentrations in both the 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP and bulk TiO<sub>2</sub> exposure groups compared to the control, however whether or not TiO<sub>2</sub> was internalised remains unclear. Ti concentrations in all TiO<sub>2</sub> exposed fish returned to control levels by the end of the recovery period. There was no statistically significant change in erythrocyte counts throughout the exposure, but there was a two-fold decline in leukocyte counts in all TiO<sub>2</sub> treatment groups compared to time-matched controls. There were no statistically significant changes in whole body Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup> and Mn concentrations, but there were some small TiO<sub>2</sub> exposure-related declines in whole body Cu and Zn concentrations at the end of the recovery period in adult zebrafish. Na<sup>+</sup>K<sup>+</sup>-ATPase activity did not change significantly

in brain, gill or liver tissues. Total glutathione (GSH) levels in brain, gill and liver tissues were significantly increased due to TiO<sub>2</sub> NP exposure, but did not change in the bulk TiO<sub>2</sub>-exposed group. Histological examination of gill, liver, brain and gonad tissues showed little evidence of treatment-related morphological change. At the end of the exposure adult zebrafish were able to reproduce, but there was a significant reduction in the number of viable embryos produced in the 1.0 mg l<sup>-1</sup> bulk and TiO<sub>2</sub> NP treatments nine days after the exposure period. Overall, this study showed limited toxicity of bulk or nano scale TiO<sub>2</sub> during the exposure apart from some oxidative stress in the latter. Reproduction was affected in both bulk and NP 1.0 mg l<sup>-1</sup> groups with a decline in the number of viable embryos produced.

#### **4.2. Introduction**

Information on the aquatic ecotoxicity of manufactured nanoparticles (NPs) is now emerging (reviews, Handy et al., 2008; Kahru and Dubourguier, 2010; Klaine et al., 2008; Klaine et al., 2012; Moore, 2006; Nowack and Bucheli, 2007), and the data generated so far generally shows that the lethal levels to aquatic species are at high mg l<sup>-1</sup> concentrations, and that sub-lethal effects can occur at around 1 mg l<sup>-1</sup> or less. However information on sublethal effects is still being collected, and the data sets, especially for fishes, remains limited (reviews, nanometals, Shaw and Handy, 2011; body systems-effects on fishes, Handy et al., 2011). While concern over the toxicity of NPs has arisen because of their nanoscale properties (Colvin 2003), there has been no consistent evidence of a relationship between any nanoscale properties of NPs and toxicological responses in fish (Petersen and Henry 2012).

TiO<sub>2</sub> NPs are used in a range of commercial products such as paints and sunscreens (Aitken et al., 2006; Hansen et al., 2008) and as such environmental exposure to NPs is increasing. The bulk form of ordinary TiO<sub>2</sub> powder has low toxicity to mammals (e.g.,

Warheit et al., 1997), and in fishes has been used as an inert marker in nutrition trials (Lied et al., 1982). In contrast, several studies with fine and ultrafine (< 100 nm) TiO<sub>2</sub> have demonstrated respiratory toxicity in rodents (e.g., Bermudez et al., 2004; Oberdorster et al., 1992; Warheit et al., 2005) and in fishes (Federici et al., 2007). A consensus view on the sublethal effects of TiO<sub>2</sub> on the body systems of fishes is still emerging, but toxic effects reported include damage to the gill epithelium and oxidative stress in adult rainbow trout (*Oncorhynchus mykiss*) exposed to up to 1 mg l<sup>-1</sup> TiO<sub>2</sub> for 14 days (Federici et al., 2007). Dietary exposure to 100 mg kg<sup>-1</sup> TiO<sub>2</sub> NPs for 8 weeks had minimal impact on the physiology of juvenile rainbow trout, however there were some biochemical disturbances in the brains of exposed fishes (Ramsden et al., 2009). Conversely, injection studies with TiO<sub>2</sub> NPs in rainbow trout showed little evidence of any toxicology and no loss of filtration rate in the kidneys of trout (Scown et al., 2009). Clearly, there are potentially toxic effects of TiO<sub>2</sub> NPs which are evident in the internal organs in fishes, but whether or not these effects translate into a subsequent decline in reproductive capacity is unclear. Whether or not TiO<sub>2</sub> NPs are absorbed also remains unclear and it is possible that the effects described above are secondary effects of NP exposure rather than direct toxicity caused by the presence of NPs in tissues. Johnston et al. (2010) used CARS imaging to demonstrate that TiO<sub>2</sub> NPs can be internalised over the gill epithelial membrane in fish, albeit at levels most likely below detection limits of other analytical instrumentation.

The effects of dissolved metals on the reproduction of fishes are well known (see Shaw and Handy, 2011 for a comparison with nanometals), and include direct toxicity and metal accumulation in the reproductive organs (Pelgrom et al., 1995; Woltering, 1984), which can result in reduced fecundity (Hatakeyama and Yasuno, 1987). Alternatively, fish may divert energy away from reproduction in favour of tissue repair or the maintenance of somatic growth during metal exposure (e.g., Cu, Handy et al.,

1999). Reproductive behaviours and endocrinology of fishes may also be affected by metal exposure (review, Scott and Sloman, 2004). However, whether or not such mechanisms apply to NPs like TiO<sub>2</sub> is unclear.

The early life stages of fishes do show toxicity during exposure to TiO<sub>2</sub> NPs (Chen et al., 2011a; Hao et al., 2009; Paterson et al., 2011; Zhu et al., 2008), albeit often at high mg l<sup>-1</sup> exposure concentrations. The zebrafish (*Danio rerio*) is an important model organism for reproductive toxicity studies, and in recent work on titania, Xiong et al. (2011) showed that the 96 h LC<sub>50</sub> for TiO<sub>2</sub> NPs in adult zebrafish was 124.5 mg l<sup>-1</sup>, far above the ng-µg l<sup>-1</sup> concentrations of TiO<sub>2</sub> predicted in surface waters (Gottschalk et al., 2009). However, the situation for chronic exposure or sublethal effects in zebrafish is less clear. Recently, Wang et al. (2011) found that a 13 week chronic exposure to 0.1 and 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NPs resulted in a decrease in egg production by adult zebrafish compared to unexposed controls. However it is not known if such effects are related to the nano scale of the material used, direct target organ toxicity in the reproductive system, or more subtle effects associated with the bioenergetics of the animal during exposure.

The main aim of this study was to test the null hypothesis that exposure to TiO<sub>2</sub> NPs would have no effect on fish health or reproductive output. The specific aims of the current study were to overview the health and any sub-lethal effects of TiO<sub>2</sub> NPs compared to bulk TiO<sub>2</sub> on adult zebrafish by assessing key aspects of physiology including osmoregulation, haematology, biochemical defences such as glutathione, and the anatomical integrity of the internal organs, including the gonads. Then, having established the effects of TiO<sub>2</sub> in the adults, breeding trials were conducted in clean water to determine whether or not the fish would show reproductive behaviour and produce gametes that resulted in viable embryos.

### 4.3. Methods

#### 4.3.1. Stock animals and experimental design

Mature adult zebrafish (*Danio rerio*) ( $n = 560$ ) were sourced from J&K Aquatics Ltd in Taunton, Somerset, UK. The fish originated from breeding stock farms in Singapore. Fish were held in re-circulating, filtered, dechlorinated, Plymouth freshwater (see below) for at least two weeks, and fed on tropical fish aquarium flake and brine shrimp (*Artemia salina*) to ensure good health before starting the experiment. Three hundred fish were then randomly allocated into 12 glass exposure tanks (45×25×25 cm), each containing 20 l of clean water to acclimate for 14 days prior to starting the exposures (25 fish/tank, mean weight at the end of the acclimation period  $\pm$  SEM,  $0.6 \pm 0.01$  g,  $n = 300$  fish). During the acclimation period, the water was aerated, filtered and re-circulated (see below for water quality), and the fish were fed twice daily on brine shrimp. The photoperiod remained fixed at a 12 h light: 12 h dark cycle. The acclimation period also enabled some pre-exposure breeding trials to ensure that the batch of fish would breed (see below).

At the start of the exposure phase, the tanks were switched from re-circulation mode to enable a semi-static exposure with 80% water changes every 24 hours to enable control of the exposure concentration. Fish were fed with a single feed of brine shrimp prior to re-dosing the tanks with the appropriate TiO<sub>2</sub> each day. Tanks were randomly allocated to treatments (in triplicate) as no-added TiO<sub>2</sub> controls, 0.1 or 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NPs, and for logistics/ethical reasons a reference at the highest concentration (1 mg l<sup>-1</sup>) for the bulk TiO<sub>2</sub> powder. These concentrations were chosen to enable comparison with our previous sublethal experiments on trout (Federici, et al., 2007).

Dosing was done once the fish had finished feeding to minimise ingestion of TiO<sub>2</sub>. After each water change the tanks were re-dosed with 80 % of the nominated concentrations to bring the final tank concentration back up to the full nominated dose

(e.g. after an 80 % water change of the 1.0 mg l<sup>-1</sup> tanks 20 % of the dose remained in the water so 1.6 ml of the 10 g l<sup>-1</sup> stock was added to bring the total concentration back to 1.0 mg l<sup>-1</sup>). After addition of the dose the water was stirred using a glass rod to ensure even mixing. Control tanks were also stirred with glass rods to standardise stress across all tanks. For water samples Ti concentrations were converted to TiO<sub>2</sub> concentrations using a molecular weight conversion. Water quality was measured daily. Parameters measured included temperature, dissolved oxygen (DO) and pH (Hach HQ40d multi meter), total ammonium, nitrite and nitrate (Hach DR 2800 portable spectrophotometer with test kits LCK 304, LCK 341 and LCK 340 for ammonium, nitrite and nitrate respectively).

Fish were sampled ( $n = 2$  or 3 fish per tank;  $n = 6-9$  fish/treatment) at the start of the exposure (initial fish, day 0), day 7 and day 14 during the exposure phase, as well as during the post-exposure breeding trials (see below) for haematology, whole body ion analysis, histology and biochemistry.

#### **4.3.2. Breeding trials**

Several preliminary trials were conducted to establish a sound reproductive study protocol and to ensure that the test fish were sexually mature. All breeding trials were carried out in clean, aerated water (no TiO<sub>2</sub>, water quality as above) using small glass breeding tanks (35×20×20 cm) containing 10 l of water. The tanks were fitted with a mesh of glass rods at the bottom to ensure that any embryos produced that normally settle at the bottom were separated from the adult fish (to prevent embryo predation). For logistical reasons, the breeding trials needed to be staggered. Breeding trials were carried out twice in the pre-exposure phase and three times post-exposure phase at days 2, 9 and 16 post-exposure (day 35). All remaining fish ( $n = 12$  per tank) at day 35 were sampled where possible for metal analysis and histology as above.

In each breeding trial 9 fish (6 female, 3 male from each treatment) were randomly selected from each of the exposure tanks and placed into a corresponding breeding tank containing clean (control) water at around 5pm. All fish used for reproduction trials were therefore allowed to breed in clean water and any embryos produced were kept free from exposure treatments. Fish were then left to settle overnight and to breed the following morning. At around 2 hours post-sunrise (10am) the fish were relocated from the breeding tanks back into the experimental tanks. Any eggs produced in the breeding tanks were collected and counted as viable (fertilised with a normal appearance), or non-viable (unfertilised and/or dead embryo). A sub-sample of viable embryos was randomly collected, cleaned by carefully moving them into a new dish with clean fresh water, and grown on in petri dishes ( $n = 50$  per dish,  $n = 3$  dishes per treatment if sufficient numbers were achieved). Water in the petri dishes was changed every 24 h and total number of healthy embryos recorded. Any dead embryos were removed at the time of the water change. Embryos were grown on to hatching to ascertain cumulative survival and hatching success.

#### **4.3.3. Titanium dioxide NP stock dispersions and dosing**

The details of the titanium dioxide NP powder used here are given in Chapter 2.

#### **4.3.4. Haematology**

The details of blood collection and analysis are given in Chapter 2.

#### **4.3.5. Whole body ion and trace metal analysis**

The details of the metal analysis protocol followed here are given in Chapter 2.

#### **4.3.6. Biochemistry**

Fish were also collected for biochemistry. Fish were terminally anaesthetised with MS222 (without blood sampling), weight and total length was recorded, then whole animals were snap frozen in liquid nitrogen and stored at -80 °C until required for biochemistry. Subsequently fish were individually dissected (over ice) to harvest the branchial basket, the whole brain and the liver. Tissues were homogenised manually (using a metal rod) in 10 volumes (100 µl for tissue weighing 10 mg) of ice-cold hypotonic buffer (in mmol l<sup>-1</sup>; 100 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-hydroxymethyl-1,3-propanediol)). Homogenates were stored at -80 °C until required. For brain and liver tissue, due to the small volumes of homogenates obtained, it was necessary to pool samples by tank (two samples pooled together resulting in  $n = 3$  samples per treatment) in order to gain sufficient volume to carry out the Na<sup>+</sup>K<sup>+</sup>-ATPase assay. In a few cases it was also necessary to pool gill homogenates by tank, but wherever possible pooling was kept to a minimum. Prior to any pooling of homogenates an aliquot (8 µl) was diluted further (1:100) for protein and total glutathione analyses to yield the highest resolution possible.

Tissue homogenates were analysed (each sample in triplicate wherever possible) for total protein content, Na<sup>+</sup>K<sup>+</sup>-ATPase activity and total glutathione as described in Smith et al. (2007) with a few minor changes. The details of these assays are given in Chapter 2.

#### **4.3.7. Histology**

The details of the histological analysis of tissues are given in Chapter 2.

#### 4.3.8. Statistical analysis

The details of the statistical analyses performed in the present study are given in Chapter 2.

#### 4.4. Results

No fish died during the experiment and no abnormal behaviours (loss of equilibrium, refusal to feed) were observed in any of the treatments. Temperature, DO, pH, ammonium, nitrite and nitrate levels over the course of the exposure were (mean  $\pm$  SEM,  $n = 42$  water samples):  $25.8 \pm 0.08$  °C,  $7.92 \pm 0.01$  mg l<sup>-1</sup> (> 95 % saturation),  $7.45 \pm 0.01$ ,  $0.08 \pm 0.01$  mg l<sup>-1</sup>,  $0.17 \pm 0.02$  mg l<sup>-1</sup> and  $< 22$  mg l<sup>-1</sup> respectively with no tank effects (pooled data).

##### 4.4.1. Confirmation of exposure

Water samples taken from the exposure tanks immediately after dosing confirmed that TiO<sub>2</sub> concentrations were initially met. Measured TiO<sub>2</sub> concentrations in the tanks were (mean  $\pm$  SEM,  $n = 3$  tanks)  $0.04 \pm 0.01$ ,  $0.87 \pm 0.01$ ,  $0.12 \pm 0.01$  and  $1.07 \pm 0.03$  mg l<sup>-1</sup> for control, bulk TiO<sub>2</sub>, 0.1 and 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NPs respectively.

However, as expected, there was some settling of TiO<sub>2</sub> from the water column over the 24 hour period between water changes. The percentage of TiO<sub>2</sub> still in suspension at the end of a 24 hour period relative to initial concentration were (% , data are mean  $\pm$  SEM,  $n = 3$  tanks per treatment at 24 h after dosing):  $68.7 \pm 1.8$ ,  $71.9 \pm 3.2$  and  $49.3 \pm 2.6$  for 0.1 mg l<sup>-1</sup> TiO<sub>2</sub> NP, 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP and 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub> tanks respectively.

Ti exposure was also confirmed by measuring the whole body (including the gastrointestinal (GI) tract) Ti levels (Figure 4.1). All the TiO<sub>2</sub> treatment groups showed clear elevations in whole body Ti compared to the no-added TiO<sub>2</sub> control. There was

also a transient concentration-effect at day 7 for the TiO<sub>2</sub> NP treatments, but this difference was lost by the end of the exposure period. No material-type effects were evident. At three weeks post-exposure, all the fish in all treatments showed a return of Ti concentration back to the pre-exposure background level.

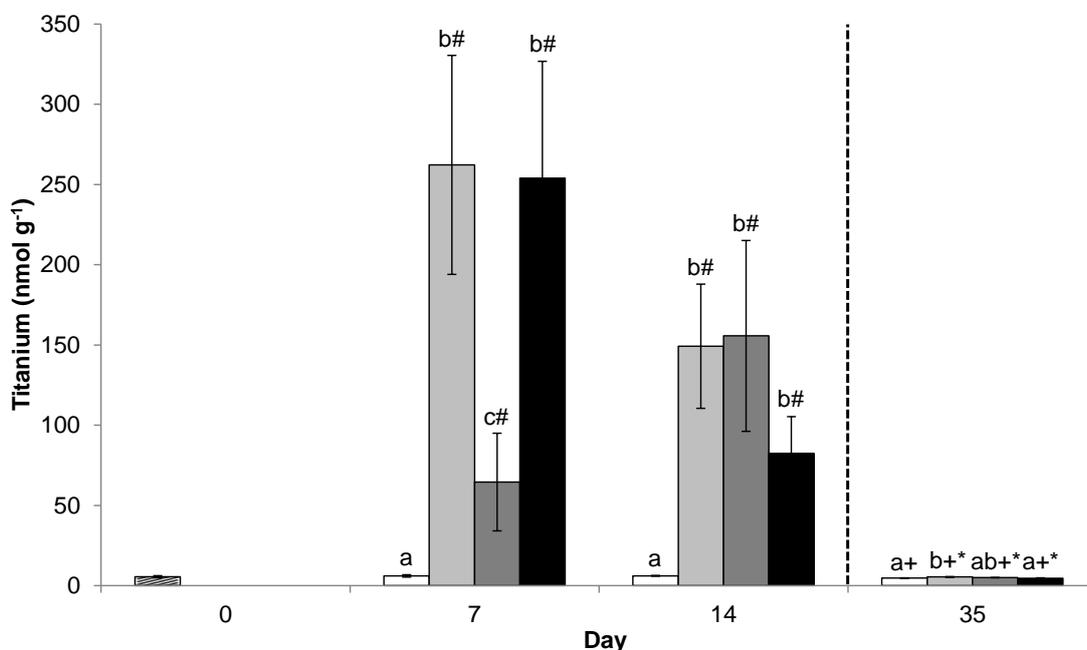


Figure 4.1. Total titanium concentration in whole zebrafish digests. Hatched bar at day 0 is initial fish, clear bars are control fish, light grey bars are 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub> control fish, dark grey bars are 0.1 mg l<sup>-1</sup> TiO<sub>2</sub> NP fish and black bars are 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP fish. Data are mean ± SEM nmol Ti g<sup>-1</sup> dry weight, *n* = 6 fish per treatment. Dashed line indicates end of exposure (start of recovery period). # indicates significant difference to initial fish (ANOVA or Kruskal-Wallis, *P* < 0.05); \* indicates significant difference to day 7 within treatment (ANOVA or Kruskal-Wallis, *P* < 0.05); + indicates significant difference to previous time point within treatment (ANOVA or Kruskal-Wallis, *P* < 0.05); different letters within a time point indicate significant differences between treatments (ANOVA or Kruskal-Wallis, *P* < 0.05).

#### 4.4.2. Haematology

Erythrocyte counts from whole blood samples at days 7 and 14 showed no obvious treatment effects with cell counts at day 14 of  $3.64 \pm 0.31$ ,  $3.82 \pm 0.46$ ,  $3.67 \pm 0.35$  and  $3.36 \pm 0.33$  (cells  $\times 10^6 \text{ mm}^{-3}$ , data are mean ± SEM, *n* = 6 fish per treatment) for control, bulk TiO<sub>2</sub>, 0.1 mg l<sup>-1</sup> and 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP respectively (ANOVA, *P* > 0.05). Total white blood cell counts at day 7 showed no significant differences but at day 14

were significantly lower for all TiO<sub>2</sub> treatments compared to the control (ANOVA,  $P < 0.05$ ) with counts of  $19.83 \pm 1.04$ ,  $9.05 \pm 1.47$ ,  $11.92 \pm 1.78$  and  $11.83 \pm 0.99$  (cells  $\times 10^3 \text{ mm}^{-3}$ , mean  $\pm$  SEM,  $n = 6$  fish per treatment) for control, bulk TiO<sub>2</sub>, 0.1 mg l<sup>-1</sup> and 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP respectively. There were no significant statistical differences between any of the TiO<sub>2</sub> treatments.

#### **4.4.3. Whole body ions and trace metals**

Metal analysis of whole zebrafish acid digests showed no discernible treatment effects for any of the other six metals analysed (Figure 4.2). There were some minor transient changes in potassium and sodium however these changes were not treatment-related. Total zinc content was lower in the bulk 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> and 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP groups at day 14 than the control and 0.1 mg l<sup>-1</sup> TiO<sub>2</sub> NP groups, but this change was only significant for the bulk group. Total copper content in whole zebrafish samples was significantly lower in all TiO<sub>2</sub> treatment groups compared to control at the end of the recovery period, but again there was no obvious treatment effect, nor were the copper levels significantly lower than those of the initial fish.

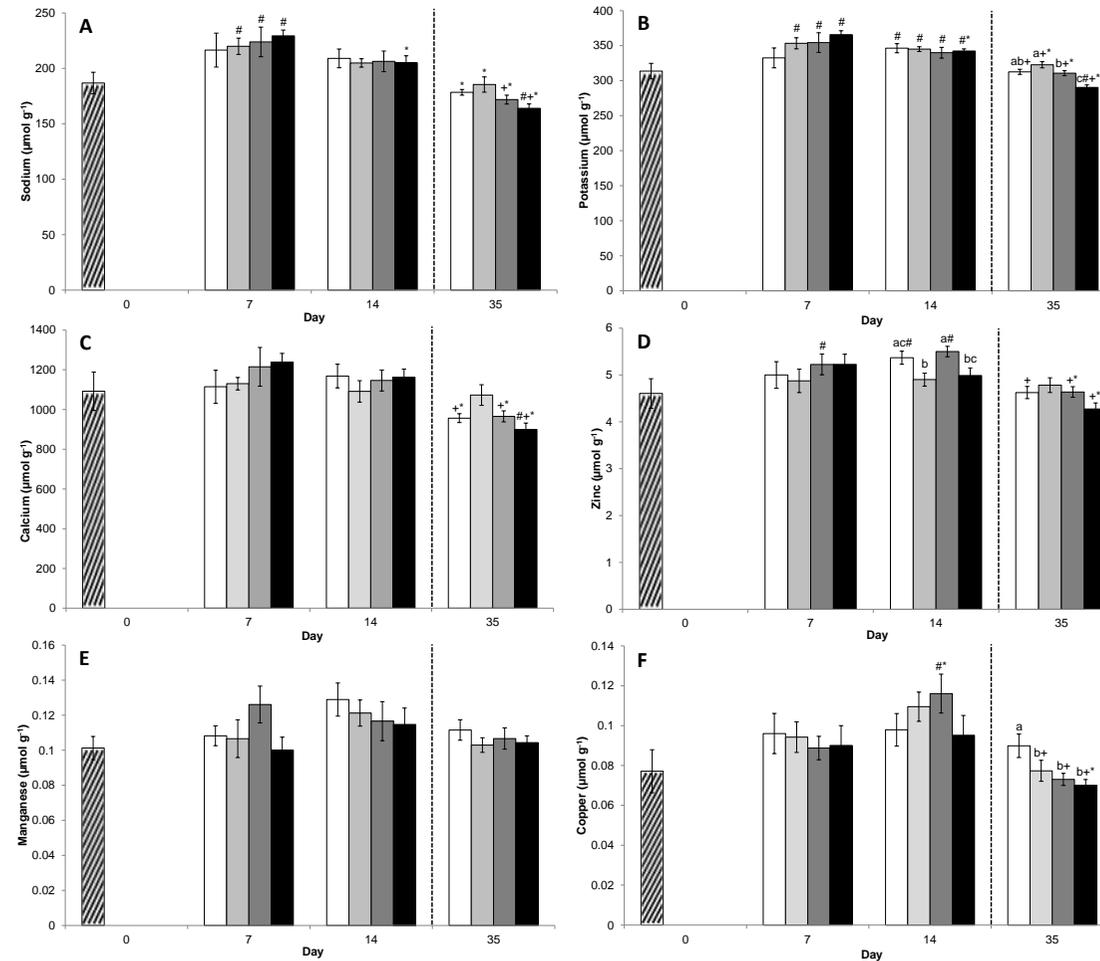


Figure 4.2. Total metal content of whole zebrafish for (A) sodium, (B) potassium, (C) calcium, (D) zinc, (E) manganese and (F) copper. Hatched bar at day 0 is initial fish, clear bars are control fish, light grey bars are 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub> control fish, dark grey bars are 0.1 mg l<sup>-1</sup> TiO<sub>2</sub> NP fish and black bars are 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP fish. Data are mean ± SEM μmol metal g<sup>-1</sup> dry weight, *n* = 6 fish per treatment. Dashed line indicates end of exposure (start of recovery period). # indicates significant difference to initial fish (ANOVA or Kruskal-Wallis, *P* < 0.05); \* indicates significant difference to day 7 within treatment (ANOVA or Kruskal-Wallis, *P* < 0.05); + indicates significant difference to previous time point within treatment (ANOVA or Kruskal-Wallis, *P* < 0.05); different letters within a time point indicate significant differences between treatments (ANOVA or Kruskal-Wallis, *P* < 0.05).

#### 4.4.4. Biochemistry

Na<sup>+</sup>K<sup>+</sup>-ATPase activities were measured in the brain, gill and liver tissues of zebrafish. There were no statistical differences in any of the tissues (ANOVA,  $P > 0.05$ ). Na<sup>+</sup>K<sup>+</sup>-ATPase activities at the end of the exposure period (day 14) are given in Table 1-1. Na<sup>+</sup>K<sup>+</sup>-ATPase activities throughout the exposure ranged from 3.05-4.73, 0.51-1.91 and 0.66-1.09  $\mu\text{mol Pi mg protein}^{-1} \text{ h}^{-1}$  for brain, gill and liver tissues respectively.

Table 4-1. Na<sup>+</sup>K<sup>+</sup>-ATPase activities at the end of the exposure period (day 14) in brain, gill and liver tissues of zebrafish.

Treatment	Na <sup>+</sup> K <sup>+</sup> -ATPase activity at day 14 ( $\mu\text{mol Pi mg protein}^{-1} \text{ h}^{-1}$ )		
	Tissue		
	Brain	Gill	Liver
Control	3.73 $\pm$ 0.15	1.91 $\pm$ 0.53	1.09 $\pm$ 0.13
1.0 mg l <sup>-1</sup> bulk TiO <sub>2</sub>	3.05 $\pm$ 0.03	1.84 $\pm$ 0.60	1.00 $\pm$ 0.00
0.1 mg l <sup>-1</sup> TiO <sub>2</sub> NP	4.23 $\pm$ 0.93	0.51 $\pm$ 0.41	0.66 $\pm$ 0.32
1.0 mg l <sup>-1</sup> TiO <sub>2</sub> NP	4.73 $\pm$ 0.93	1.36 $\pm$ 0.70	0.66 $\pm$ 0.29

Total glutathione (GSH) levels were measured in the brain, gill and liver tissues of zebrafish. NP treatment effects were apparent in brain, gill and liver tissues as both 0.1 and 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP groups had significantly higher GSH levels than the time-matched control and bulk TiO<sub>2</sub> groups (ANOVA,  $P < 0.05$ , Figure 4.3). These differences were due to the material type as the bulk TiO<sub>2</sub> control treatment did not show the same elevation in total GSH, compared to the controls, that the NP exposed treatments did.

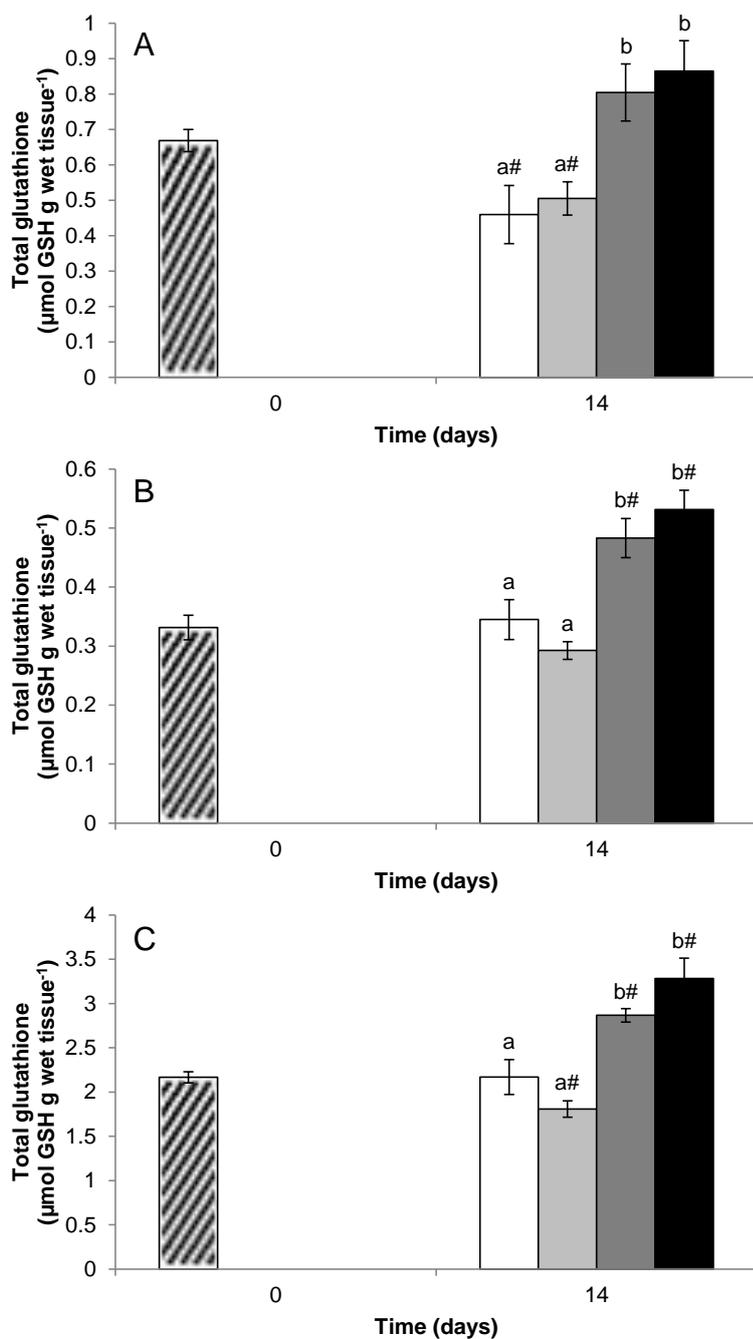


Figure 4.3. Total glutathione levels in zebrafish (A) brain, (B) gill and (C) liver tissues. Hatched bar at day 0 is initial fish, clear bars are control fish, light grey bars are 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub> control fish, dark grey bars are 0.1 mg l<sup>-1</sup> TiO<sub>2</sub> NP fish and black bars are 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP fish. Data are mean ± SEM μmol GSH g wet tissue<sup>-1</sup>,  $n \leq 6$  fish per treatment. # indicates significant difference to initial fish ( $t$ -test,  $P < 0.05$ ); different letters within a time point indicate significant differences between treatments (ANOVA or Kruskal-Wallis,  $P < 0.05$ ).

#### 4.4.5. Histology

Brain, gill, liver and gonad tissues from adult zebrafish were examined for treatment-related histological changes at the end of the exposure period, but no effects were observed in any tissues examined. The gill morphology was normal with no treatment-dependent evidence of oedema, epithelial lifting, or aneurisms on the secondary lamellae. A quantitative analysis of the gills confirmed these observations. For example, the percentage of club tips on gill secondary lamellae was calculated (data are at day 14, mean  $\pm$  SEM % club tips,  $n \leq 6$  fish per treatment):  $0.7 \pm 0.5$ ,  $2.3 \pm 0.9$ ,  $2.0 \pm 1.6$  and  $3.9 \pm 3.9$  % in control, bulk  $1.0 \text{ mg l}^{-1} \text{ TiO}_2$ ,  $0.1 \text{ mg l}^{-1} \text{ TiO}_2 \text{ NP}$  and  $1.0 \text{ mg l}^{-1} \text{ TiO}_2 \text{ NP}$  groups respectively (no significant difference between treatments, ANOVA,  $P > 0.05$ ). The liver tissue showed no overt signs of pathology, with no significant changes in the proportion of sinusoid space relative to the parenchyma. Mean percentage of sinusoid space relative to the parenchyma at day 14 was (mean  $\pm$  SEM,  $n \leq 6$  fish per treatment):  $19.7 \pm 3.9$ ,  $12.3 \pm 1.6$ ,  $17.2 \pm 2.7$  and  $11.00 \pm 1.1$  in control, bulk  $1.0 \text{ mg l}^{-1} \text{ TiO}_2$ ,  $0.1 \text{ mg l}^{-1} \text{ TiO}_2 \text{ NP}$  and  $1.0 \text{ mg l}^{-1} \text{ TiO}_2 \text{ NP}$  groups respectively. There was no evidence of abnormal lipidosis or clear treatment-related changes in apparent glycogen storage in the livers of adult zebrafish at the end of the exposure phase.

The architecture of the brain was normal for all treatment groups with a very low background level of injuries. All treatment groups displayed a rare background incidence of necrotic nerve cell bodies in the brain, but there was no treatment-related effect. The structure of gonadal tissue was also normal in all treatment groups with no occurrence of ova-testis, no evidence of reactive hyperplasia, or atrophy of ova or testis. Female gonad showed a normal spread of oocyte developmental stages in all treatments. Mean follicular size at day 14 was (mean  $\pm$  SEM,  $n \leq 4$  females per treatment):  $101 \pm 4.8$ ,  $131.5 \pm 12.5$ ,  $135.4 \pm 20.4$  and  $173.7 \pm 36.1 \text{ }\mu\text{m}$  in the control, bulk  $1.0 \text{ mg l}^{-1} \text{ TiO}_2$ ,  $0.1 \text{ mg l}^{-1} \text{ TiO}_2 \text{ NP}$  and  $1.0 \text{ mg l}^{-1} \text{ TiO}_2 \text{ NP}$  groups respectively. Only the  $1.0 \text{ mg l}^{-1}$

TiO<sub>2</sub> NP group showed a significantly higher value than the time-matched controls (ANOVA,  $P < 0.05$ ), and there was no material-type effect.

#### 4.4.6. Breeding trials

There was temporal variation in breeding success of the zebrafish, despite being from the same stock of animals. The pre-exposure trials were particularly variable with the mean number of viable embryos (< 2 hours post-fertilisation, hpf) produced being 122 with a standard deviation of 209. Each of the post-exposure trials for each treatment was compared to this pre-exposure mean value, and consequently, no significant differences were observed. However, when the individual breeding trials were analysed for treatment differences there was a significant treatment effect 9 days post-exposure (data are mean  $\pm$  SEM viable embryos,  $n \leq 3$  breeding groups per treatment):  $203 \pm 38$ ,  $27 \pm 27$ ,  $264 \pm 71$  and  $17 \pm 17$  in the control, bulk  $1.0 \text{ mg l}^{-1}$  TiO<sub>2</sub>,  $0.1 \text{ mg l}^{-1}$  TiO<sub>2</sub> NP and  $1.0 \text{ mg l}^{-1}$  TiO<sub>2</sub> NP groups respectively. The mean number of viable embryos produced by the bulk  $1.0 \text{ mg l}^{-1}$  TiO<sub>2</sub> and  $1.0 \text{ mg l}^{-1}$  TiO<sub>2</sub> NP groups were significantly lower than the control and  $0.1 \text{ mg l}^{-1}$  TiO<sub>2</sub> NP groups (ANOVA,  $P < 0.05$ ). By the time of the last breeding trial at 16 days post-exposure there were no longer any significant differences between groups.

Cumulative plots of the total number of viable embryos produced in all tanks of the exposure groups showed a similar trend to that of the mean values (Figure 4.4). The control and  $0.1 \text{ mg l}^{-1}$  TiO<sub>2</sub> NP groups show a steady cumulative production of viable embryos whereas the bulk  $1.0 \text{ mg l}^{-1}$  TiO<sub>2</sub> and  $1.0 \text{ mg l}^{-1}$  TiO<sub>2</sub> NP groups showed a markedly lower production of viable embryos. The bulk  $1.0 \text{ mg l}^{-1}$  TiO<sub>2</sub> and  $1.0 \text{ mg l}^{-1}$  TiO<sub>2</sub> NP groups produced significantly fewer eggs (both viable and non-viable) than the control and  $0.1 \text{ mg l}^{-1}$  TiO<sub>2</sub> NP groups, but there was no significant difference between the treatment groups in the number of viable embryos as a percentage of the total

number of eggs produced at day 9 post-exposure (ANOVA,  $P > 0.05$ ). The number of viable embryos as a percentage of the total number of eggs produced at day 9 post-exposure were (data are mean  $\pm$  SEM where possible,  $n \leq 3$  breeding groups per treatment):  $97.9 \pm 1.2$ ,  $97.6$ ,  $98.2 \pm 0.8$  and  $92.9$  in the control, bulk  $1.0 \text{ mg l}^{-1} \text{ TiO}_2$ ,  $0.1 \text{ mg l}^{-1} \text{ TiO}_2$  NP and  $1.0 \text{ mg l}^{-1} \text{ TiO}_2$  NP groups respectively. There was also no significant difference in the survival of viable embryos to hatching between treatment groups (data not shown).

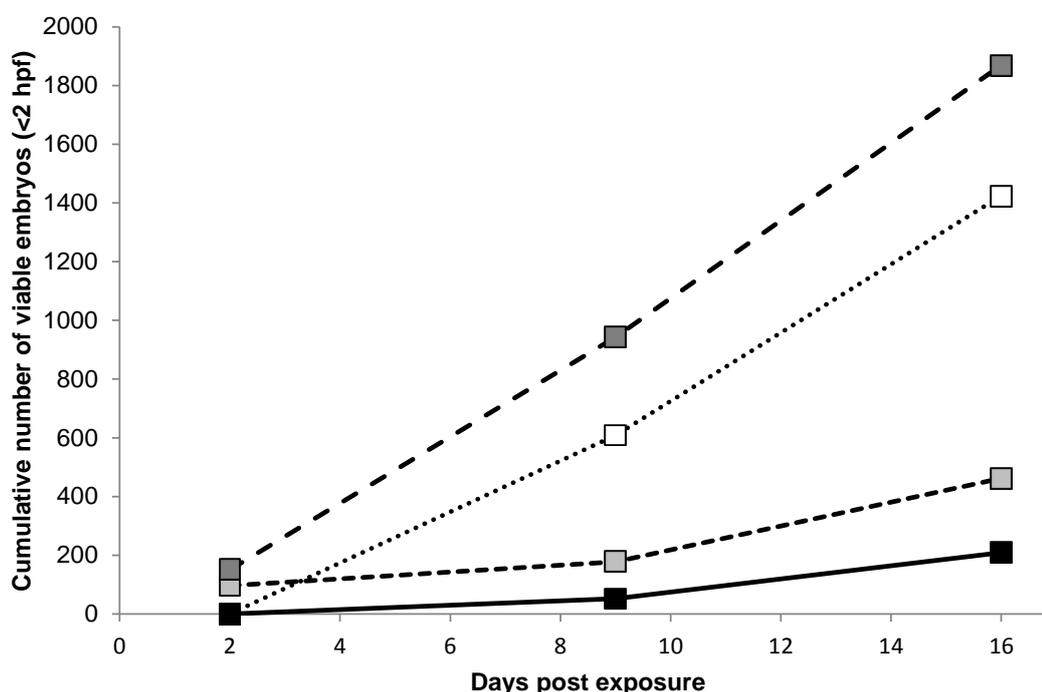


Figure 4.4. Cumulative number of viable embryos (< 2 hpf) produced by control (clear squares with dotted line),  $1.0 \text{ mg l}^{-1}$  bulk  $\text{TiO}_2$  (light grey squares with close-dashed line),  $0.1 \text{ mg l}^{-1}$   $\text{TiO}_2$  NPs (dark grey squares with dashed line) and  $1.0 \text{ mg l}^{-1}$   $\text{TiO}_2$  NPs (black squares with solid line) treatment groups of zebrafish after the exposure period.

## 4.5. Discussion

### 4.5.1. Confirming exposure to $\text{TiO}_2$

There were no mortalities in the present study and no abnormal behaviours (loss of equilibrium, refusal to feed) were observed in any of the treatments throughout both the exposure and recovery phases. The exposure was confirmed by measuring the total Ti

metal concentrations in the tanks after dosing, and over the course of 24 h the inevitable settling behaviour of the material caused this to decline to around 70 % of the initial concentration for TiO<sub>2</sub> NPs and 50 % of the initial concentration in the water column for the bulk material. The faster settling of the larger bulk material is readily explained by colloid theory (see Handy et al., 2008c). In this study we used a semi-static exposure regime to renew the test water every 24 h. More frequent water changes (e.g., every 12 h in trout studies, Federici et al., 2007) can give Ti concentrations in the tank water closer to the initial values, but at the same time this will also disturb fish behaviours, and the objective here was to ensure the fish would also be able to breed. The 24 h water change was chosen as the best compromise for this dilemma based on our pilot studies, and was also used by Wang et al. (2011).

Ti exposure was also confirmed by measured Ti concentrations in the whole bodies of the zebrafish (Figure 4.1) with Ti elevations in all treatments compared to the no-added TiO<sub>2</sub> controls. However, there was no overall material-type effect evident in the whole body accumulation with nano and bulk forms showing similar Ti levels by the end of the experiment. Notably, by the end of the recovery period the Ti concentration in both of the TiO<sub>2</sub> NP groups was back to the level found in the time matched control group. The Ti levels in the carcass from the bulk TiO<sub>2</sub> were also close to the control values. The animals were rinsed of any surface water, and although the gut was included in the whole body analysis, there was no evidence of the congestion of TiO<sub>2</sub> in the gut (observed in *Daphnia*, Zhu et al., 2010) or evidence of quantities of the material on the gut mucosa from the histological examination of exposed zebrafish. Despite these measures it remains possible that TiO<sub>2</sub> NPs were adhered to the surface layers of the fish. Drinking of exposure water and adhesion of particles to mucus within the gill baskets are other possible scenarios for Ti elevation in the TiO<sub>2</sub> exposed fish. Despite these more likely causes for the presence of Ti in whole body digests it remains possible

that the observed decrease in Ti body burden after the exposure may be from the internal tissues of the animal rather than the body surface or gut lumen. However, in the absence of a verified routine analytical method to determine NPs in complex samples (von der Kammer et al., 2012) such as acid digests of whole fish, it remains unknown whether or not the Ti was present in the tissues as particles.

#### **4.5.2. Haematology, electrolytes and trace metals**

There were no major disturbances to haematology with erythrocyte counts remaining unchanged for the duration of the exposure period, but there was a statistically significant decrease in total leukocyte counts for all three TiO<sub>2</sub> exposure groups compared to the time-matched controls. However, the white cell counts remained within the normal range for zebrafish (Velasco-Santamaría et al., 2011), and this treatment-effect is therefore of limited functional importance. This is also consistent with our previous reports on trout which show normal haematology over 2 weeks exposure to TiO<sub>2</sub> NPs (Federici et al., 2007).

Total body electrolyte and trace metal concentrations did not show any major disturbances over the duration of the exposure (Figure 4.2). There were no significant treatment-related changes in Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup> concentrations which indicates good general health and, together with the absence of any treatment-related change in Na<sup>+</sup>K<sup>+</sup>-ATPase activity, suggests that the fish were able to maintain good osmoregulatory function. This was also supported by the gill histology where there was no evidence of acute oedema or epithelial lifting, or reactive hyperplasia in the gills from any of the treatments. Notably, for exactly the same material and exposure used here, Federici et al. (2007) noted some significant oedema and epithelial damage in the trout gill. This suggests that zebrafish are more tolerant of TiO<sub>2</sub> exposure compared to trout. Chen et al. (2011b) also examined the effects of TiO<sub>2</sub> NPs in a waterborne exposure to zebrafish

and found only minor gill injury with concentrations up to 5 mg l<sup>-1</sup> TiO<sub>2</sub> NPs for up to six months.

However, some trace elements have specific functions in the reproductive systems of vertebrate animals. Zinc plays a key role in the production of egg proteins and its absence can be a good indicator of reproductive stress (Thompson et al., 2003). At the end of the exposure period there was a small but statistically significant decrease in Zn concentrations in the 1.0 mg l<sup>-1</sup> bulk and NP TiO<sub>2</sub> treatments compared to the time-matched control (Figure 4.2). Some transient tissue Zn depletion in the muscle and gill was also observed in trout exposed to the same TiO<sub>2</sub> NPs over 2 weeks (Federici et al., 2007). There were no treatment-related changes in whole body Mn or Cu during the exposure (Figure 4.2). However, in the recovery phase there was a treatment-dependent whole body Cu and K<sup>+</sup> depletion. The reason for this effect is unclear, but changes in trace metal homeostasis following metal exposure have been noted previously (e.g., dietary Cu in tilapia, Shaw and Handy, 2006).

#### **4.5.3. Oxidative stress**

Nano-scale TiO<sub>2</sub> is known to produce reactive oxygen species (ROS), especially the hydroxyl radical ( $\cdot$ OH) (Reeves et al., 2008; Xiong et al., 2011), and there are several reports of changes in oxidative stress parameters in fishes with TiO<sub>2</sub> NPs (trout, Federici et al., 2007; Reeves et al., 2008; Xiong et al., 2011). The induction of glutathione is a well-known defence against oxidative stress, and in this study there were statistically significant increases in GSH in the gill, liver and brain in association with exposure to TiO<sub>2</sub> NPs (Figure 4.3). However, this was not observed in the bulk TiO<sub>2</sub> treatment, and is consistent with historic view that bulk titania powders are not very reactive. Due to the small size of the fish only whole body Ti was measured, so it was not possible to determine if the tissue level changes in GSH were related directly to Ti in those organs, but indirect oxidative stress is well known given the high mobility of

the hydroxyl radical and, more likely, hydrogen peroxide (Halliwell and Gutteridge, 1992). An alternative hypothesis is that accumulation of TiO<sub>2</sub> on external surfaces (e.g., skin and gills) caused mild respiratory distress that led to the minor changes in GSH observed in the present study. Regardless, the induction of GSH is generally a protective effect, and this is consistent with a lack of effects on the Na<sup>+</sup>K<sup>+</sup>-ATPase which is sensitive to oxidative damage, as well as a lack of any electrolyte depletion from the tissues.

The absence of internal oxidative stress was also reflected in the normal liver and brain histology. The livers of TiO<sub>2</sub> NP-exposed fish showed no significant change in the proportions of sinusoid space, and there was no evidence of any treatment-dependent lipidosis that is often a feature of oxidative stress in the liver (see Federici et al., 2007). Interestingly, the liver of zebrafish may be less sensitive than that of rainbow trout which showed some fatty change in an identical exposure situation to the present study (Federici et al., 2007). The brain is well known for its sensitivity to ROS and some nanomaterials can cause brain pathology (see Handy et al., 2011). However, the absence of vacuole formation and other injuries in the brain of zebrafish also supports the notion of limited oxidative stress to the internal tissues in this study. Chen et al. (2011b) also reported no obvious histological changes in the brains of zebrafish exposed to TiO<sub>2</sub> NPs at concentrations up to 7 mg l<sup>-1</sup>.

#### **4.5.4. Reproductive effects of TiO<sub>2</sub> exposure**

There have been a number of studies published which have examined the toxicity of NPs to adult fish or to embryos (review in Handy et al., 2011); however there are very few which examine the effects of NP exposure on reproduction. The emerging data suggest that intact embryos are reasonably tolerant of metal NP exposure (see discussion in Handy et al., 2011 and references therein) and the data in this study (Figure 4.4) confirms that exposure of the adult fish can have a negative effect on

fecundity, without any significant changes in the condition factor of exposed fish. The cause of reduced fecundity was not a general decline in the health of the adult fish (normal haematology, generally no adverse pathology, etc., above). However, some specific trace element disturbances may have played a role. For example, zinc plays a key role in the production of egg proteins (Thompson et al., 2003) and the observed Zn depletion might contribute to decreased gamete quality. Wang et al. (2011) also recently found that 0.1 and 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP caused a decline in the number of eggs produced and in the survival rate of zebrafish embryos compared to unexposed controls (no bulk treatment in the experimental design). In the present study, for the nanoscale material, the deleterious effect on the cumulative number of viable embryos was mainly in the 1.0 mg l<sup>-1</sup> treatment, suggesting a threshold somewhere between 0.1-1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NPs for this effect in a 2 week exposure of the adults. Notably, the bulk material also caused a similar reduction in embryo viability to 1 mg l<sup>-1</sup> TiO<sub>2</sub> NPs, and identifies a latent hazard of ordinary TiO<sub>2</sub> powders to fishes.

#### **4.5.5. Environmental implications for fish populations**

One of the key parameters of fish population dynamics is reproductive success and without sufficient production of embryos the chances of long term population survival are diminished (Kooijman et al., 1999). The decrease in the cumulative number of viable embryos during TiO<sub>2</sub> exposure (Figure 4.4) may therefore be a concern for wild fish populations. Furthermore, this effect on viable embryo production was also evident for the bulk TiO<sub>2</sub> material (Figure 4.4). It would therefore be prudent to re-visit environmental risk assessments for bulk TiO<sub>2</sub> and reconsider the hazard with respect to fish populations. The changes in fecundity observed here, in the absence of overt disturbances of the physiological systems that are important for individual survival (normal osmoregulation, haematology, etc.), suggests that (like other chemicals) reproductive end points may be sensitive tools for assessing the toxicity of TiO<sub>2</sub> NPs.

The latent effect of NP exposure in adults on the next generation of fishes has not been previously documented, and even for traditional chemicals such as pesticides this phenomena has rarely been reported in zebrafish (see discussion in Velasco-Santamaria et al. 2011). The possibility of latent effects not only on the exposed population but on the health and survival of the subsequent generations should be considered in environmental risk assessments for TiO<sub>2</sub>. Simply assessing the toxic effects on the primary test organism may not be sufficient to give adequate protection against the possible effects of a compound on the reproductive success and survival of not only the F1 generation but future generations as well. In addition, the data here and in Chen et al. (2011b) suggest that adult zebrafish are more tolerant of TiO<sub>2</sub> exposures than rainbow trout (Federici et al., 2007). This implies that threshold values for effects on zebrafish reproduction may not be protective for salmonid species, and an uncertainty factor for species differences is worth including in any risk calculations for reproductive effects.

#### **4.5.6. Conclusions**

In conclusion, the present study has demonstrated that zebrafish can maintain their physiological health in terms of osmoregulatory status, with limited oxidative stress and organ pathology during 1 mg l<sup>-1</sup> exposures to either nanoscale or bulk TiO<sub>2</sub> for 14 days. The animals remained capable of reproductive behaviours, and could produce viable embryos following exposure. However, a latent effect on the cumulative production of viable embryos is observed for both types of material at 1 mg l<sup>-1</sup> TiO<sub>2</sub> exposure concentrations, indicating that the bulk material may also be hazardous and should be considered in environmental risk assessments. Further research is needed to determine whether the latent effect on embryo production is a result of subtle changes in the partitioning of the energy budget of the adults, or inadvertent carryover of Ti into the gametes with a consequent developmental toxicity to the embryos.

#### **4.6. Acknowledgments**

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# 5. Sub-lethal effects of single walled carbon nanotubes on the physiology and reproduction of zebrafish

## 5.1. Abstract

There is a lack of knowledge on the potential toxicity of carbon nanotubes (CNTs) to fish. The present study aimed to investigate the toxicity of single walled CNTs, along with relevant controls, on zebrafish using a holistic physiological approach. End points include haematology, trace metals, biochemistry, histology and reproduction. Fish were exposed for 21 days to: control (no added NPs or dispersion vehicles), carbon black NPs (0.25 mg l<sup>-1</sup> with 0.25 mg l<sup>-1</sup> SDS dispersion vehicle), sodium dodecyl sulphate (SDS) control (0.25 mg l<sup>-1</sup>), or SWCNTs (0.25 mg l<sup>-1</sup> with 0.25 mg l<sup>-1</sup> SDS). Reproductive trials were carried out before and during the exposure period to assess reproductive health. There was no significant change in either erythrocyte or leukocyte counts (ANOVA,  $P > 0.05$ ). Bulk electrolyte and trace metal analysis showed no evidence of a SWCNT effect and there was no sign of osmoregulatory dysfunction (Na<sup>+</sup>K<sup>+</sup>-ATPase activity not significantly altered (ANOVA,  $P > 0.05$ )). There was no evidence of oxidative stress in any of the treatment groups with total glutathione (GSH) levels remaining within normal limits for the duration of the exposure period. Histological examination showed no evidence of tissue damage as a result of SWCNT exposure. The number of viable embryos produced by SWCNT-exposed fish declined throughout the exposure period but this effect was not significant (Kruskal-Wallis,  $P > 0.05$ ). We

conclude that whilst the majority of end points examined showed little evidence of toxicity, exposure to SWCNTs may affect the reproductive health of zebrafish.

## 5.2. Introduction

Carbon nanotubes (CNTs) can be single-walled (SWCNT) or multi-walled (MWCNT) and their usage is steadily increasing due to their very high strength and unique electrical properties (high conductivity, low resistance). As a result of these highly desirable properties CNTs have great potential in a variety of fields including electronics, computing and aerospace, as well as possible uses in the medical field such as drug delivery mechanisms (Lam et al., 2004; Donaldson et al., 2006). SWCNTs are also used in water purification and filtration (Aitken et al., 2006). SWCNTs are typically 1-3 nm in diameter depending on the metal catalyst used in their manufacture, and can polymerise up to several micrometres in length (Aitken et al., 2006; Donaldson et al., 2006; Shvedova et al., 2007; Smith et al., 2007). These individual tubes then show a tendency to aggregate into bundles or fibres as a result of attractive van der Waals' forces, with the final product being an asbestos-like fibre (Donaldson et al., 2006). This aggregation behaviour in carbon based NPs has generated some debate involving the use of dispersion agents, particularly in aqueous solutions of carbon NPs. Henry et al. (2007) suggested that the toxicity observed in studies examining the toxic effects of C<sub>60</sub> fullerenes may be actually due to the dispersion vehicle tetrahydrofuran (THF) and its degradation products rather than the C<sub>60</sub> fullerenes themselves. This problem is something that has also been studied *in vitro* by Alpatova et al. (2010). Our laboratory has had good success with the use of the anionic surfactant SDS with which good dispersion can be achieved with minimal toxicity.

There have been very few studies investigating the effects of CNTs on aquatic animals *in vivo*. In a study carried out by Galloway et al. (2010) the marine polychaete

*Arenicola marina* was exposed to SWCNTs via the sediment. The authors found no obvious toxicity and the CNTs displayed limited bioavailability with little evidence of aggregates in the gastrointestinal tract being found. Cheng et al. (2009) exposed zebrafish (*Danio rerio*) embryos and larvae to fluorescently labelled MWCNTs via micro-injection. The authors found that whilst embryos injected either at the 1-cell stage or 72 h post fertilisation showed no evidence of toxicity, there was a potential effect on reproductive success. The injected embryos showed no significant decline in survival but their off-spring did exhibit significantly lower survival rates. The authors were unable to determine the reason for this effect but the results highlight the importance of understanding chronic exposure effects. Our laboratory has carried two studies to test the effects of SWCNTs on fish. Smith et al. (2007) exposed rainbow trout (*Oncorhynchus mykiss*) to 0-0.5 mg l<sup>-1</sup> SWCNTs for 10 days in an aqueous exposure study. The authors found evidence of respiratory toxicity, gill and liver pathology and oxidative stress as indicated by elevated total glutathione levels in gill and liver tissues. In a dietary exposure to SWCNTs Fraser et al. (2010) found little evidence of toxicity in rainbow trout but there was a transient change in thiobarbituric acid reactive substances (TBARS) levels in SWCNT exposed fish.

It is clear that there is a level of uncertainty regarding the potential toxicity of CNTs but the fact that there is some evidence of oxidative stress and the potential for reduced reproductive health necessitates further investigation. The aim of the current study was to provide one of the first toxicological observations on sub-lethal aqueous exposure to SWCNTs in zebrafish testing the null hypothesis that exposure to SWCNTs would have no effect on fish health or reproductive output. Specific aims included examining the effects of SWCNTs, along with both a carbon NP control (carbon black, CB) and a dispersion vehicle control (sodium dodecyl sulphate, SDS), on various aspects of the

physiology of adult zebrafish and, in turn, potential effects on reproduction and survival of offspring.

### 5.3. Methods

#### 5.3.1. Stock animals and experimental design

Mature adult zebrafish (*Danio rerio*) ( $n = 300$ ) were grown from stock fish in the zebrafish facility at the University of Plymouth. Fish were held in re-circulating, filtered, dechlorinated, Plymouth freshwater (see below) and fed on tropical fish aquarium flake and brine shrimp (*Artemia salina*) to ensure good health before starting the experiment. Then, 288 fish were randomly allocated into 12 glass exposure tanks (350×200×200 mm from Clear-Seal, UK) containing 10 l of clean water to acclimate for 14 days prior to starting the exposures (24 fish/tank) and to allow for pre-exposure breeding trials. Throughout the acclimatisation and exposure period the fish in exposure tanks were separated by sex. Bespoke glass rod meshes were used to divide the sexes with 12 males and 12 females on either side of the divide. The photoperiod remained fixed at a 12 h light: 12 h dark cycle.

Fish were weighed at the start of the exposure (mean  $\pm$  SEM,  $n = 24$ ,  $0.5 \pm 0.02$  g) and tanks were randomly allocated to treatments (in triplicate) as control (no added compounds), SWCNT ( $0.25 \text{ mg l}^{-1}$ ), SDS control ( $0.25 \text{ mg l}^{-1}$ ) and CB control ( $0.25 \text{ mg l}^{-1}$ ). These values were chosen after a previous aqueous study using CNTs found some toxicity without being lethal (Smith, et al., 2007). Fish were fed *Artemia salina* brine shrimp twice daily; once in the morning and once after a water change. Water changes were carried out every 24 hours with 80 % changes carried out in the afternoon. Dosing was done once the fish had finished their second feed to minimise ingestion of NPs and after the initial dose the subsequent doses were 80 % of the nominated concentrations to

allow for 20 % of the dose left in the water after the water change (i.e. 0.2 mg l<sup>-1</sup> added to the 0.25 mg l<sup>-1</sup> tanks after an 80 % water change). Water quality was measured daily. Parameters measured included temperature, dissolved oxygen (DO) and pH (Hach HQ40d multi meter), total ammonium, nitrite and nitrate (Hach DR 2800 portable spectrophotometer with test kits LCK 304, LCK 341 and LCK 340 for ammonium, nitrite and nitrate respectively).

Fish were sampled ( $n = 2$  fish per tank;  $n = 6$  fish/treatment) at the start of the exposure (initial fish, day 0), and then on days 7, 14 and 21 for haematology, whole body ion analysis, histology and biochemistry.

### **5.3.2. Breeding trials**

Breeding trials were carried out in individual 2 l polycarbonate zebrafish spawning chambers (220×100×100 mm from Aquatic Habitats, USA) with an internal chamber with a mesh flooring to separate fish from freshly spawned eggs. All breeding trials were carried out using control water (no added compounds, water quality as above). Fish were bred on five separate occasions; twice before and three times during the exposure period at days -7, 0, 7, 14 and 21 (before or during exposure period). For each breeding trial three pairs of fish (3 females, 3 males) were randomly selected from each of the 12 exposure tanks and placed into separate breeding chambers (one pair per chamber, 9 chambers per treatment) at around 5pm. Fish were then left to settle and breed the following morning. The rest of the fish in the exposure tanks were also allowed to breed at this point to ensure the breeding regime remained constant. The glass mesh dividers in the exposure tanks were re-positioned in a horizontal plane at the bottom of the tanks to separate the fish from any eggs produced so as not to introduce any unfair nutritional gain from ingested eggs. At around 2 hours post-sunrise (10am)

the breeding pairs were sampled for one of three end points (one pair per end point; haematology and metal analysis, biochemistry and histology at days 0, 7, 14 and 21).

Any eggs produced in the breeding chambers were collected and counted as viable (fertilised with a normal appearance), or non-viable (unfertilised and/or dead embryo). A sub-sample of viable embryos was randomly collected, cleaned by carefully moving them into a new dish with clean fresh water, and grown on in petri dishes ( $n = 50$  per dish,  $n = 3$  dishes per chamber if sufficient numbers were achieved). Water in the petri dishes was changed every 24 h and total number of healthy embryos recorded. Any dead embryos were removed at the time of the water change. Embryos were grown on to hatching to ascertain cumulative survival and hatching success. Any eggs produced in the exposure tanks were siphoned out and the fish were re-sexed and separated by replacing the glass mesh dividers in the vertical position.

### **5.3.3. Carbon nanotube and carbon black stock solutions**

The single-walled carbon nanotubes used here were from the same batch previously characterised and used by our laboratory (Smith, et al., 2007). Stock suspension preparation and confirmation of dispersion were also carried out as in Smith et al. (2007) with minor modifications. Briefly, the powder form of single-walled carbon nanotubes was obtained from Cheap Tubes Inc. (Vermont, USA), and had (manufacturer's information) 1.1 nm mean outside diameter, 5-30  $\mu\text{m}$  length, and the powder was a minimum of 96.3 % carbon, (maximum impurities were: Al 0.08, Cl 0.41, Co 2.91, and S 0.29 %). However, analysis of stock suspensions revealed these impurities were below detection and the batch purity was high (Smith, et al., 2007).

Stock suspensions of dispersed SWCNTs were prepared using a combination of dispersion vehicle (SDS, sodium dodecyl sulphate) and mechanical shaking after considering the recommendations of the manufacturer, the solubility of CNTs in various

dispersion vehicles (Hamet al., 2005), and the potential toxicity of these vehicles to fish. SDS was selected because the chemistry indicated moderately good dispersion of carbon nanotubes with SDS (Ham, et al., 2005) while the maximum concentration of carbon nanotubes with SDS (Ham, et al., 2005) while the maximum concentration of SDS we used ( $0.25 \text{ mg l}^{-1}$  in the fish tank for vehicle control and both carbon NP concentrations) was an order of magnitude below the lethal threshold for trout (Abel, 1976). A  $0.5 \text{ g l}^{-1}$  stock suspension of SWCNTs was prepared by adding dry SWCNT powder to a small volume of SDS at  $3 \text{ g l}^{-1}$  in ultrapure (Millipore) water and then diluting to give a final concentration of  $0.5 \text{ g l}^{-1}$  of both SWCNT and SDS. An SDS vehicle control was made at a concentration of  $0.5 \text{ g l}^{-1}$ . In order to control for a carbon-based NP effect a carbon black (CB) control group was included in the experimental design. The CB powder used here was the same batch previously characterised by Stone et al. (1998) and Brown et al. (2000). The carbon NP control was made as described for SWCNT using CB suspended in SDS solution at a final concentration of  $0.5 \text{ g l}^{-1}$  for both CB and SDS. Particle analysis of the prepared stock suspensions allowed primary bundle and particle sizes to be determined using transmission electron microscopy TEM (JEOL 1200EXII) (Figure 5.1). The mean bundle diameter of SWCNTs was  $14.6 \pm 1.6 \text{ nm}$  ( $n = 102$ ) and the mean diameter of CB particles was  $17.4 \pm 0.8 \text{ nm}$  ( $n = 55$ ), (data = mean  $\pm$  SEM). However, due to the carbon-based nature of these NPs no concentration data were able to be collected from water or tissue digest samples.

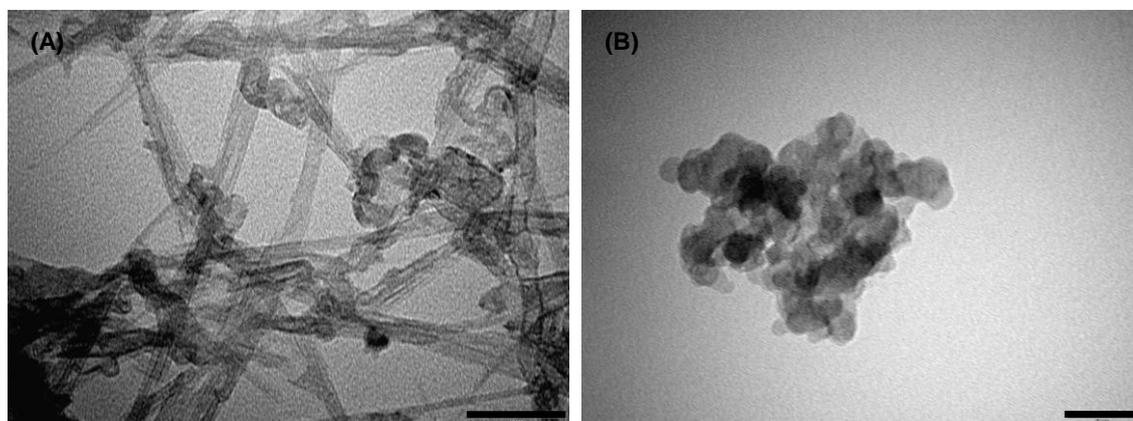


Figure 5.1. TEM micrographs of (A) SWCNTs and (B) CB stock suspensions. Scale bars are 50 nm.

#### **5.3.4. Haematology**

The details of blood collection and analysis are given in Chapter 2.

#### **5.3.5. Metal analysis**

Following blood sampling fish were placed onto slides and then processed for ion analysis as described in Ramsden et al. (2009) with minor modifications. Briefly, fish were oven dried to a constant weight, then digested in 4 ml of concentrated nitric acid at 70 °C for 2 hours and allowed to cool. Each sample was then diluted with ultrapure water to achieve a final volume of 16 ml and analysed for Ca, Na, K, Mn, Cu and Zn by inductively coupled plasma optical emission spectrometry (ICP-OES, Varian 725 ES). Analytical grade standards were used throughout, and the acidity and matrix of the standards was matched to the samples.

#### **5.3.6. Biochemistry**

Fish were also collected for biochemistry. Fish were terminally anaesthetised with MS222 (without blood sampling), weight and total length was recorded, then whole animals were snap frozen in liquid nitrogen and stored at -80 °C until required for biochemistry. Subsequently fish were individually dissected (over ice) to harvest the branchial basket, the whole brain and the liver. Tissues were homogenised manually (using a metal rod) in 10 volumes (100 µl for tissue weighing 10 mg) of ice-cold hypotonic buffer (in mmol l<sup>-1</sup>; 100 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-hydroxymethyl-1,3-propanediol)). Homogenates were stored at -80 °C until required. For brain and liver tissue, due to the

small volumes of homogenates obtained, it was necessary to pool samples by tank (two samples pooled together resulting in  $n = 3$  samples per treatment) in order to gain sufficient volume to carry out the  $\text{Na}^+\text{K}^+$ -ATPase assay. In a few cases it was also necessary to pool gill homogenates by tank, but wherever possible pooling was kept to a minimum. Prior to any pooling of homogenates an aliquot (8  $\mu\text{l}$ ) was diluted further (1:100) for protein and total glutathione analyses to yield the highest resolution possible.

Tissue homogenates were analysed (each sample in triplicate wherever possible) for total protein content,  $\text{Na}^+\text{K}^+$ -ATPase activity and total glutathione as described in Smith et al. (2007) with a few minor changes. The details of these assays are given in Chapter 2.

### **5.3.7. Histology**

The details of the histological analysis of tissues are given in Chapter 2.

### **5.3.8. Statistical analysis**

The details of the statistical analyses performed in the present study are given in Chapter 2.

## **5.4. Results**

There were no mortalities of adult zebrafish throughout the exposure period and no abnormal behaviours (loss of equilibrium, refusal to feed) were observed in any of the treatments. Temperature, DO, pH, ammonium, nitrite and nitrate levels over the course of the exposure were (mean  $\pm$  SEM,  $n \leq 42$ ):  $27.1 \pm 0.04$  °C,  $7.22 \pm 0.01$  mg  $\text{l}^{-1}$  (> 90 % saturation),  $7.29 \pm 0.01$ ,  $1.05 \pm 0.09$  mg  $\text{l}^{-1}$ ,  $0.17 \pm 0.02$  mg  $\text{l}^{-1}$  and  $< 22$  mg  $\text{l}^{-1}$  respectively with no tank effects (pooled data).

#### 5.4.1. Haematology

There were some minor transient changes in both red and white blood cell counts over the course of the 21 day exposure period but there were no significant treatment effects (ANOVA,  $P > 0.05$ ). Erythrocyte counts from whole blood samples at days 7, 14 and 21 showed no obvious treatment effects with cell counts at day 21 of  $3.49 \pm 0.33$ ,  $3.00 \pm 0.36$ ,  $2.98 \pm 0.18$  and  $2.52 \pm 0.42$  (cells  $\times 10^6 \text{ mm}^{-3}$ , data are mean  $\pm$  SEM,  $n = 3$ ) for control,  $0.25 \text{ mg l}^{-1}$  CB,  $0.25 \text{ mg l}^{-1}$  SDS and  $0.25 \text{ mg l}^{-1}$  SWCNT groups respectively. Total leukocyte counts from whole blood samples at days 7, 14 and 21 also showed no obvious treatment effects with cell counts at day 21 of  $13.13 \pm 0.56$ ,  $5.70 \pm 1.49$ ,  $15.70 \pm 8.40$  and  $10.62 \pm 1.73$  (cells  $\times 10^3 \text{ mm}^{-3}$ , data are mean  $\pm$  SEM,  $n = 3$ ) for control,  $0.25 \text{ mg l}^{-1}$  CB,  $0.25 \text{ mg l}^{-1}$  SDS and  $0.25 \text{ mg l}^{-1}$  SWCNT groups respectively.

#### 5.4.2. Whole body ions and trace metals

Metal analysis of whole zebrafish acid digests showed no discernible treatment effects for any of the other six metals analysed (Figure 5.2). Concentrations of the bulk electrolytes  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$  and the trace metal zinc all remained within normal limits throughout the exposure period with no significant treatment effects (ANOVA,  $P > 0.05$ ). There was a degree of variation in both manganese and copper concentrations for the duration of the exposure; however these changes were not treatment related when compared to time-matched controls.

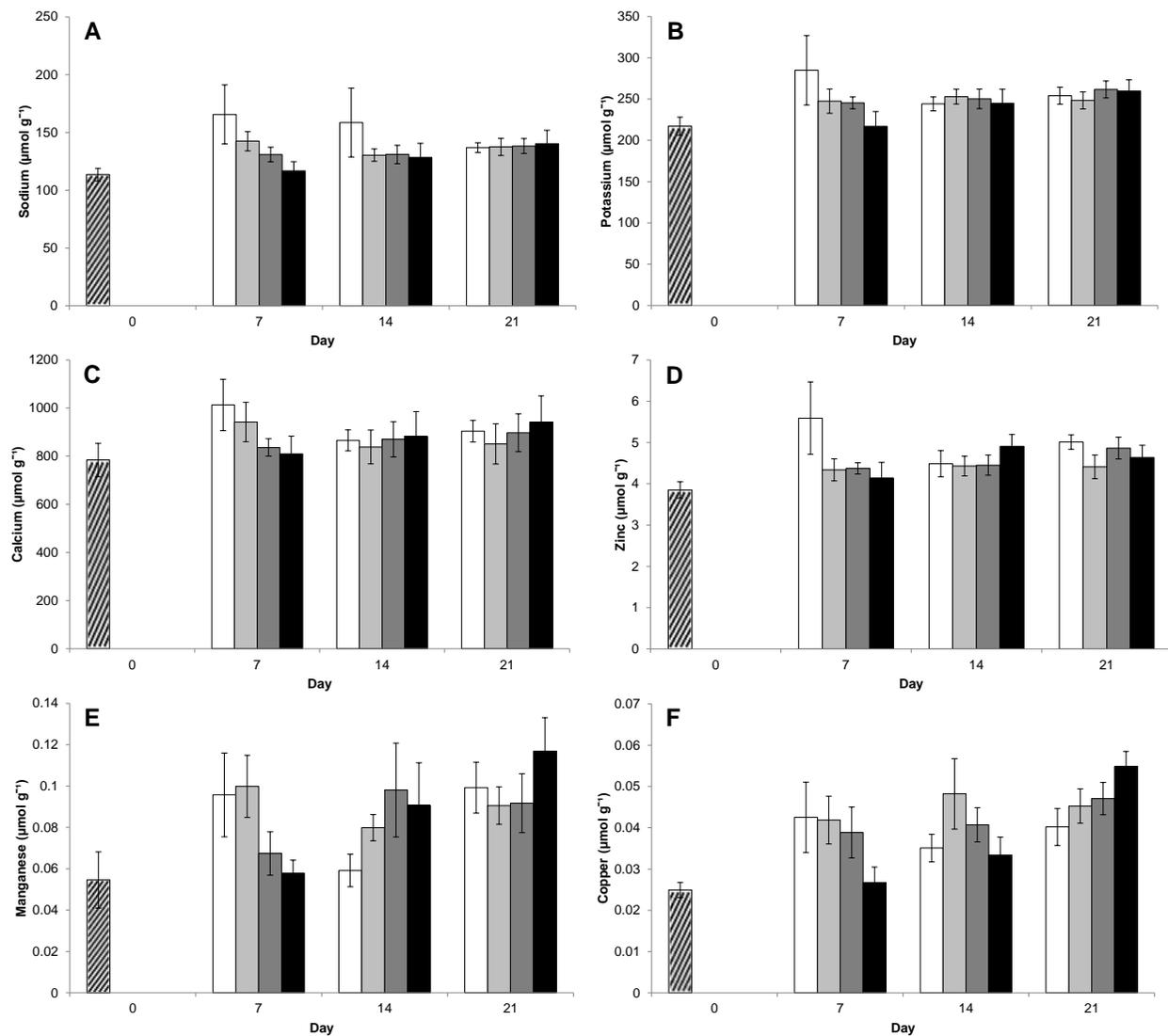


Figure 5.2. Zebrafish metal analysis for (A) sodium, (B) potassium, (C) calcium, (D) zinc, (E) manganese and (F) copper in zebrafish exposed to control (clear bars), 0.25 mg l<sup>-1</sup> CB (light grey bars), 0.25 mg l<sup>-1</sup> SDS (dark grey bars), or 0.25 mg l<sup>-1</sup> SWCNT (black bars) for 21 days. Hatched bar at day 0 is initial fish. Data are mean  $\pm$  SEM  $\mu\text{mol metal g}^{-1}$  dry weight,  $n = 6$  fish per treatment. There were no significant differences for any of the metals analysed (ANOVA or Kruskal-Wallis,  $P > 0.05$ ).

### 5.4.3. Biochemistry

The level of variation in Na<sup>+</sup>K<sup>+</sup>-ATPase activities was moderately high for all three tissues measured for the duration of the exposure period (Figure 5.3). Na<sup>+</sup>K<sup>+</sup>-ATPase activities in brain tissue showed no significant differences in time or treatment effects (ANOVA,  $P > 0.05$ , Figure 5.3A). Both gill and liver tissue Na<sup>+</sup>K<sup>+</sup>-ATPase activities showed a large amount of variation and there were no significant differences in either tissue (ANOVA,  $P > 0.05$ ). Na<sup>+</sup>K<sup>+</sup>-ATPase activities in the gill tissue of the SDS-exposed fish showed a decrease compared to initial levels but this effect was not significant. There were no clear trends in liver Na<sup>+</sup>K<sup>+</sup>-ATPase activities.

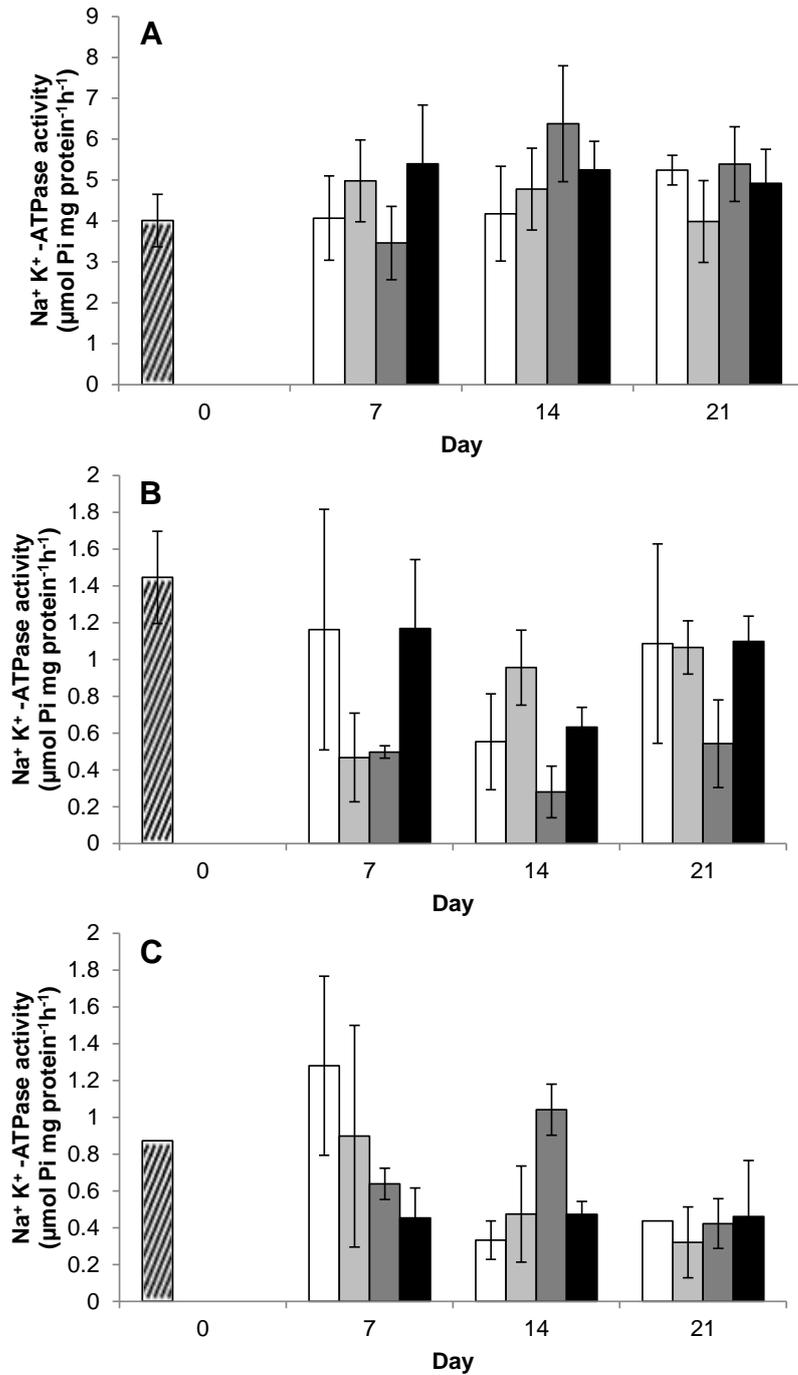


Figure 5.3. Na<sup>+</sup>K<sup>+</sup>-ATPase activity in crude homogenates of the (A) brain, (B) gill and (C) liver tissues of zebrafish exposed to control (clear bars), 0.25 mg l<sup>-1</sup> CB (light grey bars), 0.25 mg l<sup>-1</sup> SDS (dark grey bars) and 0.25 mg l<sup>-1</sup> SWCNT (black bars) for 21 days. Data are mean ± S.E.M., *n* = 3 pooled samples of 2 fish each for brain and liver samples, and *n* = 3-6 mixed pooled and individual fish for gill samples. Hatched bars are the initial fish at time zero collected immediately prior to starting the experimental exposures. There were no significant differences in any of the tissues analysed (ANOVA or Kruskal-Wallis, *P* > 0.05).

Total glutathione (GSH) levels were measured in the brain, gill and liver homogenates of all fish sampled for biochemical analysis (Figure 5.4). GSH levels in

both brain and liver tissue samples showed no significant treatment effects for the duration of the exposure period (ANOVA,  $P > 0.05$ ). Gill GSH levels showed some significant differences (ANOVA,  $P < 0.05$ ). GSH levels were significantly lower in SWCNT-exposed fish at days 14 and 21 than some of the other time-matched treatment groups; however there was no clear treatment effect as the GSH levels for the SWCNT-exposed fish were not significantly different to the control group on either day.

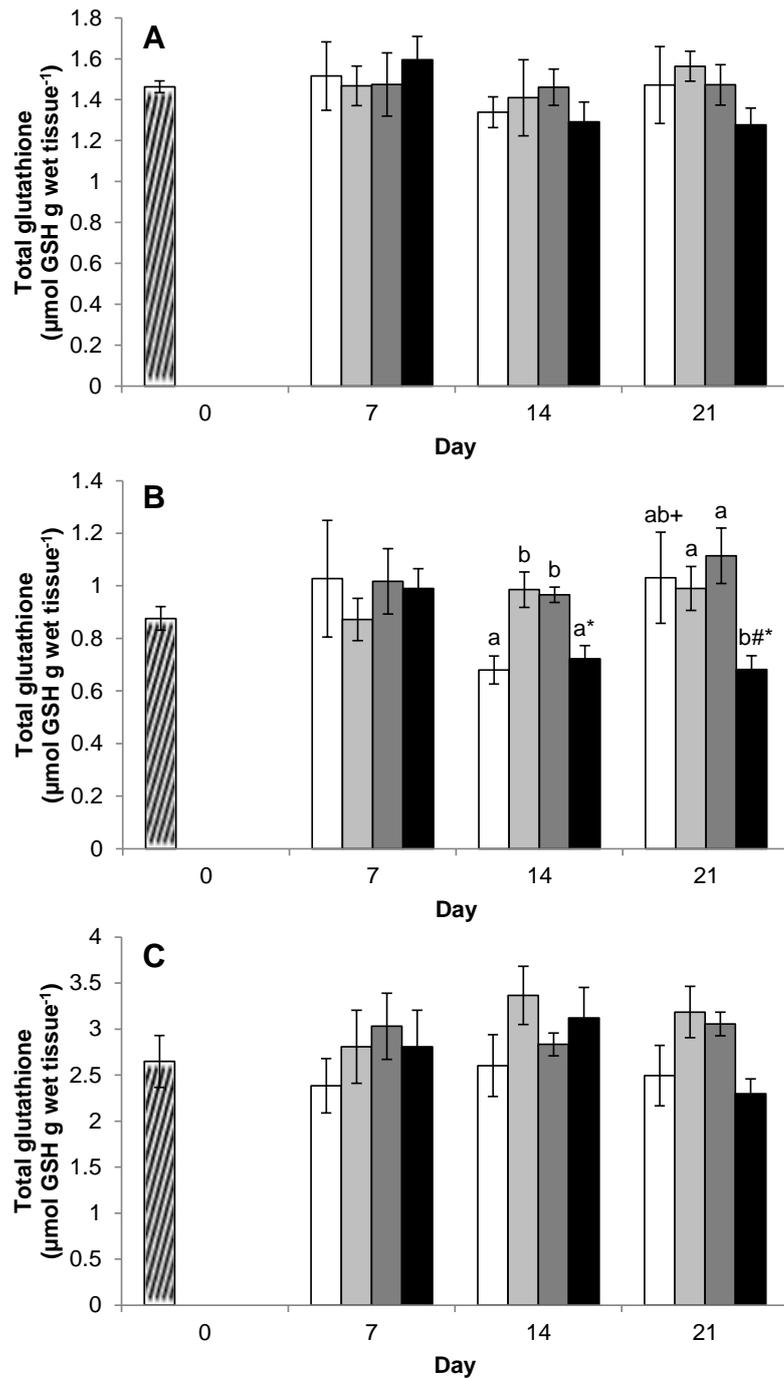


Figure 5.4. Total glutathione (GSH) levels in crude homogenates of the (A) brain, (B) gill and (C) liver tissues of zebrafish exposed to control (clear bars), 0.25 mg l<sup>-1</sup> CB (light grey bars), 0.25 mg l<sup>-1</sup> SDS (dark grey bars) and 0.25 mg l<sup>-1</sup> SWCNT (black bars) for 21 days. Data are mean  $\pm$  S.E.M.,  $n = 6$  fish for all tissues. Hatched bars are the initial fish at time zero collected immediately prior to starting the experimental exposures. # indicates significant difference to initial fish (ANOVA or Kruskal-Wallis,  $P < 0.05$ ); \* indicates significant difference to day 7 within treatment (ANOVA or Kruskal-Wallis,  $P < 0.05$ ); + indicates significant difference to previous time point within treatment (ANOVA or Kruskal-Wallis,  $P < 0.05$ ); different letters within a time point indicate significant differences between treatments (ANOVA or Kruskal-Wallis,  $P < 0.05$ ).

#### 5.4.4. Histology

Brain, gill, liver and gonad tissues were analysed for any evidence of pathological change. There were no significant changes in any tissues examined. Quantitative data was obtained where possible; for example percentage of club tips on gill secondary lamellae was calculated but there were no significant differences:  $0.35 \pm 0.35$ ,  $1.12 \pm 0.77$ ,  $0.53 \pm 0.53$  and  $0.33 \pm 0.33$  % (data are at day 21, mean  $\pm$  SEM % club tips,  $n \leq 6$  fish per treatment) in control,  $0.25 \text{ mg l}^{-1}$  CB,  $0.25 \text{ mg l}^{-1}$  SDS and  $0.25 \text{ mg l}^{-1}$  SWCNT groups respectively. The liver tissue showed no overt signs of pathology, with no significant changes in the percentage of sinusoid space relative to the parenchyma. Mean percentage of sinusoid space relative to the parenchyma at day 21 was:  $14.6 \pm 2.0$ ,  $14.6 \pm 2.0$ ,  $15.8 \pm 0.7$  and  $14.9 \pm 0.9$  (data are mean  $\pm$  SEM proportion sinusoid space relative to parenchyma,  $n \leq 6$  fish per treatment) in control,  $0.25 \text{ mg l}^{-1}$  CB,  $0.25 \text{ mg l}^{-1}$  SDS and  $0.25 \text{ mg l}^{-1}$  SWCNT groups respectively. There were no differences in the level of lipid peroxidation in liver tissue with signs of lipid peroxidation in all of the exposure groups including the control group.

The architecture of the brain was normal for all treatment groups with a very low level of tissue damage. All treatment groups displayed a small incidence of necrotic bodies and one or two examples of blood vessel abnormalities in fish from both control and treatment groups. The structure of gonadal tissue was also normal in all treatment groups with no occurrence of ova-testis, no evidence of reactive hyperplasia, or atrophy of ova or testis. Female gonad showed a normal spread of oocyte developmental stages in all treatments. Mean follicular size was calculated and there were no significant differences:  $220.3 \pm 5.4$ ,  $257.3 \pm 9.4$ ,  $254.5 \pm 7.5$  and  $277.0 \pm 32.3$   $\mu\text{m}$  (data are at day 21, mean follicular size  $\pm$  SEM,  $n \leq 4$  females per treatment group) in control,  $0.25 \text{ mg l}^{-1}$  CB,  $0.25 \text{ mg l}^{-1}$  SDS and  $0.25 \text{ mg l}^{-1}$  SWCNT groups respectively.

### 5.4.5. Breeding trials

The level of variance in the breeding trials was moderately high but a clear trend was apparent in the number of viable embryos produced over the course of the exposure period. Of the four treatment groups only the SWCNT group exhibited a decline in the total number of viable embryos produced throughout the exposure period. This trend for a decline in total number viable embryos produced is apparent when standardised as a mean number of viable embryos per pair (Figure 5.5). At day 21 the SWCNT exposed fish produced fewer viable embryos than the other treatments, however this trend was not significant (Kruskal-Wallis,  $P = 0.09$ ).

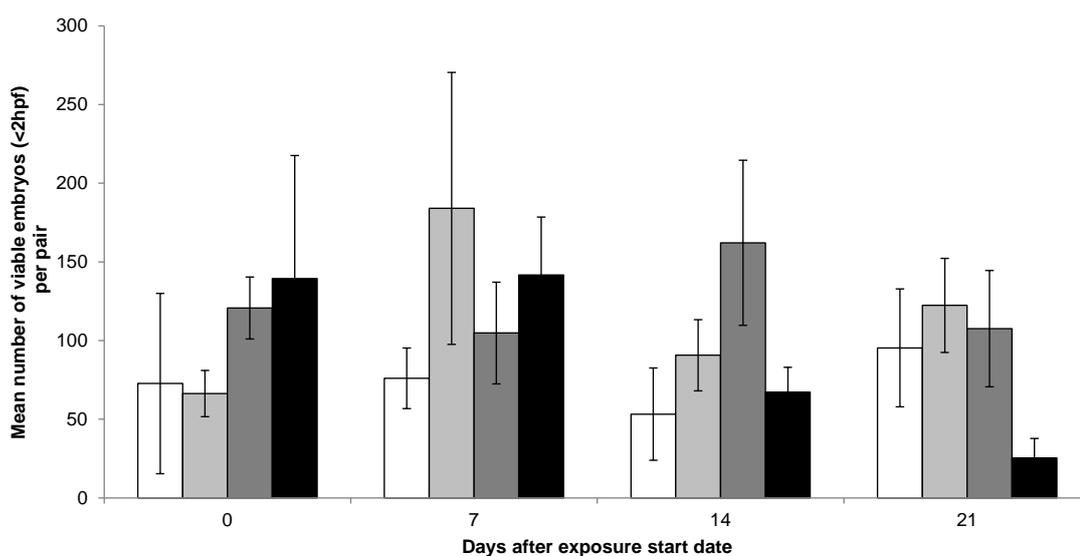


Figure 5.5. Mean number of viable embryos produced in each breeding trial during the exposure period for zebrafish exposed to control (clear bars), 0.25 mg l<sup>-1</sup> CB (light grey bars), 0.25 mg l<sup>-1</sup> SDS (dark grey bars) and 0.25 mg l<sup>-1</sup> SWCNT (black bars) for 21 days. Data are mean  $\pm$  SEM number of viable embryos for each treatment. There were no significant differences (Kruskal-Wallis,  $P > 0.05$ ).

Whilst the number of viable embryos was declining over the exposure period for the SWCNT exposed fish there was no significant effect on the survival of the viable embryos that were produced (Figure 5.6). At day 14 the survival of the SWCNT group

embryos was lower than the previous breeding event at day 7, but this trend did not continue to day 21.

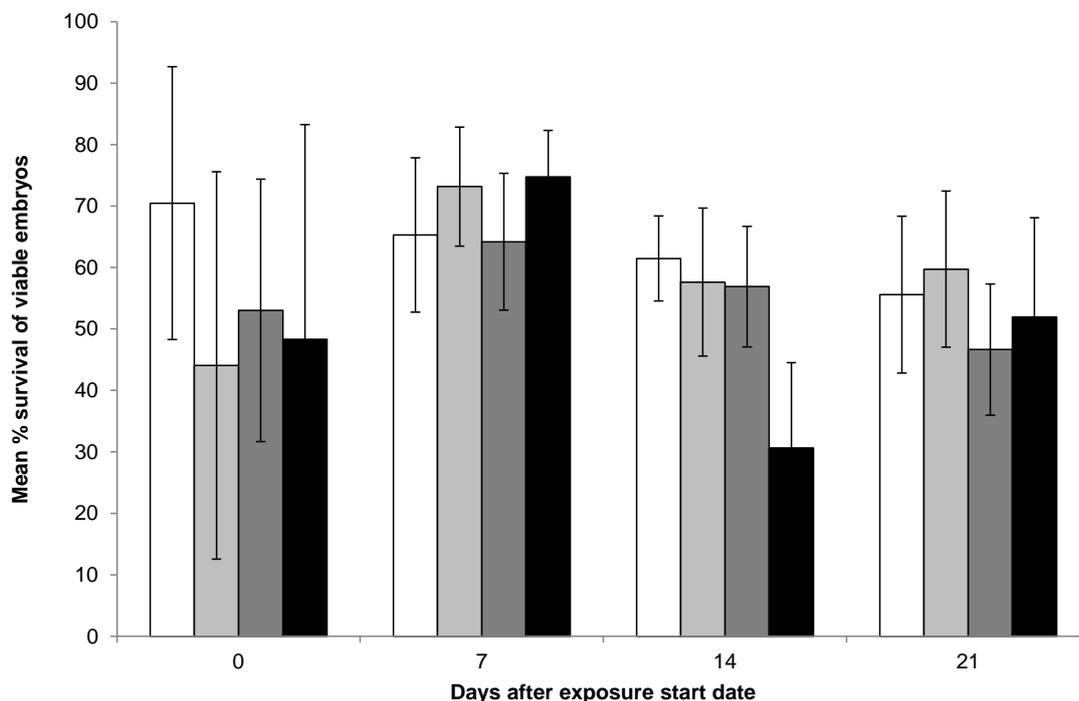


Figure 5.6. Percent survival of viable embryos produced in each breeding trial during the exposure period for zebrafish exposed to control (clear bars), 0.25 mg l<sup>-1</sup> CB (light grey bars), 0.25 mg l<sup>-1</sup> SDS (dark grey bars) and 0.25 mg l<sup>-1</sup> SWCNT (black bars) for 21 days. Data are mean  $\pm$  SEM % survival of viable embryos to hatching for each treatment. Data were arcsine transformed before statistical analysis. There were no significant differences (ANOVA,  $P > 0.05$ ).

## 5.5. Discussion

### 5.5.1. Haematology, electrolytes and trace metals

There were no mortalities in the present study and all fish exhibited normal behaviour throughout both exposure and recovery phases. Blood cell counts showed no obvious effects of SWCNT exposure on the oxygen carrying potential of the exposed fish. There were no major disturbances to haematology with erythrocyte counts (RBC) remaining unchanged for the duration of the exposure period. Leukocyte (WBC) counts did not exhibit any significant differences to the controls and are within the normal range for these fish (Velasco-Santamaría et al., 2011). There were no signs of cell

damage for either erythrocytes or leukocytes. It has been reported that CNTs can stimulate macrophage production in rainbow trout cell cultures (Klaper et al., 2010), however in the present study there were no significant changes in leukocyte production over the course of the exposure period.

Total body electrolyte and trace metal analysis did not show any major disturbances over the duration of the exposure (Figure 5.2). There were no significant treatment-related changes in  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$  concentrations which indicates good general health and, together with the absence of any treatment-related change in  $\text{Na}^+\text{K}^+$ -ATPase activity, suggests that the fish were able to maintain good osmoregulatory function. As we were interested in the reproductive effects of SWCNT exposure it was necessary to monitor total body Zn concentrations. Zinc plays a key role in the production of egg proteins and its absence can be a good indicator of reproductive stress (Thompson et al., 2003). There were no significant changes in the zinc concentrations of any of the exposure groups throughout the exposure period. The other two whole body trace metals tested were manganese and copper. Manganese is important in switching on inflammatory responses and copper plays many roles in protein structure and regulating other metals (Handy, pers. comm.) Neither of these two metals showed any treatment-related changes throughout the course of the exposure period indicating that there was no evidence of immunosuppression caused by SWCNTs. Both manganese and copper concentrations increased in all treatments at day 21 compared to the start of the exposure. This increase was more pronounced in the SWCNT exposed fish but concentrations were not significantly higher than the time-matched controls.

#### **5.5.2. Osmoregulation and oxidative stress**

There were no clear significant treatment effects in  $\text{Na}^+\text{K}^+$ -ATPase activities (Figure 5.3).  $\text{Na}^+\text{K}^+$ -ATPase activities were similar to those obtained in a similar study examining the effects of  $\text{TiO}_2$  NPs on zebrafish detailed in Chapter 4. Smith et al.

(2007) reported some evidence of up-regulation of branchial  $\text{Na}^+\text{K}^+$ -ATPase activity in rainbow trout exposed to 0.1 and 0.25  $\text{mg l}^{-1}$  SWCNTs. The authors suggested that this allowed the fish to maintain plasma  $\text{Na}^+$  and  $\text{K}^+$  levels and as such the fish were able to maintain good osmoregulatory control. In the present study there was no sign of either an increase or inhibition of  $\text{Na}^+\text{K}^+$ -ATPase activity in any of the three tissues examined. This result, along with the unchanged bulk electrolyte concentrations in whole body digests, suggests that exposure to SWCNTs does not generate osmoregulatory dysfunction in zebrafish at the exposure concentration used herein. Fraser et al. (2010) came to a similar conclusion in their study on the effects of SWCNTs on zebrafish in a dietary exposure.

Oxidative stress has been suggested as a possible cause of CNT toxicity in both *in vitro* (Cui et al., 2005; Tian et al., 2006; Pulskamp et al., 2007) and *in vivo* (Smith et al., 2007; Fraser et al., 2010) studies. In the present study total glutathione was measured as a proxy for oxidative stress (Figure 5.4). Gill tissue GSH levels showed some transient decreases in total GSH in SWCNT-exposed fish but these changes were not significantly different to the time-matched control group on either day. Total GSH levels reported in the present study are similar to those reported by Fraser et al. (2010) and also to those obtained in a similar study by our laboratory examining the effects of  $\text{TiO}_2$  NPs on zebrafish detailed in Chapter 4, and as such seem to be within the normal range for zebrafish. The apparent lack of oxidative stress in the present study is consistent with the absence of fatty change in the liver and the normal haematology of all SWCNT-exposed fish.

### **5.5.3. Histology**

The histopathological effects of CNT exposure have been reported in a few studies on mammals (Lam et al., 2004; Warheit et al., 2004) and fish (Smith et al., 2007; Fraser

et al., 2010). The pathological effects of aqueous SWCNT-exposure in rainbow trout reported by Smith et al. (2007) included gill injuries (oedema in the secondary lamellae, mucocyte morphological changes and hyperplasia of the primary lamellae), condensed nuclear bodies in liver cells, aneurisms or swelling of blood vessels on the ventral surface of the cerebellum, and areas of fusion of the intestinal villi. In the present study there was no evidence of any significant pathology in any of the tissues examined. There were no obvious signs of respiratory distress; gill morphology was normal and there were no treatment effects on the incidence of gill injuries. The reason for the difference in the level of gill injury between the present study and that carried out by Smith et al. (2007) is likely due to the difference in sensitivities of the two species used. Rainbow trout inhabit clean, flowing fresh water whereas zebrafish are able to cope with the much poorer water quality found in stagnant water bodies. The effective dose of the NP at the gills of zebrafish is also likely to be lower than that in trout due to the lower ventilation volume of the smaller fish. Unlike the results reported in Smith et al. (2007) there was no evidence of stress-induced drinking of SWCNTs as evidenced by an absence of SWCNTs in the guts of exposed fish. Livers of fish exposed to all treatments showed normal levels of morphological variation and there was no evidence of energetic imbalance in SWCNT-exposed fish.

#### **5.5.4. Reproductive effects of SWCNT exposure**

To the best of our knowledge there have been very few studies done on the reproductive effects of NPs in fish. In a study by Cheng et al. (2009) zebrafish embryos and larvae were exposed to fluorescently labelled MWCNTs via micro-injection. The injected embryos showed no significant decline in survival but their off-spring did exhibit significantly lower survival rates. In the present study there was a trend for a decline in the number of viable embryos produced by SWCNT-exposed fish (Figure 5.5). Over the course of the exposure period there appeared to be some evidence of

reproductive stress but the reasons behind this are unclear. Whilst the number of viable embryos produced declined in SWCNT-exposed fish there was no significant change in the survival of those embryos (Figure 5.6). It seems that SWCNT exposure may influence the reproductive health of exposed adults but the off-spring they produce remain unaffected. Histological examination of gonadal tissue from SWCNT-exposed fish showed no clear evidence of compromised gametogenesis or major morphological change so the reason for the decline in fecundity remains unclear. It is apparent that there may be some challenges to SWCNT-exposed fish beyond those posed to the other treatment groups which result in a shift of energetic expenditure away from reproduction. The reasons behind this also remain unclear but this evidence, combined with the findings of Cheng et al. (2009), highlights the importance of chronic reproduction testing in order to gain a better understanding of the total effect of NP exposure.

#### **5.5.5. Toxicity of dispersion vehicle and carbon controls**

There has been some debate in recent literature about the toxicity of vehicles used to disperse NPs (Henry et al., 2007; Alpatova et al., 2010). In the present study SDS was used as a dispersion vehicle for both SWCNTs and CB treatments after a previous study in our laboratory by Smith et al. (2007) found that SDS produced very little toxicity in rainbow trout. To monitor the potential for vehicle-induced toxicity there was also an SDS-only control treatment group. There was no evidence of SDS-related toxicity in any of the end points tested in the present study with no treatment-related effects in haematology, biochemistry, histology or reproduction. The concentration of SDS used was  $0.25 \text{ mg l}^{-1}$  which is an order of magnitude below the lethal threshold for trout (Abel, 1976) and as such the absence of any toxicity was to be expected. The third control treatment used in the present study was  $0.25 \text{ mg l}^{-1}$  carbon black (CB). Again there is differing evidence with regards to the toxicity of CB NPs with reports of both

toxic (Hussain et al., 2009) and non-toxic (Lam et al., 2004; Fraser et al., 2010) effects. In the present study there was no evidence of CB-related toxicity in any of the end points tested.

### **5.5.6. Conclusions**

The present study demonstrates that, at the concentrations and exposure duration used, SWCNTs are relatively non-toxic to zebrafish. The main physiological end points tested were unchanged by exposure to SWCNTs and there was little evidence of either osmoregulatory dysfunction or oxidative stress. Histological examination of the main target organs showed little evidence of any major tissue injury and the fish appeared to be in good energetic health for the duration of the exposure period. Reproduction appears to be a potential mechanism for chronic toxicity. Whilst the present study carried out a 21 day exposure it is possible that with prolonged exposure to the concentrations of NPs used there may be a threat to the reproductive status of exposed fish. Future studies should focus on chronic exposures in order to investigate the potential harm of NPs to reproductive health and the potential costs of NP exposure to the bioenergetic budget of exposed fish.

### **5.6. Acknowledgments**

This research was funded by a grant to R. Handy from the Natural Environment Research Council UK (NE/G001812/1) and carried out whilst C. Ramsden was studying for a PhD at the University of Plymouth. C. Ramsden was partially supported by a grant from the States of Jersey. The author would like to thank Dr. David Boyle and Dr. Andrew Fisher for their guidance and expertise. Mike Hockings, Stan McMahon and Andrew Atfield are also thanked for their technical assistance.

# 6. Sub-lethal effects of titanium dioxide nanoparticles on the physiology and reproduction of the three-spined stickleback

## 6.1. Abstract

There are limited data on the sub-lethal physiological effects of titanium dioxide nanoparticles (TiO<sub>2</sub> NPs) in adult fishes, and the consequences for reproduction are also unclear. This study aimed to examine the sub-lethal effects of TiO<sub>2</sub> NPs on the physiology and reproductive health of the three-spined stickleback. Fish were exposed to 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NPs compared to a no added TiO<sub>2</sub> control and a 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub> powder for 40 days. Fish were examined for haematology, electrolyte and trace metal profiles, biochemistry and histology. During the exposure males were assessed for the ability to build a nest and recordings were made of reproductive behaviour between males and females at the end of the exposure. Ti exposure was confirmed in water samples and gill tissue acid digests showed some evidence of increased Ti in the 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> bulk and NP exposure groups compared to the control. There were no significant treatment related changes in either erythrocyte or leukocyte counts throughout the exposure. Trace metal analysis of gill, liver, muscle and kidney tissue showed no significant treatment related changes in any of the metals analysed (Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Mn, Mg, Cu and Zn). Total glutathione (GSH) levels in brain, gill, liver and muscle tissues were unchanged in all of the treatment groups. Muscle lactic acid (lactate) and lactate dehydrogenase (LDH) was also unaffected by TiO<sub>2</sub> exposure.

Histological examination of gill, liver, brain and gonad tissues showed little evidence of treatment-related morphological change. At day 29 of the exposure male adult sticklebacks were provided with nest building materials. All males in all treatment groups displayed some nest building behaviours and all males in both TiO<sub>2</sub> exposure groups built nests. Courtship behaviours were recorded at the end of the exposure period and there were no significant differences between groups in any of the behaviours analysed. Overall, this study showed limited toxicity of bulk or nano scale TiO<sub>2</sub> during the exposure and reproductive behaviour and output was unaffected by either treatment.

## **6.2. Introduction**

Details on NPs and the current status of TiO<sub>2</sub> NP toxicity can be found in Chapters 1 & 4.

The effects of dissolved metals on the reproduction of fishes is well known, and include direct toxicity and metal accumulation in the reproductive organs (Woltering, 1984; Pelgrom et al., 1995), which can result in reduced fecundity (Hatakeyama and Yasuno, 1987). Alternatively, fish may divert energy away from reproduction in favour of tissue repair or the maintenance of somatic growth during metal exposure (Alquezar et al., 2006). Reproductive behaviours and endocrinology of fishes may also be affected by metal exposure (review, Scott and Sloman, 2004). However, whether or not such mechanisms apply to nano metals like TiO<sub>2</sub> is unclear. The early life stages of fishes do show signs of toxic effects during exposure to TiO<sub>2</sub> NPs (Zhu et al., 2008; Hao et al., 2009; Chen et al., 2011a; Paterson et al., 2011), albeit often at high mg l<sup>-1</sup> exposure concentrations.

The three-spined stickleback (*Gasterosteus aculeatus*) is widely distributed throughout boreal and temperate regions of the northern hemisphere and inhabits a

variety of aquatic systems ranging from marine coastal areas to freshwater streams and ponds (Bell and Foster, 1994). The species complex includes marine, anadromous and resident freshwater populations. The species has well documented unique reproductive behavioural traits which it displays throughout its life history (reviews, Bell and Foster, 1994; Foster, 1994; Wootton, 1994). These behavioural traits may also make the three-spined stickleback more susceptible to bioenergetic disruption as a result of NP exposure. In zebrafish a prolonged exposure to TiO<sub>2</sub> NPs caused reduced fecundity at concentrations down to 0.1 mg l<sup>-1</sup> (Wang et al., 2011). It seems more likely that bioenergetic disturbances will become apparent after a longer term exposure than they would in short term acute studies.

A variety of work has been done on the three-spined stickleback including studies on endocrine disruption (Bell, 2004; Hahlbeck et al., 2004); trace metal toxicity (Wibe et al., 2001; Roussel et al., 2007), and the effects of stress and/or contaminants on behaviour (Bell, 2004; Craig and Laming, 2004). There is also a growing body of work using the reproductive traits of the stickleback to examine the effects of endocrine disrupting chemicals (EDCs) (Katsiadaki et al., 2007; Sebire et al., 2008; Sebire et al., 2011). Reproductive behaviour has been shown to be a sensitive tool in ecotoxicological testing in the stickleback (Sebire et al., 2008) and may prove to be more sensitive than simple physiological end points. In contrast, studies on the toxic effects of NPs on the three-spined stickleback are scarce; indeed the recent study by Sanders et al. (2008) examining the effects of cadmium sulphide NPs seems to be the only study on NPs in sticklebacks currently available for review.

The aim of the current study was to test the null hypothesis that exposure to TiO<sub>2</sub> NPs would have no effect on health or reproductive behaviours of exposed sticklebacks. The specific aims of the current study were to determine the sublethal effects of TiO<sub>2</sub> NP exposure on key aspects of physiology in the three-spined stickleback including

haematology, biochemical defences such as glutathione, energetic condition factors such as muscle lactate and the anatomical integrity of the internal organs. Then, at the end of a prolonged exposure period, to determine whether or not any sublethal effects could alter the reproductive behaviour (nest building and courtship behaviours) and spawning success of exposed adults.

### **6.3. Methods**

#### **6.3.1. Stock animals and experimental design**

Wild three-spined sticklebacks (*Gasterosteus aculeatus*) ( $n = 100$ ) were collected by netting from two river catchments in South West England (the river Erm and the river Clyst) in January and February 2011. Both sites were selected on the basis of having no significant pollution problems and where routine water quality monitoring by the Environment Agency, UK showed compliance with water quality objectives to protect the health of freshwater fish. Fish were held in re-circulating, filtered, dechlorinated, Plymouth freshwater for at least four weeks and fed daily on frozen bloodworm which was obtained from Tropical Marine Centre (Chorleywood, Hertfordshire, UK) to ensure good health before starting the experiment. The electrolyte composition of the dechlorinated tap water used for the experiments was 0.3, 0.1 and 0.4 mmol l<sup>-1</sup> for Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup> respectively. Fish were kept at 12 °C with a photoperiod of 12L:12D initially to keep the fish quiescent, then temperature and the photoperiod was gradually increased over four weeks to simulate the natural temperature increase of winter into spring and summer. The increase in temperature to 16 ± 1 °C, and the elongation of the daylight hours to 16L:8D, was also carried out to stimulate the sexual maturation of the fish so that the fish were in a desirable condition for the behavioural aspects of the experiment.

Two weeks prior to the start of the experiment the fish were moved into the experimental tanks to acclimate to the new surroundings. At the start of the experiment (day 0) fish were randomly sampled for T0 control measurements (initial fish) and the remaining 48 fish were assigned to 24 glass exposure tanks (45×25×25 cm) containing 20 l of clean water (2 fish/tank; 1 male & 1 female). Tanks were individually aerated with glass tubing to ensure good oxygenation and to provide a source of mechanical mixing of the water to help reduce the level of deposition of the test suspensions. Fish were allocated into tanks based on their river of origin; males from the river Erm were paired with females from the river Clyst to avoid sibling effects on reproductive behaviour (Frommen and Bakker, 2006; Sebire et al., 2011). The male and female in each tank were separated to ensure control over reproductive end points. Transparent screens (acrylic plastic, Liteglaze<sup>®</sup>) with 30, 5 mm diameter holes were situated in the centre of each tank to allow equal space for each fish and also to allow free movement of the water to ensure a homogeneous dispersion of the test suspensions as well as good oxygenation throughout the tanks. The clear screens also allowed both visual and chemical stimulus of the fish which is an important aspect of reproductive behaviour (Sebire et al., 2008). Solid screens were placed between tanks to ensure that fish from different tanks could not see each other thereby preventing inter-tank behaviours.

The exposure period lasted for 40 days with 80 % water changes every 24 hours to enable control of the exposure concentration. Fish were fed daily with a single feed of frozen bloodworm prior to re-dosing of the tanks to ensure minimal ingestion of TiO<sub>2</sub>. Tanks were allocated to treatments ( $n = 8$  tanks per treatment) as no-added TiO<sub>2</sub> controls, 1 mg l<sup>-1</sup> bulk TiO<sub>2</sub> powder and 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NPs. These concentrations were chosen to enable comparison with our previous sublethal experiments on trout (Federici et al., 2007) and zebrafish and Chapter 4. After each water change the tanks were re-dosed with 80 % of the nominated concentrations to bring the final tank concentration

back up to the full nominated dose (e.g. after an 80 % water change of the 1.0 mg l<sup>-1</sup> tanks 20 % of the dose remained in the water so 1.6 ml of the 10 g l<sup>-1</sup> stock was added to bring the total concentration back to 1.0 mg l<sup>-1</sup>). After addition of the dose the water was stirred on both sides of the screens using a glass rod to ensure even mixing. Control tanks were also stirred with glass rods to standardise stress across all tanks.

Water quality was measured daily. Parameters measured included temperature, dissolved oxygen (DO) and pH (Hach HQ40d multi meter) and total ammonia (HI 95715, Hanna Instruments).

Fish were sampled ( $n = 2$  fish per tank;  $n = 4-6$  fish/treatment) at the start of the exposure (initial fish, day 0) and day 40 for haematology, whole body ion analysis, histology and biochemistry.

### **6.3.2. Reproductive behaviour trial**

Several trials were conducted to establish a sound reproductive study protocol and to ensure that the test fish were sexually mature. For the stickleback trial there was the added complication of understanding the reproductive behaviour of the species. Observations were made over several months and trials examining the nest building behaviour of males were conducted to optimise the materials and methods used in the final trial.

After 28 days of exposure (day 29) males were provided with nest building materials in a similar fashion to that detailed by Barber et al. (2001). Briefly, 500 ml fine gravel substrate was placed over one half of the male's section of the tank. Then 250 ml fine sand was put on top of the gravel and 200, 60 mm long green polyester strands (false weed) were placed in a bundle into the sand and gravel. Nest building behaviours (digging activity, gluing activity, movement of sand and gravel and fanning) were noted throughout the exposure period. Six days after the addition of the nest building material

(day 35) the female was placed in with the male for 5 minutes to further stimulate the nest building of the males. Three days after this (day 38) the females were placed in with the male for 20 minutes for the recording of the reproductive behaviour with a digital video camera (Sony Handycam HDR-CX360VE). Recordings of two tanks per treatment (treatments were rotated to minimise time effects) were taken at the same time to minimise the total time taken to make all of the recordings (maximum of 4 hours between first and last recordings) and recordings were taken from 11am until 3pm after the fish had been fed. Previous observations indicated that there was little difference in activity levels during these four hours therefore time was not considered to be a variable for behaviour.

Any pairs that did not successfully spawn on day 38 were put together for a further 20 minutes the following day (day 39) to allow them a second chance for spawning. The pairs were not recorded this time but observations were made. The video footage was subsequently analysed for various behavioural end points using Noldus Observer XT (version 7) behavioural analysis software. End points analysed were both point and duration (state) behaviours and included dorsal pricking, leading to nest, biting, zigzag swimming, chasing and breeding (more details on behaviours can be found in Bell and Foster, 1994 and Sebire et al. 2008).

### **6.3.3. Titanium dioxide NP stock dispersions and dosing**

The details of the titanium dioxide NP powder used here are given in Chapter 2.

### **6.3.4. Haematology**

The details of blood collection and analysis are given in Chapter 2.

### **6.3.5. Whole body ion and trace metal analysis**

The details of the metal analysis protocol followed here are given in Chapter 2 with minor modifications in the samples preparation steps. Briefly, following blood sampling, fish were dissected for the gill basket, liver, muscle (flank muscle, de-skinned) and kidney. Tissues were placed onto clean glass slides and oven dried to a constant weight, then digested in 0.5 ml of concentrated nitric acid at 70 °C for 2 hours, and allowed to cool. Then, a 10 % solution of Triton X-100 was slowly added to each of the digested tissue samples to achieve a final concentration of 2 % Triton X-100 in each tube (0.4 ml of the 10 % Triton X-100 solution). Each sample was then diluted with ultrapure water to achieve a final volume of 2 ml (adding 1.1 ml ultrapure water) and analysed for Ti, Cu, Zn, Mn, Mg, Ca, Na and K by inductively coupled plasma optical emission spectrometry (ICP-OES, Varian 725 ES). Further details of the protocol are detailed in Chapter 2.

### **6.3.6. Biochemistry**

Fish were also collected for biochemistry. Fish were terminally anaesthetised with MS222 (without blood sampling), weight and total length was recorded, and then fish were dissected for brain, gill basket, liver and muscle (flank muscle, de-skinned). Tissues were immediately snap frozen in liquid nitrogen and stored at -80 °C until required for biochemistry. Tissues were homogenised (Pellet pestle, Sigma-Aldrich Company Ltd, Gillingham, Dorset, UK) in 10 volumes (100 µl for tissue weighing 10 mg) of ice-cold hypotonic buffer (in mmol l<sup>-1</sup>; 100 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-hydroxymethyl-1,3-propanediol). Homogenates were stored at -80 °C until required. Tissue homogenates were analysed (each sample in triplicate wherever possible) for total protein content and

total glutathione as described in Smith et al. (2007) with a few minor changes, the details of which are given in Chapter 2. Muscle homogenates were also analysed in triplicate for lactate dehydrogenase (LDH) activity and lactic acid (lactate) concentrations.

Lactate dehydrogenase (LDH) activity in muscle tissue was determined according to Bergmeyer and Bernt, (1974). Briefly, 40  $\mu\text{l}$  of diluted (final dilution of 1:20) tissue homogenate was added to a cuvette containing 2.8 ml assay buffer (50  $\text{mmol l}^{-1}$  sodium dihydrogen orthophosphate dehydrate and 0.6  $\text{mmol l}^{-1}$  sodium pyruvate) and 100  $\mu\text{l}$  of 9  $\text{mmol l}^{-1}$  NADH. Each sample was analysed using a Helios  $\beta$  UV-Vis Spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA) at 340 nm for 5 minutes. Change in absorbance (negative slope) per minute was recorded and units of LDH were determined (change in absorbance per min / (extinction coefficient of NADH ( $6.3\text{mM}^{-1} \text{cm}^{-1}$ )  $\times$  path length (cm)) = concentration ( $\text{mmol l}^{-1}$ )). Samples were analysed in triplicate where possible.

Lactic acid (lactate) content of muscle tissue was determined according to Gutmann and Wahlefeld, (1974) and as modified by Engle and Jones, (1978) with some minor modifications. Briefly, raw homogenates were deproteinated in an equal volume of 5 % trichloroacetic acid (TCA), centrifuged at 13,000 rpm for 3 minutes at 4  $^{\circ}\text{C}$ , and the resulting supernatant was pH neutralised with 1  $\text{mol l}^{-1}$  sodium bicarbonate. 10  $\mu\text{l}$  of tissue homogenate, blank or standard (0-8  $\text{mmol l}^{-1}$  L-lactic acid) was added in triplicate to wells of a 96-well plate containing 200  $\mu\text{l}$  glycine-hydrazine buffer (0.5  $\text{mol l}^{-1}$  glycine and 0.4  $\text{mol l}^{-1}$  hydrazine sulphate), 10  $\mu\text{l}$  of 9  $\text{mmol l}^{-1}$  NAD<sup>+</sup> and 1 U of L-lactate dehydrogenase. Plates were then covered and incubated at 37  $^{\circ}\text{C}$  for 2 h. Absorbance at 340 nm was measured using a microplate reader (as detailed above) and tissue lactate content ( $\mu\text{mol g}^{-1}$  wet weight tissue) determined using the standard calibration curve.

### **6.3.7. Histology**

The details of the histological analysis of tissues are given in Chapter 2.

### **6.3.8. Statistical analysis**

The details of the statistical analyses performed in the present study are given in Chapter 2.

## **6.4. Results**

There were no mortalities of adult sticklebacks throughout the exposure period and no abnormal behaviours (loss of equilibrium, refusal to feed) were observed in any of the treatments. Gross condition indices (condition factor, gonadosomatic index and splenosomatic index) showed no statistically significant differences for same-sex treatment comparisons (Figure 6.1 for *K* and GSI). Temperature, DO, pH and ammonia levels over the course of the exposure were (mean  $\pm$  SEM,  $n = 40$ ):  $16.1 \pm 0.1$  °C,  $10.2 \pm 0.1$  mg l<sup>-1</sup> (> 95 % saturation),  $7.4 \pm 0.1$  and  $0.1 \pm 0.02$  mg l<sup>-1</sup> respectively with no tank effects (pooled data).

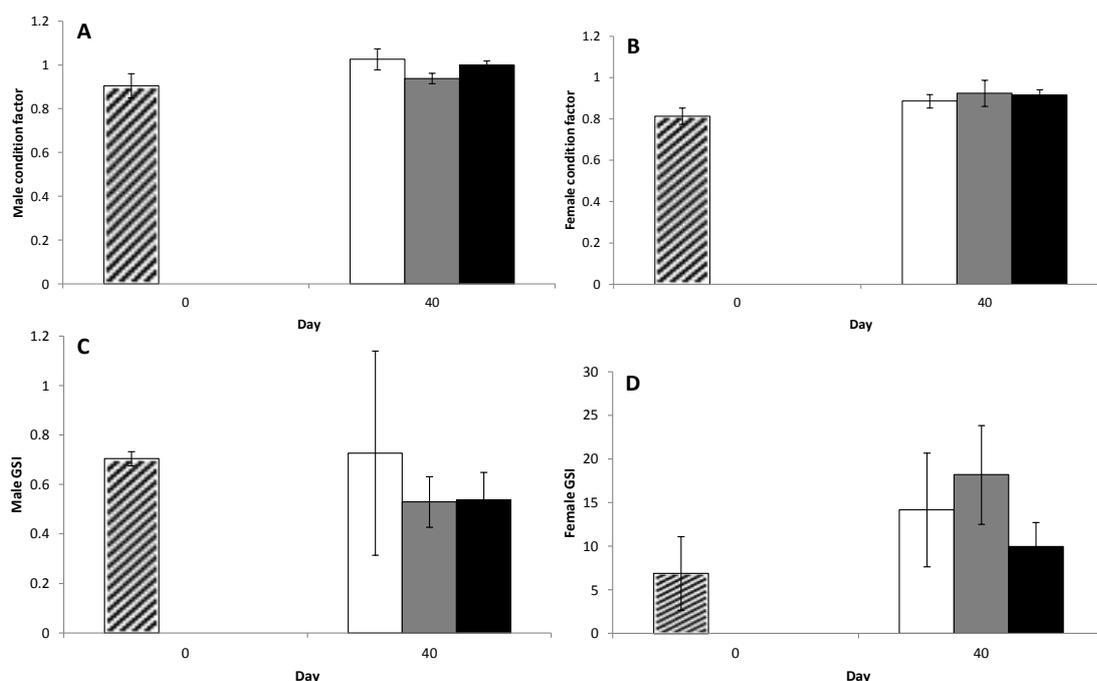


Figure 6.1. Condition factor in male (A) and female (B) sticklebacks and gonadosomatic index in male (C) and female (D) sticklebacks. Hatched bar at day 0 is initial fish, clear bars at day 40 are control fish, grey bars are 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub> fish and black bars are 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP fish. Data are mean ± SEM,  $n \leq 6$  fish per treatment. There were no significant differences (ANOVA,  $P > 0.05$ ).

#### 6.4.1. Confirmation of exposure

Water samples taken from the exposure tanks immediately after dosing confirmed that TiO<sub>2</sub> concentrations were initially met. Measured TiO<sub>2</sub> concentrations in the tanks immediately after addition were (mean ± SEM,  $n = 16$ ) < 0.004,  $1.03 \pm 0.01$  and  $0.95 \pm 0.02$  mg l<sup>-1</sup> for control, 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub> and 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NPs respectively. Measured TiO<sub>2</sub> concentrations in the tanks after 24 hours (prior to water changes) were (mean ± SEM,  $n = 16$ ) < 0.004,  $0.64 \pm 0.02$  and  $0.56 \pm 0.02$  mg l<sup>-1</sup> for control, 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub> and 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NPs respectively. As expected, there was some settling of TiO<sub>2</sub> from the water column over the 24 hour period between water changes. The percentage of TiO<sub>2</sub> still in suspension at the end of a 24 hour period relative to initial concentration were (% , data are mean ± SEM,  $n = 8$  tanks per treatment at 24 h after dosing):  $62.8 \pm 2.0$  and  $58.9 \pm 1.7$  for 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub> and 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP tanks respectively.

Tissue titanium content did not show evidence of any major accumulation of Ti from the water (Figure 6.2). Gill Ti content appeared to be higher for both the TiO<sub>2</sub> exposed groups but there were no significant differences to the control fish at day 40 or the initial fish at day 0 (Kruskal-Wallis,  $P > 0.05$ ). The other three tissues analysed (liver, muscle and kidney) all showed slight decreases in tissue Ti content from day 0 to day 40 (time, but no treatment, effects). This decrease was significant in the muscle tissue of all three exposure groups at day 40 compared to the initial fish (ANOVA,  $P < 0.05$ ).

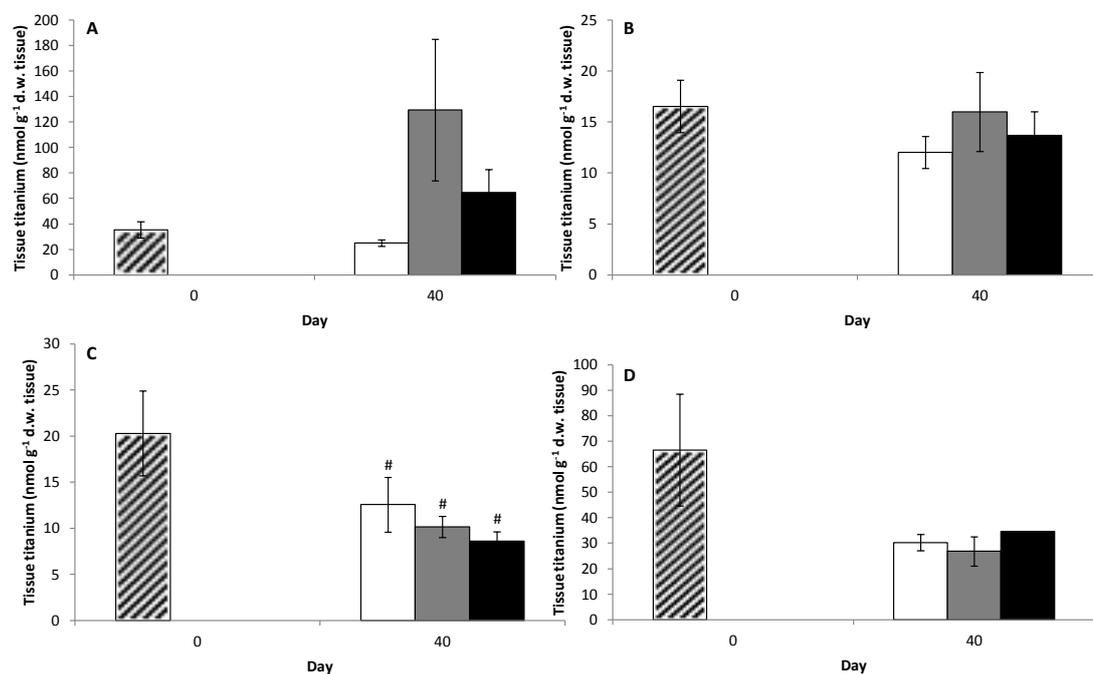


Figure 6.2. Total titanium concentration in stickleback (A) gill, (B) liver, (C) muscle and (D) kidney tissue digests. Hatched bar at day 0 is initial fish, clear bars at day 40 are control fish, grey bars are 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub> fish and black bars are 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP fish. Data are mean  $\pm$  SEM nmol Ti g<sup>-1</sup> dry weight,  $n \leq 6$  fish per treatment. # indicates significant difference to initial fish (ANOVA,  $P < 0.05$ ).

#### 6.4.2. Haematology

Erythrocyte counts from whole blood samples at day 40 were significantly lower in bulk TiO<sub>2</sub> exposed fish than NP exposed fish (ANOVA,  $P < 0.05$ ). However neither TiO<sub>2</sub> group was significantly different to the controls. Erythrocyte counts at day 40 were  $2.68 \pm 0.56$ ,  $1.50 \pm 0.55$  and  $3.31 \pm 0.35$  (cells  $\times 10^6$  mm<sup>-3</sup>, data are mean  $\pm$  SEM,  $n$

= 6 fish per treatment) for control, 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub>, and 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP respectively. Total white blood cell counts at day 40 were significantly lower for all treatments compared to the initial fish (ANOVA,  $P < 0.05$ ) with counts of  $8.9 \pm 1.25$ ,  $7.7 \pm 1.20$  and  $13.8 \pm 3.04$  (cells  $\times 10^3$  mm<sup>-3</sup>, mean  $\pm$  SEM,  $n = 6$  fish per treatment) for control, 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub>, and 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP respectively. There were no significant statistical differences in total leucocyte counts between any of the treatments at day 40.

#### **6.4.3. Whole body ions and trace metals**

Metal analysis of stickleback tissue acid digests showed no discernible treatment effects for any of the other seven metals analysed in any of the tissues examined. In the main tissue electrolytes there were two minor changes worthy of note. Gill potassium content was significantly lower in all three treatment groups at day 40 compared to initial fish (ANOVA,  $P < 0.05$ , Figure 6.3A). Liver calcium content was very high in three of the six fish analysed from the NP group at day 40, however there was no significant difference between treatments at day 40 (Kruskal-Wallis,  $P > 0.05$ , Figure 6.3B). There was no obvious sex difference in the liver calcium content for the NP group and the reason for the wide range in calcium content remains unclear. Total copper and zinc content in all tissue samples showed no obvious signs of disturbance with no statistical differences between treatments groups or over time ( $P > 0.05$ , data not shown). The same was true for both magnesium and manganese content with no significant differences either due to time or treatment ( $P > 0.05$ , data not shown).

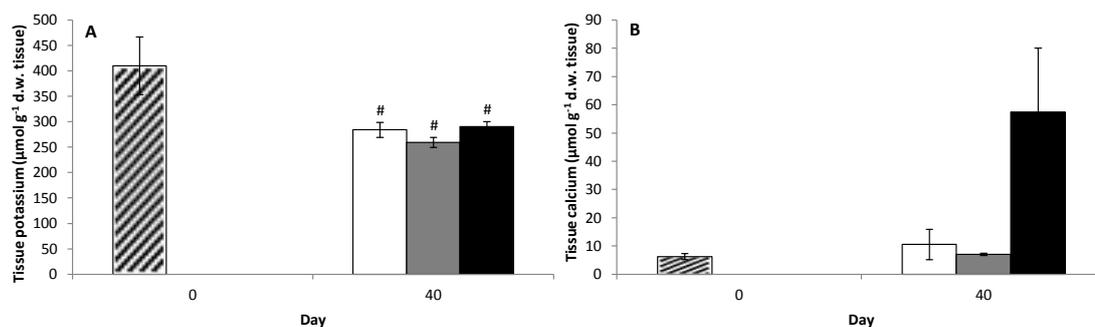


Figure 6.3. Total gill potassium (A) and liver calcium (B) concentration in stickleback tissue digests. Hatched bar at day 0 is initial fish, clear bars at day 40 are control fish, grey bars are 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub> fish and black bars are 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP fish. Data are mean ± SEM nmol Ti g<sup>-1</sup> dry weight, *n* ≤ 6 fish per treatment. # indicates significant difference to initial fish (ANOVA, *P* < 0.05).

#### 6.4.4. Biochemistry

Total glutathione (GSH) levels were measured in the brain, gill, liver and muscle tissues of sticklebacks. There were no significant time or treatment effects in any of the tissues measured (ANOVA, *P* > 0.05, Table 6-1).

Table 6-1. Total glutathione (GSH) at the end of the exposure period (day 40) in brain, gill, liver and muscle tissues of sticklebacks.

Treatment	Total glutathione (GSH) at day 40 (µmol GSH g wet tissue <sup>-1</sup> )			
	Tissue			
	Brain	Gill	Liver	Muscle
Control	2.17 ± 0.12	1.85 ± 0.06	1.92 ± 0.31	0.42 ± 0.04
1.0 mg l <sup>-1</sup> bulk TiO <sub>2</sub>	1.98 ± 0.07	1.52 ± 0.07	1.62 ± 0.31	0.57 ± 0.09
1.0 mg l <sup>-1</sup> TiO <sub>2</sub> NP	1.94 ± 0.12	1.81 ± 0.16	2.19 ± 0.48	0.55 ± 0.04

Lactate dehydrogenase (LDH) and muscle lactic acid (lactate) were measured in the muscle tissue of sticklebacks. There were no significant time or treatment effects in either of the two end points (ANOVA, *P* > 0.05, Figure 6.4).

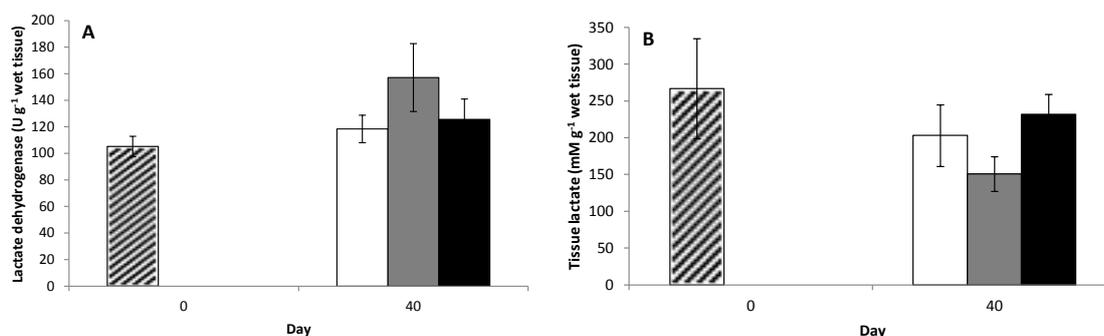


Figure 6.4. Lactate dehydrogenase (LDH) (A) and lactic acid (lactate) (B) content in stickleback muscle homogenates. Hatched bar at day 0 is initial fish, clear bars at day 40 are control fish, grey bars are 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub> fish and black bars are 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP fish. Data are (A) mean ± SEM LDH U g<sup>-1</sup> wet tissue and (B) mean ± SEM lactate mM g<sup>-1</sup> wet tissue, *n* = 6 fish per treatment. There were no significant time or treatment differences in either assay (ANOVA, *P* > 0.05).

#### 6.4.5. Histology

Gill, liver, gonad and muscle tissues were analysed for any evidence of pathological change. There were no significant changes in any tissues examined. Quantitative data was obtained where possible; for example the percentage of club tips on secondary lamellae of the gills were calculated but there were no significant differences:  $4.94 \pm 1.23$ ,  $5.43 \pm 1.83$  and  $2.36 \pm 0.81$  % (data are at day 40, mean ± SEM % club tips, *n* ≤ 6 fish per treatment) in control, 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub> and 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP groups respectively. The liver tissue showed no overt signs of pathology, with no significant changes in the percentage of sinusoid space relative to the parenchyma. Mean percentage of sinusoid space relative to the parenchyma at day 40 was:  $11.4 \pm 1.3$ ,  $8.5 \pm 1.9$  and  $9.3 \pm 2.5$  (data are mean ± SEM, *n* ≤ 6 fish per treatment) in control, 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub> and 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP groups respectively. There were no differences in the level of lipid peroxidation in liver tissue with signs of lipid peroxidation in all of the exposure groups including the control group. There were no differences in the level of lipid peroxidation in liver tissue with signs of lipid peroxidation in all of the exposure groups including the control group.

The architecture of the muscle was normal for all treatment groups with no evidence of muscle atrophy, hydropic change in the muscle fibres, inflammation or necrosis. There was no loss of structure of the fibre bundles (fibrosis) compared to the controls, although this was difficult to discern due to some background dehydration artefacts within the skeletal muscle fibres (not treatment-related). The structure of gonadal tissue was also normal in all treatment groups with no occurrence of ova-testis, no evidence of reactive hyperplasia, or atrophy of ova or testis. Female gonad showed a normal spread of oocyte developmental stages in all treatments. Mean follicular size was calculated and there were no significant differences between treatments or over time (ANOVA,  $P > 0.05$ ). Mean follicular diameters at day 40 were:  $326.5 \pm 50.5$ ,  $430.5 \pm 12.5$  and  $329.0 \pm 14.0$   $\mu\text{m}$  (data are mean follicular size  $\pm$  SEM,  $n \leq 3$  females per treatment group) in control,  $1.0 \text{ mg l}^{-1}$  bulk  $\text{TiO}_2$  and  $1.0 \text{ mg l}^{-1}$   $\text{TiO}_2$  NP groups respectively.

#### **6.4.6. Reproductive behaviour trial**

Sexual maturation was apparent in all pairs of fish in both the control and  $1.0 \text{ mg l}^{-1}$  bulk  $\text{TiO}_2$  group. Only one female in the  $1.0 \text{ mg l}^{-1}$   $\text{TiO}_2$  NP group did not show any signs of interest in the male, or any obvious gamete production. Males from all treatment groups showed some level of nuptial colouration and there were no apparent differences between either the number of males displaying colouration or the level of colouration displayed between treatment groups.

Nest building was monitored once the nest building material had been provided (Figure 6.5). Of the eight males in the control group six built good quality normal nests (clear evidence of the use of gravel, sand and weed materials and a clear entrance present) whilst the other two males displayed digging and gluing activity but did not produce a normal nest structure. All of the males in the  $1.0 \text{ mg l}^{-1}$  bulk  $\text{TiO}_2$  group displayed all elements of nest building behaviours and produced good quality normal

nests. Of the eight pairs in the  $1.0 \text{ mg l}^{-1} \text{ TiO}_2 \text{ NP}$  group one pair appeared not to show any of the nest building behaviour. It was confirmed upon sampling that the pair consisted of two females; therefore all behavioural data for the NP group is based on  $n = 7$ . All of the seven males in the NP group displayed all elements of nest building behaviour and produced good quality normal nests. There were no apparent differences in nest quality or structure between any of the treatment groups.

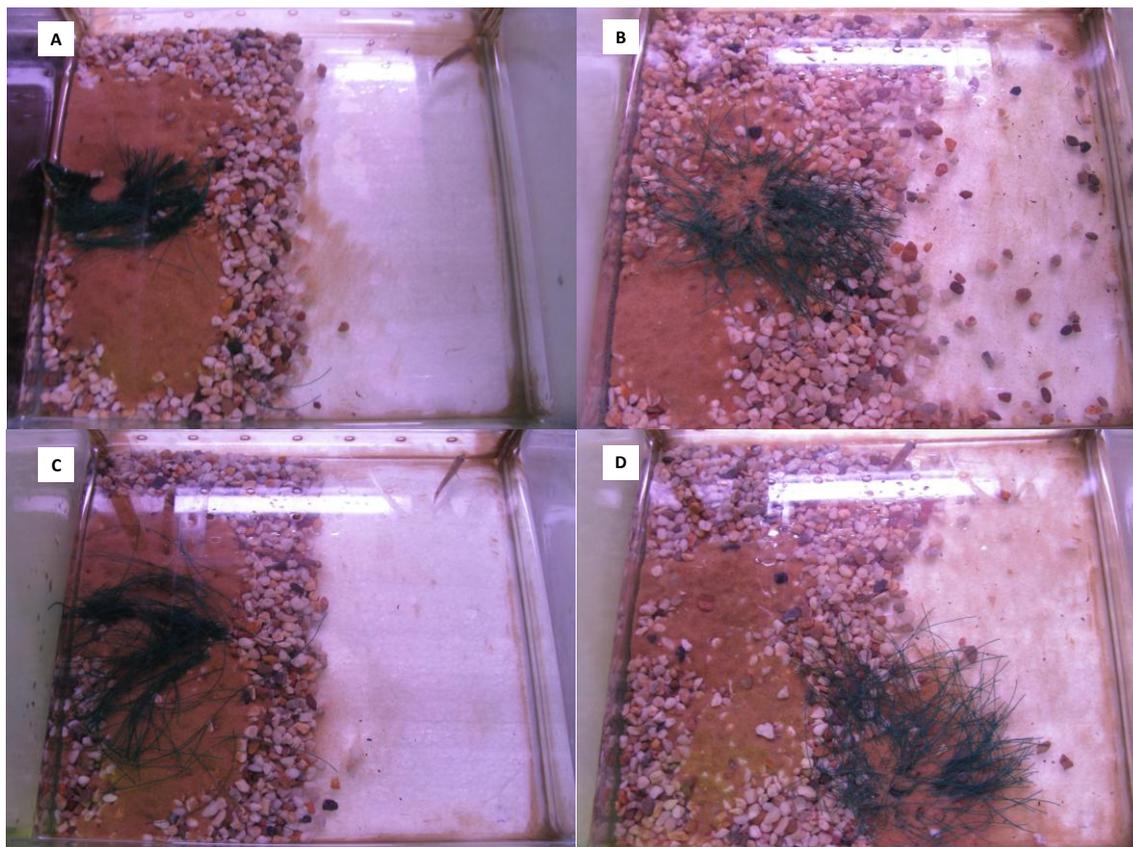


Figure 6.5. Examples of nest material position in control (A) and  $\text{TiO}_2 \text{ NP}$  (C) tanks immediately after addition of the nest building material and in control (B) and  $\text{TiO}_2 \text{ NP}$  (D) tanks six days after the addition of the material. The gravel and sand has been moved, the green false weed has been repositioned and there is a clear entrance to the nests.

The 20 minute behavioural recordings were analysed for number of bites (male behaviour), number of chasing events (male behaviour) and number of zigzag bouts (male behaviour). There were no significant differences between treatment groups for any of these end points (ANOVA,  $P > 0.05$ , Figure 6.6). The number of chasing events and the number of zigzag bouts both appeared to show a treatment related decline however this trend was not significant. There were no differences between the number

of successful spawning events between treatment groups with three of eight, one of eight and two of seven pairs successful in the control, 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub> and 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP groups respectively.

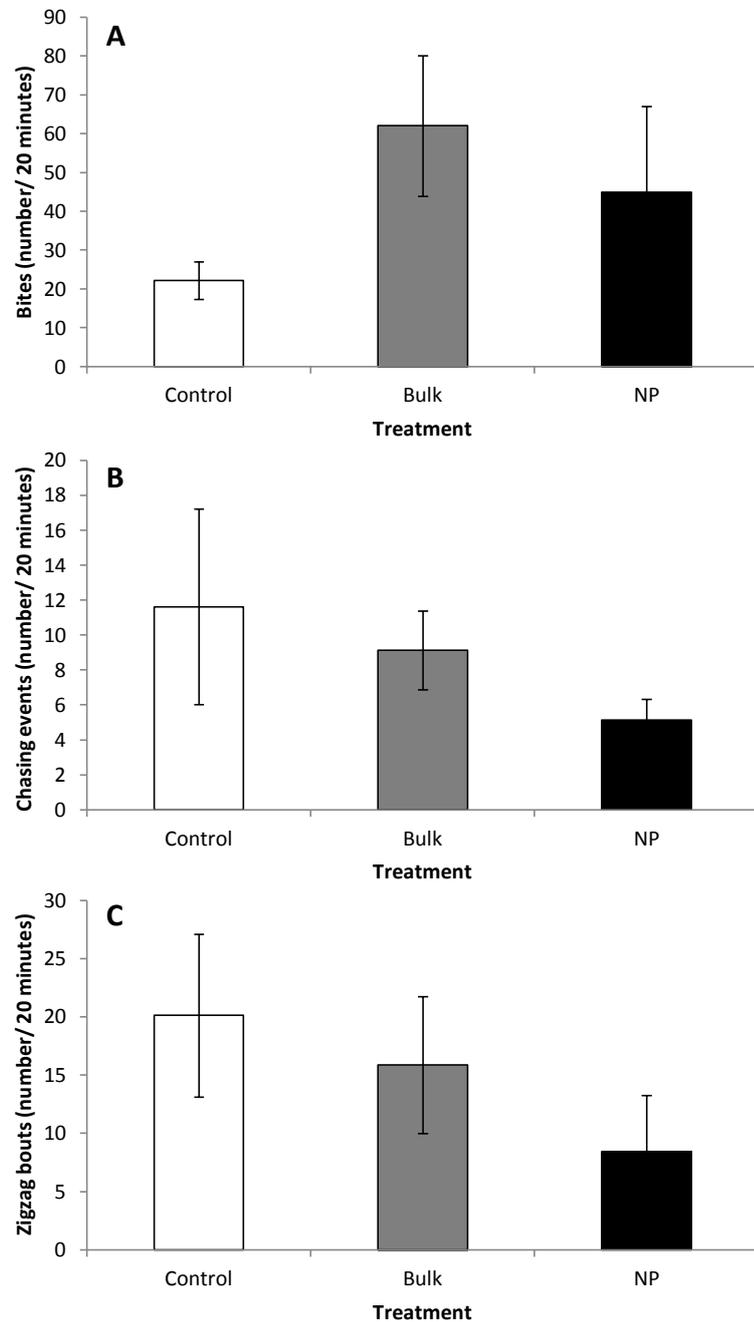


Figure 6.6. Reproductive behaviour analysis of (A) number of bites, (B) number of chasing events and (C) number of zigzag bouts in sticklebacks (day 38). Clear bars are control fish, grey bars are 1.0 mg l<sup>-1</sup> bulk TiO<sub>2</sub> fish and black bars are 1.0 mg l<sup>-1</sup> TiO<sub>2</sub> NP fish. Data are mean  $\pm$  SEM,  $n \leq 8$  pairs per treatment. There were no significant treatment differences in any of the end points tested (ANOVA,  $P > 0.05$ ).

## 6.5. Discussion

### 6.5.1. Confirming exposure to TiO<sub>2</sub>

There were no mortalities in the present study and all fish exhibited normal behaviour throughout the exposure period, including the behavioural phases at the end of the exposure. The exposure was confirmed by measuring the total Ti metal concentrations in the tanks after dosing, and over the course of 24 h the inevitable settling behaviour of the material caused this to decline to around 60 % of the initial dose for both TiO<sub>2</sub> bulk and NP material types. In this study we used a semi-static exposure regime to renew the test water every 24 h. More frequent water changes (e.g., every 12 h in our trout studies, Federici et al., 2007) can give Ti concentrations in the tank water closer to the initial values, but at the same time this will also disturb fish behaviours, and our objective was to ensure the fish would also be able to breed. The 24 h water change was chosen as the best compromise for this dilemma based on our pilot studies, and was also used by Wang et al. (2011).

Ti concentrations in gill, liver, muscle and kidney tissue were measured (Figure 6.2) with Ti elevations in gill tissue of both TiO<sub>2</sub> treatments compared to the no-added TiO<sub>2</sub> controls. There was however a large amount of variation in Ti concentration in the gill tissue and as a result there was no significant difference between the TiO<sub>2</sub> treatments and the controls. The variation is likely due to differing levels of aggregation of TiO<sub>2</sub> particles in the gill baskets rather than uptake through the gill membrane. The absence of clear Ti elevation in gill tissue of fish exposed to TiO<sub>2</sub> has also been reported by our laboratory previously (Federici et al., 2007). Looking at the other tissues measured it seems that there was no internalisation of the TiO<sub>2</sub> as Ti concentrations in liver, muscle and kidney tissues showed no significant differences between treatment groups. Ti concentrations actually decreased significantly from the initial fish concentrations in muscle tissue (Figure 6.2C). Depuration of tissue Ti has been shown previously in

rainbow trout intestine and spleen tissues (Ramsden et al., 2009) and is in contrast to elevated gonadal Ti in zebrafish exposed to TiO<sub>2</sub> NPs (Wang et al., 2011). It appears that TiO<sub>2</sub> NPs are not able to cross the gills into the circulatory system; or if they are the fish can either excrete them or sequester them in other tissues not measured in the present study. Whether or not TiO<sub>2</sub> NPs can be internalised and, in the event that they are, the exact mechanism involved in Ti distribution within the fish remains unclear.

### **6.5.2. Haematology, electrolytes and trace metals**

There were no major disturbances to haematology with no significant treatment effects for either erythrocyte or leukocyte counts. This is consistent with our previous reports on trout and zebrafish which both show normal haematology over 2 weeks exposure to TiO<sub>2</sub> NPs (see Federici et al., 2007 and Chapter 4 respectively). Tissue electrolyte and trace metal concentrations did not show any major disturbances over the duration of the exposure. There were no significant treatment-related changes in Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup> concentrations which indicates good general health and suggests that the fish were able to maintain good osmoregulatory function (Figure 6.3). The decrease in gill potassium for all treatments at day 40 compared to initial fish (Figure 6.3A) is likely a result of a change in water chemistry between stock and experimental tanks. The increase in liver calcium of TiO<sub>2</sub> NP exposed fish (Figure 6.3B) was not significant. Therefore there is no obvious evidence for any osmoregulatory dysfunction or inhibition of oocyte production in TiO<sub>2</sub> exposed fish. Some trace elements have specific functions in the reproductive systems of vertebrate animals. Zinc plays a key role in the production of egg proteins and its absence can be a good indicator of reproductive stress (Thompson et al., 2003). However in the present study there were no changes in zinc concentrations in any of the tissues measured. There were also no treatment-related changes in tissue Mn, Mg or Cu during the exposure (data not shown).

### **6.5.3. Biochemistry**

Nano-scale TiO<sub>2</sub> is known to produce reactive oxygen species (ROS), especially the hydroxyl radical ( $\cdot$ OH) (Reeves et al., 2008; Xiong et al., 2011), and there are several reports of changes in oxidative stress parameters in fishes with TiO<sub>2</sub> NPs (trout, Federici et al., 2007; Reeves et al., 2008; Xiong et al., 2011). The induction of glutathione is a well-known defence against oxidative stress, but in contrast to the above findings and those in Chapter 4, in the present study there were no significant changes in GSH in any of the tissues measured (Table 6-1). It is possible that during the exposure period glutathione levels did change but by the end of the exposure period of 40 days the fish had regained oxidative control.

Metal exposure can lead to decreased metabolic scope for swimming activity (Campbell et al., 2002; 2005) and muscle lactic acid (lactate) and lactate dehydrogenase (LDH) can give an insight into the energetic status of a fish. In the present study both lactate and LDH were measured in the muscle tissue of sticklebacks and the results showed no significant differences between any of the treatments (Figure 6.4). These results, together with the absence of any significant weight loss and the presence of nest building behaviour and spiggin production suggests that the fish were able to maintain sufficient energy stores for all aspects of energetic expenditure, including reproduction.

### **6.5.4. Histology**

The histopathological effects of TiO<sub>2</sub> NP exposure have been reported in both mammals (Bermudez et al., 2004; Warheit et al., 2007) and fishes (Federici et al., 2007; Chen et al., 2011b). Gill injuries reported include oedema and fusion of the secondary lamellae, swollen mucocytes and hyperplasia of the primary lamellae (Federici et al.,

2007; Chen et al., 2011b). In the present study there was no evidence of acute oedema or epithelial lifting, irritation of the mucocytes or reactive hyperplasia in the gills from any of the treatments. Notably, for exactly the same material and exposure used here, Federici et al. (2007) noted some significant oedema and epithelial damage in the trout gill. This suggests that sticklebacks are more tolerant of TiO<sub>2</sub> exposure compared to trout which is a similar finding to a study on zebrafish carried out by our laboratory (Chapter 4). Chen et al. (2011b) also examined the effects of TiO<sub>2</sub> NPs in a waterborne exposure to zebrafish and found only minor gill injury with concentrations up to 5 mg l<sup>-1</sup> TiO<sub>2</sub> NPs for up to six months.

Other organs commonly examined in toxicity tests are the brain, intestine and liver. Chen et al. (2011b) found no obvious histological changes in the brains, hearts or livers of the zebrafish exposed to TiO<sub>2</sub> NPs at concentrations up to 7 mg l<sup>-1</sup>. These findings seem to agree with the results of the present study as there was no evidence of any significant pathology in livers or brains of any TiO<sub>2</sub>-exposed fish. The livers of TiO<sub>2</sub> NP-exposed fish showed no significant change in the proportions of sinusoid and somatic space and there were no clear signs of energetic stress. It seems that, even at concentrations an order of magnitude above environmentally relevant concentrations, sticklebacks do not show any obvious signs of tissue damage, which is in contrast to the more sensitive rainbow trout which can suffer both gill damage and minor fatty change of the liver in a similar exposure situation to the present study (Federici et al., 2007).

#### **6.5.5. Effects of TiO<sub>2</sub> exposure on reproductive behaviour**

There have been a number of studies published which have examined the toxicity of NPs to adult fish or to embryos (review in Handy et al., 2011); however there are very few which examine the effects of NP exposure on reproduction. With regards to the three-spined stickleback there are currently no reports of the effects of NPs on

reproductive behaviour and only one brief report on the physiological effects of CdS NPs (Sanders et al., 2008). The majority of studies using the stickleback have examined EDCs. In the present study there was no evidence of any morphological change in the gonadal tissue of TiO<sub>2</sub> exposed sticklebacks and there were no differences in the mean follicular size in ovarian tissue samples.

There were no significant differences in the male reproductive behaviour end points tested herein (Figure 6.6). In their study examining the effects of CdS NPs on three-spined sticklebacks, Sanders et al. (2008) noted an absence of nest building behaviour in all treatment groups of one of the replicates. The authors suggested that this absence of reproductive behaviour was likely due to the fish not being in the correct physiological status for spawning. This was not the case for the present study as all males in both TiO<sub>2</sub> treatment groups displayed nest building behaviours and produced good quality nests. This suggests that exposure to TiO<sub>2</sub> in both bulk and nano form does not impair the ability of sexually mature males to build nests. The next stage of reproductive behaviour in males after a nest has been built is courtship. Courtship behaviours were recorded on day 38 of the exposure period and there were no significant differences between treatment groups in any of the behaviours tested (Figure 6.6). There is a possibility of a subtle treatment effect as both the number of chasing events and the number of zigzag bouts was lower in the TiO<sub>2</sub> NP exposed males than the control and bulk TiO<sub>2</sub> groups. These results were not statistically significant but it is possible that after a longer exposure period these behavioural differences may become more pronounced. It would appear that the reproductive behaviour of the three-spined stickleback is not overtly sensitive to TiO<sub>2</sub> NP exposure. The efficacy of this assay remains good, however for this species at the exposure concentrations and periods used herein more sensitive assays or end points may be necessary to monitor any toxicological effects that may occur.

### **6.5.6. Conclusions**

In the present study we demonstrate that even at concentrations an order of magnitude above environmentally relevant concentrations TiO<sub>2</sub> NPs do not elicit overt toxicological changes in three-spined sticklebacks. All of the main physiological end points examined showed little evidence of any treatment effects in either the material types. Histological examination of the main target organs showed little evidence of any major tissue injury and the fish appeared to be in good energetic health for the duration of the exposure period. The use of the stickleback reproductive behaviour assay proved to be an effective tool in assessing the impact of an environmental pollutant on life history traits; but in the case of TiO<sub>2</sub> NPs there appears to be little detriment to the reproductive health of the stickleback. It is however possible that under more prolonged circumstances there may prove to be more significant changes in these behavioural end points which may become problematic to the future health of an exposed population. We suggest that future focus should be on the biochemical effects of environmentally relevant concentrations of TiO<sub>2</sub> NPs and that chronic exposure studies should continue to investigate the potential harm of NPs to reproductive health and bioenergetic costs.

### **6.6. Acknowledgments**

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## 7. General discussion

This thesis aimed to examine a few key areas relevant to NP toxicity. The aims were firstly to improve the detection and characterisation of NPs in stock suspensions and more complex matrices such as tissues. The second aim was to examine the effects of TiO<sub>2</sub> NPs and SWCNTs on the general physiology and reproductive output of the zebrafish. The third aim was to examine the effects of NPs on the reproductive behaviour of the three-spined stickleback. Lastly the thesis aimed to put these findings into context in relation to the possible effects that NP exposure may have on the bioenergetics of fish and the knock-on effects on the survival of the population. These aims were in place to address the main hypothesis that exposure to manufactured NPs would result in disturbances to general physiology, reproduction and the behaviours of fish.

### 7.1. Detection and characterisation of NPs

One of the key issues in testing the effects of NPs on organisms is being able to characterise the NPs being used. This step is highly important to ensure that the investigator can put the appropriate material type and size controls in place in order to offer evidence of specific material type effects, and to confirm the details of the exposure. The other reason for needing this characterisation is to be able to compare between studies so that an understanding of species specific differences can be gained and the possibility of underestimation of the toxicity of a NP is less likely.

The data in Chapter 3 shows good progress in the ability to measure Ti from TiO<sub>2</sub> NPs in complex tissue and whole fish acid digests. In spike recovery tests at the start of the investigation the recoveries of Ti from a known spike were as low as 3 % and typically averaged 20 %. After several series of tests and trials this recovery was

improved to up to approximately 85 % with an average of around 55 %. The three fold increase in recovery is good but clearly there is still room for improvement. Whilst other laboratories claim to achieve better recoveries than this the methods they employ are either more costly both in time and money, or simply not safe/practical for general ecotoxicological testing in volume (high throughput analysis). The other novel contribution that the work detailed in Chapter 3 makes is the possibility of being able to characterise the particle size distribution of TiO<sub>2</sub> NPs in complex matrices like fish tissue digests. Whilst the approach of single particle ICP-MS for tissues is only in its infancy there have been a small number of proof-of-principle studies for characterising NPs by using single particle ICP-MS in water (see Chapter 3 for a brief review). The study detailed in Chapter 3 is currently the first contribution to this field with regards to characterising TiO<sub>2</sub> NPs in tissues.

## **7.2. A comparison of nanoparticle type effects in the zebrafish**

The work in Chapters 4, 5 and 6 enables some comparison between materials, and across species of fish. Comparing firstly the effects of NP type in the same species (Chapters 4 and 5 on zebrafish, TiO<sub>2</sub> NPs versus SWCNTs) there are some clear similarities. Haematology of zebrafish appears to be unaffected by exposure to either TiO<sub>2</sub> NPs or SWCNTs. The minor transient changes that were observed in both trials were not significantly treatment related and there were no obvious signs of haematological disturbance after exposure to either nanomaterial. These results are amongst the first such reports but they are in agreement with findings in rainbow trout after TiO<sub>2</sub> NP and SWCNT exposure (Federici et al. 2007 and Smith et al. 2007 respectively).

Trace metal analysis of the zebrafish was carried out using the whole zebrafish. As such no tissue specific effects could be analysed, however there were some subtle shifts

if whole body trace metals in both experiments. The only statistically significant change was in the zebrafish exposed to both TiO<sub>2</sub> bulk and NP treatments at 1 mg l<sup>-1</sup>. These fish showed a decrease in total zinc content at the end of the exposure period (day 14). The fish in both of these treatment groups also showed decreased fecundity after the exposure period during the reproductive trials. Whether these two facts are linked remains unverified but zinc is known to be important in the production of egg proteins (Thompson et al. 2003). There were no other significant changes in any of the bulk electrolytes or the other trace elements that were tested in both experiments. This indicates that for both of the two nanomaterials, at the concentrations tested, there is little toxicity caused to metal homeostasis in zebrafish.

There is little toxicity to the osmoregulatory function of zebrafish caused by exposure to either type of nanomaterial. The Na<sup>+</sup>K<sup>+</sup>-ATPase activity in all of the tissues tested (brain, gill and liver) was not changed as a result of TiO<sub>2</sub> NP exposure and there were also no significant changes in the same tissues tested in the fish exposed to SWCNTs. Total glutathione (GSH) is a marker of oxidative stress and it is here that there was a difference between the two nanomaterials. The GSH levels in fish exposed to TiO<sub>2</sub> NPs at both 0.1 and 1 mg l<sup>-1</sup> were significantly higher in all three tissues tested than the time-matched bulk and control groups after 14 days exposure. This was the only nano-specific effect observed in the entire experiment (Chapter 4) and is different to the SWCNT exposure where there was no significant treatment effect on GSH levels across the whole 21 exposure period. It seems that TiO<sub>2</sub> NPs have a significantly higher capacity for ROS generation than both the bulk TiO<sub>2</sub> powder and SWCNTs.

The effects of the two nanomaterials of interest on the gross histology of the zebrafish was investigated after previous studies reported evidence of pathology as a result of exposure to both of these nanomaterials (see Chapter 1 for a review). In the present studies however there was no evidence of any gross pathology caused by either

of the two exposure regimes. Being aqueous exposures the most likely source of pathology would have been the gill tissue, but at the concentrations used there was no evidence of any major gill tissue damage. These findings seem to agree, at least for TiO<sub>2</sub> NPs, with a recent report in the literature where significant gill pathology in zebrafish was only seen in fish exposed to 5 mg l<sup>-1</sup> or more for a period of six months (Chen et al. 2011b). Certainly it seems unlikely that zebrafish are the most sensitive species for histological end points in ecotoxicological testing. The salmonids remain the species of choice in order to properly assess the potential for morphological damage as a result of nanomaterial exposure.

In contrast, the results of the reproductive end points that were investigated in the present studies suggest that there is scope for the reproductive assay in ecotoxicological testing with nanomaterials. After exposure to both TiO<sub>2</sub> and SWCNTs there was some evidence of stress to the reproductive output of the zebrafish. At this point it is worth comparing the designs of the reproductive studies in Chapters 4 and 5. Chapter 4 outlines the idea of group spawning events before and after the exposure period. This approach was chosen for the benefits of higher potential embryo yield and higher likelihood of successful pairings, but after the experiment it became clear that there were some flaws in the design. The fish that were left at the end of the exposure period were quite stressed after all the netting of the sampled fish. Therefore on the first post-exposure spawning event there was very little successful reproduction. At day 9 post-exposure there was a significant difference between the two high dose TiO<sub>2</sub> groups (both bulk and NP) and the low dose NP and controls groups in the number of viable embryos produced (Figure 4.4). By the time of the last spawning event at 16 days post-exposure all groups seemed to be on the recovery and there was no longer any significant difference in embryo production. It was clear that there was a small window

where differences could be seen but the level of variability in the reproduction data called for a more robust approach.

The new design using individual breeding pairs in Chapter 5 yielded a much higher  $n$ -value (increasing from  $n = 3$  to  $n = 9$  from Chapter 4 to Chapter 5) and as such more robust data was attained. The other main difference in design was the change from spawning events before and after the exposure period (Chapter 4), to spawning events before and during the exposure (Chapter 5). This approach meant that the decline (if any) in reproductive output could be monitored whilst the fish were being exposed to the nanomaterial; the level of stress was also lower thereby reducing the number of variables to only the treatment effect. The results of this modified design were less variable than the previous experiment however it was still sufficiently high to give a non-significant analysis of the reproduction data (Figure 5.5). There was however a clear trend observed in the SWCNT exposed fish with a reduction in the number of viable embryos produced during the exposure period. It seemed likely that this trend would have continued if the exposure had been prolonged.

Despite the level of variation which seems to be inevitable in reproduction studies it was apparent that the reproductive end points were the most sensitive of all those tested for zebrafish exposed to both TiO<sub>2</sub> NPs and SWCNTs. The controls that were used to control for particle size and material type also proved very useful in either confirming lack of toxicity caused by dispersants (SDS) or indeed in putting the assumed safety of TiO<sub>2</sub> bulk particles in doubt. As a result of these relatively successful reproductive studies the three-spined stickleback was chosen as a key local species to investigate further into the possible reproductive toxicity of TiO<sub>2</sub> NPs.

### **7.3. A comparison between the zebrafish and the three-spined stickleback**

Following on from the previous two Chapters (4 and 5), Chapter 6 investigated the effects of TiO<sub>2</sub> NP exposure on the three-spined stickleback. The experimental design was developed after the results of the previous studies had been analysed. The modifications for the experiment included prolonging the exposure period after the relatively low level of sublethal toxicity seen in Chapter 4 and increasing the focus on the reproductive end points, including reproductive behaviour. The design involved the use of pairs of fish (one male and one female) in each tank and as such the *n*-value of the design had to be increased in order to make the statistical analysis of simple effect/no-effect comparisons more robust.

The general physiology of the sticklebacks after a 40 day exposure was investigated to allow direct comparison between species. The results given in Chapter 6 outline the main findings. Briefly, there were similarities between the stickleback and the zebrafish in many end points. Haematology and trace metal analyses showed very little evidence of any toxicity caused by a 40 exposure to TiO<sub>2</sub> NPs. There was little evidence of any Ti accumulation in the tissues tested; even the gill tissue increase in Ti content was not significantly higher than the controls. Biochemical analysis also showed very little disturbance to the sticklebacks in terms of oxidative stress and bioenergetic costs. This is where a species difference was seen as the stickleback showed no elevation in GSH after 40 days compared to the nano-specific elevation seen in the zebrafish exposed for only 14 days. There are two possible reasons for this difference; the stickleback is less sensitive to oxidative stress than the zebrafish, or the prolonged exposure period allowed the stickleback to adjust to the increased oxidative stress so the GSH levels had returned to normal levels by the time of sampling.

Histological analysis of the sticklebacks showed similarity to the zebrafish experiments with very little evidence of any pathology in any of the tissues examined. Again there was little sign of tissue damage in the gill and gonad morphology appeared

normal. It was apparent that, much like the zebrafish, the three-spined stickleback was less sensitive to nanomaterial exposure than rainbow trout (e.g. gill pathology observed by Federici et al. 2007).

Due to the design of the stickleback experiment and the different life history traits of the stickleback compared to the zebrafish it was not possible to monitor the cumulative production of embryo production in any meaningful way. The benefit of the design did however allow for a sensitive examination of reproductive behaviours, in particular in the male stickleback. The results detailed in Chapter 6 outline the effect of TiO<sub>2</sub> exposure on the behaviour of the stickleback with very little evidence of any perturbation to the reproductive behaviours of the fish. It is possible that under more chronic exposure conditions the small differences observed in male behaviour may become more apparent, however at the concentrations and periods used in the experiment there was no significant effect of TiO<sub>2</sub> exposure on the three-spined stickleback. This conclusion is similar to those outlined above for the zebrafish, with only minor differences between the species indicating that perhaps the zebrafish reproduction model is the more sensitive tool for assessing nanomaterial toxicity.

#### **7.4. Nanomaterials and environmental protection**

The potential for unique physicochemical properties causing toxicity to wildlife is the reason for the recent surge of interest in nanomaterials. At the same time there has been a similar surge in research to ensure that the natural environment is adequately protected against the potential problems these unique properties may cause. There is a growing body of research which has investigated a wide range of nanomaterials and their toxicity to a similarly wide range of test species. It seems that there may be a need to make some adjustments to the studies that are carried out in order to bring some form of standardisation to tests so that the most species are protected for in the environment.

For example, the work in this thesis has shown that TiO<sub>2</sub> NPs have the ability to generate a low level of sublethal toxicity to zebrafish, but without the wide range of end points that were tested these effects may have been overlooked. There is also a clear difference between species in their sensitivity to nanomaterials and it is important that the ecotoxicological tests that are used to assess nanomaterial toxicity in the future are tailored to use the most sensitive species in the most sensitive testing regimes possible.

It will also be very important to be able to properly characterise the nanomaterials being tested so that true comparisons between species can be made. The range of primary particle size and dispersion steps for TiO<sub>2</sub> alone (Table 1-1) is such that it is difficult to come to a conclusion on their toxicity. Added to this is the complexity of the manner in which the particles behave in various aqueous environments. There remains one main benefit of studies using TiO<sub>2</sub> or any other metal-based nanomaterial over carbon-based ones – there are currently more reliable techniques for analysing metals than there are for distinguishing between natural and man-made carbons. It was not possible to determine the exact concentration of SWCNTs in the exposure with zebrafish in Chapter 5. Improvements must be made if accurate, repeatable ecotoxicological tests continue to be carried out with carbon-based nanomaterials (see Petersen and Henry 2012 for a review). It is currently possible to radio- or fluorescently- label carbon nanomaterials (see Chapter 1 for a discussion) but in doing this there is the possibility of altering the surface properties of the particles, thereby potentially affecting their toxicity.

The take-home message must be that there is a need to standardise the properties of, and protocols applied to, nanomaterials when carrying out ecotoxicological studies. There is also a need to focus studies on particular species which have particular sensitivity to certain assays. The current framework for material testing concerns only the individuals being tested. The work carried out in this thesis has demonstrated the

importance of considering not only the exposed individuals but also the potential effects to the wider population. With nanomaterials accumulating in the environment a chronic exposure to multiple generations will be the most likely scenario to generate significant toxic effects to the detriment of the populations. Taking a worst-case scenario approach to testing will ensure that any legislation applied to the use and disposal of nanomaterials is as safe and robust as possible. Whilst nanomaterials will continue to improve various aspects of human life it remains vital to protect the natural environment.

## **7.5. Conclusions and future work**

The work in the current collection of studies has produced important novel information regarding the characterisation and toxicity of TiO<sub>2</sub> NPs and SWCNTs. An improved method for measuring fish tissues dosed with Ti from TiO<sub>2</sub> has been described and the first steps in particle size characterisation in complex matrices have been made. The studies have also demonstrated the comparative differences in toxicity between TiO<sub>2</sub> NPs and SWCNTs, as well as species differences between the zebrafish and the three-spined stickleback. It is clear that for the two nanomaterials herein, there are certain biological end points which have higher sensitivity and should be focussed on in the future. In particular the reproductive end points seem to be the most promising and sensitive in determining the possible toxicological effects of nanomaterials which do not show overt acute toxicity. It has been shown that there are still uncertainties relating to NP toxicity, but there are areas in which future work can focus. The effects of nanomaterials on the population level of fish species is of high importance, as is continuing the drive to better characterise nanomaterials in both simple and complex matrices. Whilst it seems that there are many nanomaterials which may induce more

overt toxicity than the two used herein it is vital not to ignore the subtleties of toxicity that have been identified.

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