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HISTOPATHOLOGICAL EFFECTS OF METAL AND METALIC NANOPARTICLES ON THE BODY SYSTEMS OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

by

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

DOCTOR OF PHILOSOPHY

School of Biomedical and Biological Sciences

Faculty of Science and Technology

June 2013

Histopathological effects of metal and metalic nanoparticles on the body systems of rainbow trout (Oncorhynchus mykiss)

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Abstract

Histopathology studies of metal nanoparticles (NPs) compared to traditional forms of metal in fish are scarce. Additionally, it is unclear whether metal nanoparticles cause greater or different pathologies compared to other forms of metal. The current study aimed to assess the pathological effects of Cu-NPs and TiO_2 NPs on rainbow trout via various routes of exposure and, where appropriate, to compare them to either the equivalent dissolved metal salts or bulk powder forms. The first experiment showed that waterborne exposure to Cu-NPs and $CuSO_4$ caused similar types of organ pathologies and alteration in the spleen content, however there were some material-type effects in the incidence injuries; with Cu-NPs in some organs by causing more injury in the intestine, liver, and brain when compared to effects caused by the equivalent concentration of $CuSO_4$. Lowering water pH did have an effect on the toxicity of Cu-NPs and dissolved Cu in trout, and the results illustrated that both Cu treatments are more toxic at pH 5 than pH 7 by causing more physiological and pathological changes, although both $CuSO_4$ and Cu-NP treatments showed similar types of organ lesions. Waterborne exposure to TiO_2 NPs and bulk forms of TiO_2 showed similar types of organ pathologies and alteration in the spleen contents, but there was a material-type effect in some organs (more injury with the bulk treatment than the NP form). After 96 h following intravenous injections of bulk or TiO_2 NPs in trout, organs showed similar types of pathologies; except the spleen and kidney which showed a material-type effects (more injury with NPs than the bulk forms). This could be attributed to the highest Ti accumulation from the TiO_2 NP treatment in the kidney and spleens, or to the role of these organs in filtrating the circulating blood. Overall, this thesis demonstrates that metal-NPs produced similar types of organ pathologies to traditional forms of metals through different routes of exposure, but there were some material-type effects on the incidence of injuries in some organs. The results have also added some understanding on the fate, and effects of NPs by identifying the target organs involved. Some of the nano-specific effects may need to be given extra consideration in environmental and human health risk assessments.

Dedication

This work is dedicated to my husband (Dr. Mohammed Al-Timimi), my daughters (Noor and Malak), mum and dad with my love

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Acknowledgements

First of all, I commend almighty Allah for his support and help in satisfying this thesis. I would like to acknowledge my sincere gratitude to my first supervisor Professor Richard Handy for his help, support, encouragement and advice from the beginning up to the completion of this study. Richard always gave me all the required time to discuss any details about my research at anytime I need it. I would also like to say a huge thanks to my second supervisor doctor Theodore Henry for his motivation, observation and suggestions at all stages of my research.

Many thanks go to the Ministry of Higher Education and Scientific Research-Republic of Iraq for providing the financial support which made my PhD study in the UK possible. I would also like to thank the Iraqi cultural attaché in London for their kind support. At the University of Plymouth, many people have also helped me along the way of my research; in particular, thanks to William Vevers, Andrew Atfield, Angela Harrop and Michael Hocking for technical assistance. Huge thanks to Dr. Andy Fisher who helped me with analytical techniques (*i.e.* ICP-OES). I would also like to send my thanks to Peter Bond, who's helped me in TEM work. A big thank to Binjamin Eynon for helping me during the exposure period to maintain the fish stock in the aquarium.

Next, offer my thanks and appreciation to my husband Dr. Mohammed Al-Timimi, who gave me the time and encouragement to complete my thesis. He was always tolerance, patience and supportive of me through my research. Many thanks to my lovely daughters Noor and Malak, who always make me happy and strong. A big thank to my dad, mum, sisters and brothers who were always with me and I hope that I made them proud.

My particular thanks go to my friends and colleagues at the University of Plymouth for helping me to design the experiment, collect samples and support during my research, especially Benjamin Shaw, David Boyle. I would also like to send my thanks to Aliaa Al-Jubory, Kasim Abass and others for advices and help. Thank you all and I apologise if I have missed anyone.

List of Publications

(Please refer to Appendix C for the abstract only)

Handy, R. D.; **Al-Bairuty, G.**; Al-Jubory, A.; Ramsden, C. S.; Boyle, D.; Shaw, B. J. and Henry, T. B. (2011). Effects of manufactured nanomaterials on fishes: a target organ and body systems physiology approach. Journal of Fish Biology, 79**,** 821-853.

Shaw, B. J.; **Al-Bairuty, G.** and Handy, R. D. (2012). Effects of waterborne copper nanoparticles and copper sulphate on rainbow trout, (*Oncorhynchus mykiss*): Physiology and accumulation. Aquatic Toxicology, 116-117, 90-101.

Al-Bairuty, G. A.; Shaw, B. J.; Handy, R. and Henry, T. B. (2013). Histopathological effects of waterborne copper nanoparticles and copper sulphate on the organs of rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology, 126, 104-115.

Boyle, D.; **Al-Bairuty, G.A**.; Ramsden, C.S.; Sloman, K. A.; Henry, T. B. and Handy, R. D. (2013). Subtle alterations in swimming speed distributions of rainbow trout exposed to titanium dioxide nanoparticles are associated with gill rather than brain injury. Aquatic Toxicology, 126, 116-127.

Platform presentation

1. Al-Bairuty, G. A. (2010). Histopathological effects of metal and metal Nanoparticles on organ integrity in fish. General Teaching Associates Course from 12-16th April 2010, University of Plymouth. Presentation for 10 min.

2. Al-Bairuty, G. A. (2010). Histopathological effects of copper and copper nanoparticles on organs of rainbow trout. Nanoparticles meeting, 25th February 2010, University of Plymouth. Presentation for 15 min.

3. Al-Bairuty, G. A. (2011). Histopathological effects of waterborne and injection of either bulk TiO_2 or TiO_2 NPs on organs of rainbow trout. Nanoparticles Meeting, 10th February 2011, University of Plymouth. Presentation for 15 min

4. Al-Bairuty, G. A. (2011). The effects of waterborne exposure to TiO_2 NPs compared to bulk TiO_2 on some organs of rainbow trout. Training school for young researchers in Impact assessment of nanomaterials *in vitro* and *in vivo* testing strategies from 12-14th October 2011, Bratislava, Slovakia. Presentation for 5 min

5. Al-Bairuty, G. A. (2012). The effects of pH on the sub-lethal toxicity of either dissolved copper or copper nanoparticles on some organs of rainbow trout

(*Oncorhynchus mykiss*). Nano team meeting, 6th December 2012, University of Plymouth. Presentation for 15 min

Conferences Attended:

Poster presentations:

1. **Al-Bairuty, G. A.**; Shaw, B. J. and Handy, R. D. (2010). Effects of dissolved copper and nanocopper on histopathology and haematopoietic organs of rainbow trout (*Oncorhynchus mykiss*). Society of Environmental Toxicology and Chemistry, U.K. branch annual meeting, London, 13-14th September 2010. Abstract book, SETAC. 30, P 25.

2. **Al-Bairuty, G. A.**; Shaw, B. J. and Handy, R. D. (2010). Histopathological and Haematopoietic Study of the Effect of Dissolved copper and Copper Nanoparticles on some organs of rainbow trout (*Oncorhynchus mykiss*). 3rd Annual Conference, Spirit of Discovery Plymouth's diverse marine and maritime research, 20th December 2010, University of Plymouth, Roland Levinsky Building. Abstract book p 31.

3. **Al-Bairuty, G. A.;** Shaw, B. J. and Handy, R. D. (2011) Effect of dissolved copper and nano copper on histopathology and haematopoietic organs of rainbow trout (*Oncorhynchus mykiss*). 3rd NanoImpactNet Conference, "Building a Bridge from NanoImpactNet to Nanomedical Research", 14-17th February 2011, Lausanne, Switzerland. Abstract book, 4.2.2, p 66.

4. **Genan A. Al-Bairuty**; Benjamin J. Shaw; Richard D. Handy and Theodore B. Henry (2011). Histopathological study of the effect of dissolved copper and copper nanoparticles on some organs of rainbow trout (*Oncorhynchus mykiss*). The postgraduate Society conference series.17th march 2011. University of Plymouth, Roland Levinsky.

5. **Al-Bairuty, G.**; Boyle, D.; Handy, R.D. and Henry, T.B. 2011. Histopathological study of the effect of bulk and titanium dioxide nanoparticles on some organs of rainbow trout (*Oncorhynchus mykiss*). Ecotoxicology Research and Innovation Centre, University of Plymouth, 1st Annual meeting 2011. Environmental pollution: Chemical and Biological Approches for protecting organisms. 4th April 2011, Plymouth lecture theatre, Portland square building, University of Plymouth. (ERIC. 13).

6. **Al-Bairuty, G.**; Boyle, D.; Handy, R.D. and Henry, T.B. 2011. Histopathological study of the effect of bulk or titanium dioxide nanoparticles on some organs of rainbow trout (*Oncorhynchus mykiss*). 6th International Conference on the Environmental Effects on Nanoparticles and Nanomaterials, The Royal Society, London, from 19th-21st September [N1.99].

7. **Al-Bairuty, G.**; Handy, R.D. and Henry, T.B. 2012. The effect of pH on copper accumulation from copper nanoparticles and $CuSO_4$ in rainbow trout (*Oncorhynchus mykiss*). Ecotoxicology Research and Innovation Centre, University of Plymouth, 2nd Annual Conference. 13th July 2012, Plymouth lecture theatre in the Portland Square building, Plymouth Campus, Plymouth, U.K. Abstract book [ERIC.16].

8. **Al-Bairuty, G.**; Handy, R.D. and Henry, T.B. 2012. The effect of pH on copper accumulation from copper nanoparticles and copper sulphate in rainbow trout (*Oncorhynchus mykiss*). The Postgraduate Society Conference Series, 21st November 2012, Rolle Building, University of Plymouth, U.K.

9. **Al-Bairuty, G.** and Handy, R.D. 2013. Effects of waterborne exposure to dissolved copper or copper nanoparticles on the spleen of juvenile rainbow trout (*Oncorhynchus mykiss*). Society of Environmental Toxicology and Chemistry, SETAC Europe 23rd annual meeting, "Building a better future: Responsible innovation and environmental protection", 12 -16th May 2013, Glasgow, U.K. Abstract book, No. WE058, p299.

10. **Al-Bairuty, G.**; Handy, R.D. and Henry, T.B. 2013. The effect of pH on copper accumulation from copper nanoparticles and copper sulphate in rainbow trout (*Oncorhynchus mykiss*). Annual Marine Institute Research Centre Conference, 4th June 2013, Plymouth lecture Theatre, Portland Square Buliding, Plymouth University, U.K.

Conferences where PhD work was presented (not attended By G. Al-Bairuty):

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

2. Shaw, B.; **Al-Bairuty, G.** and Handy, R. 2010. The effects of nanocopper compared to aqueous copper sulphate on juvenile rainbow trout (*Oncorhynchus mykiss*). SETAC Europe 20th Annual Meeting, 23-27 May 2010, Seville, Spain. (Poster)

3. Boyle, D.; **Al-Bairuty, G.**; Ramsden, C. S.; Sloman, K. A.; Henry, T. B. and Handy, R. D. (2010). Effects of TiO_2 nanoparticles on locomotor and social behaviour in rainbow trout. SETAC North America 31st Annual Meeting, 7-11th November, 2010, Portland, Oregon, USA, Abstract book number 522. 20.

4. Boyle, D.; **Al-Bairuty, G.**; Ramsden, C.S.; Sloman, K.A.; Henry, T.B. and Handy, R.D. 2010. Effects of TiO_2 Nanoparticles on Locomotor and Social Behaviour in Rainbow Trout. Spirit of Discovery Plymouth's diverse marine and maritime research, 3rd Annual conference .20th December 2010, Roland

Levinsky Building, University of Plymouth, Plymouth, Devon, U.K., abstract book p:17. (Presentation)

5. Boyle, D.; **Al-Bairuty, G.**; Ramsden, C.S.; Sloman, K.A.; Henry, T.B. and Handy, R.D. 2011. Effects of TiO_2 Nanoparticles on Locomotor and Social Behaviour in Rainbow Trout. Ecotoxicology research and innovation centre, University of Plymouth, 1st Annual meeting 2011. Environmental pollution: Chemical and Biological Approches for protecting organisms. 4th April 2011, Plymouth lecture theatre, Portland square building, University of Plymouth. (ERIC. 10). (Presentation)

6. 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." 4th NanoImpactNet Integrating Conference, 27th February-2nd March 2012, University College Dublin, Ireland. Abstract book p57.

7. Handy, R. D.; Windeatt, K. M.; **Al-Bairuty, G.**; Ramsden, C. S.; Boyle, D. B.; Sloman, K. A. and Henry, T. B. (2012). Concerns for Nano Effect on Nerves, and Brain Pathologies From Different Nanomaterials (TiO_2, SWCNT, C_{60}, Cu-NPs)."From theory to practice-development, training and enabling Nanosafety and health research." 4th NanoImpactNet Integrating Conference, 27th February-2nd March 2012, University College Dublin, Ireland. Abstract book p56.

8. Shaw, B.J.; **Al-Bairuty, G.A.**; Henry, T.B. and Handy, R.D. 2012. The effects of waterborne copper nanoparticles and copper sulphate on rainbow trout (*Oncorhyncus mykiss*). BANFF, 7th International Conference on the Environmental Effects of Nanoparticles and Nanomaterials, 10-12th September 2012, The Banff Centre, Banff, Alberta, Canada. (Poster)

Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee. This study was financed with the aid of The Ministry of Higher Education and Scientific Research (MOHESR), Iraq. Relevant scientific seminars, workshops and conferences were attended at which work was presented and papers have been prepared for publication.

Word account of main body of thesis: 76,557 words

Signed. G. A. AL-Bairuty

Date.....

Abbreviations

Abbreviation	Glossary
%	Percent
l	Litre
µl	Microlitre
µg	Microgram
µM	Micromole
nmol	Nano mole
nm	nanometre
dm^3	Cubic decimetre
Kg	Kilogram
°C	Centigrade (Temperature unites)
ANOVA	Analysis of variance
ATP	Adenosine triphoshate
ADME	Absorption, distribution, metabolism and excretion
ALT	Alanine transaminase
AST	Aspartate transaminase
AP	Alkaline phosphatase
ALA-D	Delta- aminolevulinic dehydratase
Ag	Silver
Ag-NPs	Silver nanoparticles
Al-NPs	Aluminium nanoparticles
AS	Arsenine
BBB	Blood brain barrier
C_{60}	Carbon 60 or fullerenes

Ca	Calcium
CAT	Catalase
Cd	Cadmium
Cl	Chloride
CNS	Central nervous system
Cr	Chromium
Cu	Copper
Cu-NPs	Copper nanoparticles
$CuSO_4$	Copper sulphate
Ctr1	Cu transporter 1
d	Day
DLVO	Refers to the names of the original authors of the theory (Derjaguin and Landau in 1941 and Verwey and Overbeck in 1948)
Ec_{50}	Effective concentration
Fe	Iron
GIT	Gastrointestinal tract
GSH	Glutathione (reduced)
GPX	Glutathione peroxidase
G6PH	Glucose 6 phosphate dehydrogenase
h	Hour
Hb	Haemoglobin
Hct	Haematocrit
H&E	Haematoxylin and eosin stain
Hg	Mercury

Hpx	Hypoxia
ICP-OES	Inductively coupled plasma optical emission spectrometer
LC_{50}	Lethal concentration
LDH	Lactate dehydrogenase
LPO	Lipid peroxidation
LSD	The least significant difference
MDA	Melanodialdehyde
Mg	Magnesium
MMC	Melanomacrophage centres
Mn	Manganese
mRNA	Messenger ribonucleic acid
MS222	Tricaine methane sulphonate
MT	Metalothionine
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium bicarbonate
Ni	Nickel
NNPC	Nigerian National Peteroleum Corportion
NMs	Nanomaterials
NPs	Nanoparticles
NTA	Nanoparticle tracking analysis, Nano Sight
P	Statistical probability
Pb	Lead
PCV	Pack cell volume
pH	Potential hydrogen ion
ppm	Part per million

ppt	Part per thousand
RBC	Red blood cell
ROS	Reactive oxygen species
SDS	Sodium dodecylsulphate
S.E.M.	Standard error mean
SO_4	Sulphate
SOD	Superoxide dismutase
SSA	Specific surface area
SWCNTs	Single walled carbon nanotubes
THF	Tetrahydrofuran
TBARS	Thiobarbituric acid reactive substance
TEM	Transmission electron microscopy
TiO_2	Titanium dioxide
WBC	White blood cell
Zn	Zinc
ZnO	Zinc oxide

Glossary

Milli-Q water: a water that has been purified and deionised to a high degree of resistance (18.2 MΩ·cm at 25 °C) by a water purification system manufactured by Millipore.

Material-type effect: a difference between the nanoform and dissolved form of metal in the case of copper and nanocopper, or between the nano and bulk forms in the case of TiO_2.

Occasional lesion: a histologically term that indicates the frequency of a lesion as less common in a tissue sample, but not rare or absent.

Swollen red blood cells: an enlargement of red blood cell, usually associated with the osmotic influx of water to increase the cell volume.

Oxidative stress: is defined as an imbalance between the systemic appearance of reactive oxygen species (ROS) and a biological system's capacity to detoxify the reactive intermediated or to repair the resulting damage.

Chapter 1

General introduction and literature review

1.1 Introduction

Environmental pollution is any discharge of substances or energy into water, land, or air that causes acute (short-term) or chronic (long-term) detriment to the earth`s ecological balance, or that lowers the quality of life (Hill, 1997). Human activities have led to the accumulation of potentially toxic chemical substances in the environment (Shi *et al*., 2008). The toxic effects of pollution depend on many factors, including concentrations per unit time (*ie*., dose), health status, age, and sex of the organisms being exposed, and the specific conditions of exposure (McKim and Nichols, 1994; Connell *et al*., 1999). There are many different sources of chemical pollution including, domestic sewage, industrial discharges, atmospheric deposition, mining discharges and agricultural run-off (Zhen-Gang, 2008); and many different types of chemicals including organochlorines pesticides (DDT), industrial organic chemicals (polychlorinated biphenyls), and trace metals (silver, copper, *etc.*, Phillips, 1980). Some trace elements are essential nutrients (*e.g*., Cu, Zn) that are required for healthy growth and metabolism of organisms (Stillman and Presta, 2000; Wöhrle and Pomogailo, 2003). However, higher concentration of these essential metals can lead to toxicity (Donkin *et al*., 2000; De Smet and Blust, 2001; Oner *et al*., 2009). More recently attention has focused on new environmental pollutants including engineered nanoparticles, which include many different types of materials made from metals as well as organic chemicals (see below). Both metals and nanometals may reach to the aquatic ecosystems as a result of natural and human activities, leading to concerns about the toxic effects of these materials in different aquatic organisms (Bury and Handy, 2010; Shaw and Handy, 2011). Fishes are useful indicator organisms of pollution as they are sensitive to their environment and have a relatively long lifespan, allowing the

identification of both acute and chronic effects after exposure to pollutants (Di Giulio and Hinton, 2008).

Bio-monitor specifically refers to the use of living organisms in monitoring procedures (Peakal, 1992). Zhou *et al*. (2008) illustrated that the algae, zooplankton, bivalve molluscs, fish, amphibian and others can be used in the aquatic environment monitoring of metals. Modern bio-monitoring techniques include the use of biomarkers (Di Giulio and Hinton, 2008) which are measures of cellular modification, biochemical, molecular, or physiological changes in an organism that indicate exposure to environmental contaminants (Peakal, 1992). Nonetheless, biomarkers have the potential to play an important role in hazard assessment because they can provide information on the impact of contaminants, and can determine the early biological, biochemical or physiological alteration resulting from the exposure (Peakall and Walker, 1994). Biomarkers are generally classified into three groups: (a) Biomarkers of exposure, (b) Biomarkers of effect, and (c) Biomarkers of susceptibility (Silbergeld and Davis, 1994; Walker *et al.*, 1996; Scklenk *et al.*, 2008). Biomarkers of exposure are used for detecting exposure to exogenous materials (pollutants) and relies on the interaction of the substance (or its metabolites) with a target molecule or cell which is measured in organs within the organism. Biomarkers of effect measure the biological response of the organism and this may be at different levels of biological organisation including biochemical, physiological, or at the tissue and organ level (*e.g*., histopathology), or the whole organism (*e.g.*, animal behaviour). Biomarkers of susceptibility are also sometimes used to infer a genetic factor or health status (*e.g.*, poor immune response) that would increase the disposition of the organism towards adverse effects. The

histopathological analysis, DNA integrity and detoxification enzyme status in fish tissue have been used as such biomarkers in environmental monitoring (Nogueir *et al.*, 2010).

Histological biomarkers which can provide a description and quantitative measurementof lesions have been used as a bio-monitoring tools in fish (Handy *et al.*, 2002c). The advantages of histological biomarkers include the ability to examine the specific target organs and cells that are affected under either *in vivo* or *in vitro* conditions (Bernet *et al.*, 1999). In fish, the histopathological biomarkers present a number of practical advantages over other biomarker approaches that included ease of sample collection and storage, the ability to estimate many body systems and cell types from the same fish, as well as the opportunity to examine very small fish that could be too small to dissect for biochemistry (Hinton and Laurén, 1990). Several studies have shown that hisopathlogic biomarkers are very sensitive, and can elucidate toxicant aetiology consistent with the type of environmental pollution (Norrgren *et al.*, 2000; Handy *et al.*, 2002c).

Exposure to the toxic substance in the environment may cause changes in the histological structure of tissues and cells which can lead to pathological changes and modified the function of tissues or organs (Scklenk *et al.,* 2008). Histopathological changes and alteration in the size of tissue cells have been reported in the gill, liver, and kidney of different types of fish (Camargo and Martinez, 2007; Figueiredo-Fernandes *et al.*, 2007; Handy *et al.*, 2002c). Different types of pathology can also be observed in different target organs in fish following exposure to metal and metal nanoparticles. For example, exposure to Cu-NPs showed significantly wider gill filaments in zebrafish than those from soluble Cu (Griffitt *et al.*, 2009).

Histological biomarkers, while relatively new (the last 60 years) have their foundations in a well-established field of science. Histology is a very old technique, and this branch of science was originally called "microscopic anatomy" (Bracegirdle, 1977). The term "histology" comes from the Greek istos which means "tissue" (Tortora and Derrickson, 2007). In the 19th century, developments in microscope construction and the introduction of staining procedures led to the rise of the field initiated earlier by Malpighi and Bichat (Bracegirdle, 1977). In the late 19th century, the progresses in histology were largely driven by the development of experimental and histochemical staining procedures (Clark and Kasten, 1983), including embedding media, microtomes and staining. Important improvements were also made in the light microscopes, leading to phase contrast microscopy, differential interference microscopy, fluorescence microscopy, and confocal microscopy. While the electron microscope was invented in the mid- 20th century, and developed into transmission and scanning electron microscopes that allowed studies of the fine structure of cells and organelles (Telford and Bridgman, 1990; Woods and Stirling, 2008). Preparation of sectioning slices involves different methods that include paraffin wax and resin for light and electron microscopy respectively.

1.2 Metal toxicity

1.2.1 Lethal toxicity of metals in fish

A number of studies have measured lethal toxic effects of dissolved metals and metal salts on fishes Table 1.1. There are several routine parameters used to measure acute toxicity such as severe biological harm effects or death. The lethal concentration (LC_{50}) is defined as the concentration of the test substance estimated to cause mortality

in 50 % of the experimental organisms (fish) over a 96 hour period. While the median effective concentration (EC_{50}) is defined as the concentration of the test substance estimated to produce a specific non-lethal effect (*e.g.*, changes in biochemistry, physiology or behaviour) in 50 % of the organism after specified duration exposure (Connell *et al.*, 1999; Rand, 2008). EC_{50} therefore occur at lower concentrations than the LC_{50} as impairment of function would occur before death. Recently, a study by Shaw *et al.* (2012) found that waterborne exposure to 100 μg l^{-1} of $CuSO_4$ caused 80 % mortality to juvenile rainbow trout (*Oncorhynchus mykiss*) after 96 h and this concentration is considered toxic to trout.

Toxicity of material can be affected by many different factors, such as the route of exposure (waterborne, dietary, injected), the physical form of the toxin (solid, liquid, gas), an individual's overall health, and numerous aspects of the environmental chemistry. The interaction between a metal and an environmental parameter such as pH, temperature, hardness, salinity, and dissolved oxygen can also influence LC_{50} values (Hansen *et al.*, 2002a; Brinkman and Woodling, 2005; Haltink *et al.*, 2006). For example, the toxicity of Cu was approximately 20 times more in soft water than in hard water for 1 to 2 g trout (Taylor *et al.*, 2000). Hansen *et al.* (2002b) found that higher hardness and lower pH water produced lower toxicity and slower rates of toxicity of both Cd and Zn in salmonid fish (*Salvelinus confluentus* and *Oncorhynchus mykiss*).

The pH value of water is also known to alter the toxicity of dissolved Cu. At low pH there are more free Cu ions (Cu^{2+} dominates chemical speciation) which are toxic to fishes (Bury and Handy, 2010), whereas at higher pH, copper hydroxide [$Cu(OH)_2$] is released as a dominant species (Tao *et al.*, 2001). Copper toxicity is expected to be

higher in acidic pH (increased solubility and the bioavailability of free metals ion) than alkaline pH from a water chemistry perspective. However, bioavailability should also be considered. The study by Takasusuki *et al.* (2004) found an inverse correlation between Cu concentration in water and toxicity in *Prochilodus scrofa*. The low toxicity of Cu in acidic water could occur due to the competition of H^+ and Cu^{2+} ions on the same sites in the gill epithelium (Laurén and McDonald, 1985), which is the main body surface for water-blood diffusion. In contrast, increasing the toxicity of Cu in alkaline water could occur due to the reduced H^+ concentration and low Ca^{2+} levels in soft and ion-poor waters that may help Cu^{2+} binding to the gill surface membrane receptors (Meador, 1991).

Table 1.1 Lethal concentration (LC_{50}) values and acute effects of metals on fishes

Metals	LC_{50} / Time	Species	The toxic effects	Authors
Cr VI	43.7 mg l^{-1} for 96 hours	Nile tilapia *Orechromis spp.*	Aqueous exposure to Cr caused a decrease in the total glycogen, lipid and protein levels in the liver, muscle, and gill and organs pathologies after 24 and 96 hour of exposure.	Abbas and Ali (2007)
Cr VI	85.7 mg l^{-1} for 96 hours	Gold fish *Carassius auratus*	Exposure to Cr caused oxidative stress, genotoxicity and histopathology in the tissues of liver and kidney.	Velma and Tchounwou (2010)
Pb Cd	482 nmol l^{-1} for 96 hours Low, 6.7 nmol l^{-1} for 96 hours	Rainbow trout *Oncorhynchus mykiss*	Pb was more toxic in soft, acid water than hard water. The LC_{50} for Cd was low, 6.7 nmol l^{-1}.	Birceanu *et al.* (2008)
$CuSO_4$	Fresh water 18 µg l^{-1} Sea water 294 µg l^{-1} Intermediate salinities 10 ppt > 963 µg l^{-1} for 96 hours	Killifish *Fundulus heteroclitus*	Sensitivity was highest in fresh water, followed by sea water with fish at intermediate salinities being the most tolerant.	Grosell *et al.* (2007)

Continued

Table 1.1

$CuSO_4$	340 and 568 µM for 96 hours	Gulf toadfish *Opsanus beta*	Fish survived 96 h of exposure to 350 µM Cu but caused 100 % mortality at 568 µM. Both acute (96 h) and prolonged (30 d) Cu exposure caused osmoregulatory disturbance and impaired nitrogenous waste excretion in the marine teleost, this type of fish was found to be more tolerant to acute copper exposure with a 96 h LC_{50} exceeding 340 µM but showed disturbed mineral balance in response to both acute and prolonged exposure to about 12 µM copper.	Grosell *et al.* (2004)
$CuSO_4$	185.75 mg l^{-1} for 96 hours	Nile tilapia *Oreochromis niloticus*	Exposure to Cu induced changes in the morphology of gill, liver and kidney tissues.	Kosai *et al.* (2009)
$CuSO_4$	0.75 mg l^{-1} for 24 hours	Fingerlings *Catla catla*	Copper exposure caused behavioural changes (erratic swimming, restlessness and surfacing).	Patel and Bahadur (2010)
$CuSO_4$	5.5 mg l^{-1} for 96 hours	Flying barb *Esomus danricus*	Exposure to Cu induced changes in the gill morphology and inhibition in the activity of	Vutukuru *et al.* (2005)

Continued

Table 1.1

			superoxide dismutase and catalase.	
$CuSO_4$	100 µg l^{-1} for 96 hours	Juvenile rainbow trout *Oncorhynchus mykiss*	100 µg l^{-1} dissolved Cu caused 80 % mortality in trout.	Shaw *et al.* (2012)
$CuCl_2$	32.8 µg l^{-1} for 48 hours and 72 hours	Dolphinfishes *Coryphaena hippurus* (embryos)	The treatment induced abnormalities such as: yolk sac swelling, spinal deformities, decrease hatch rate.	Adema- Hannes and Shenker (2008)
$AlCl_3$	12.5 mg Al l^{-1} for 48 hours	Zebrafish *Danio rerio*	Exposure to Al caused 100 % mortality to fish	Griffitt *et al.* (2011)
$ZnCl_2$	4.3 mg l^{-1} for 48 hours 10.1 mg l^{-1} for 96 hours	Red sea bream *Pagrus major* (emberyo) (larvae)	The embryos were more sensitive to Zn exposure than larvae.	Huang *et al.* (2010)
$HgCl_2$	100000 µg l^{-1} for 4 hours 5000 µg l^{-1} for 14 hours	Yellowfin sea bream *Acanthopagrus latus*	All this concentration caused 100 % mortality.	Hedayati *et al.* (2010)

Continued

Table 1.1

	2000 µg l^{-1} for 24 hours 1000 µg l^{-1} for 54 hours			
$AgNO_3$	25 µg l^{-1} for 48 hours	Zebrafish *Danio rerio*	Signs of stress were observed by the fish that exhibiting increased swimming activity and attempts to escape from the tank as well as elevated mucus secretion with strands of sloughed mucus appeared in the tank with higher concentrations.	Bilberg *et al.* (2012)

1.2.2 The accumulation of metal in target organs

Target organs are specific organs of the body that accumulate or are particularly affected by the pollutant. The accumulation of different metals in different organs such as the liver, kidney, gill, intestine of fish are shown in Table 1.2. Metal accumulation and toxicity depends not only on the total metal concentration in the environment but also on how fish can absorb these different metal species at the gill, across the skin, or the digestive tract; and on how metal speciation affects distribution of metal throughout the organism (Erickson *et al.*, 2008). The accumulation of metal in target organs is dependent on the route of metal exposure. Fish can take up metals via different routes such as by water passage through the gills during respiration, and reaches to the internal organs through the circulatory system (Evans, 1987). Another way is by food through the mouth into the gastrointestinal tract (GIT). Chowdhury *et al.* (2005) showed that the largest Cd concentration was found in the kidney followed by the gill and liver of the fish exposed to Cd via water, while in dietary exposing, the Cd found in the gut tissues followed by the kidney, liver and gills. Handy *et al.* (1999) found that exposure of rainbow trout to copper via the oral route caused greater Cu concentration in the intestine and liver (16 and 2.5 times respectively) than in controls, while Cu concentrations in the gill remained low.

Metals reach the target organs, and the accumulation in the organ is the sum of several internal processes, including: absorption, tissue distribution, metabolism or storage, and excretion (ADME) (Fig. 1.1). Metal uptake mechanisms across biological membranes involve carrier-mediated transport on metal ion transporters (Handy and Eddy, 2004; Bury and Handy, 2010). The major barrier to the absorption of metals is

the external epithelia (*e.g.* gill, skin and digestive tract), including movement across diffusion barriers such as the mucus on the epithelial surfaces (Handy *et al.*, 2005). For example, Cu ions can be taken up across the apical membrane by facilitated diffusion through either Cu transporter 1 (Ctr 1) or via epithelial Na channels, before being chaperoned through the cell into the blood or other extracellular fluid compartment (Bury and Handy, 2010). Briefly, the external Cu^{2+} is reduced to Cu^{+} before being transported inside the cell. Once inside the cell, Cu^{+} is bound to metallochaperones (*e.g.*, polypeptides) and delivered to the golgi vesicles against the concentration gradient via the Cu^{+}-ATPase (Bury *et al.*, 2003). Golgi vesicles then traffic the Cu to the serosal membrane for release via exocytosis (Bury and Handy, 2010). Copper anion (Cu/Cl^{-}) symporter is another way for intestinal Cu efflux into the blood which is dependent partly on both luminal and serosal Cl^{-} concentration (Handy *et al.*, 2000). Copper transfers to many locations inside the cell including Cu-dependent enzymes in different parts of the cell, or biochemical processes that require a particular Cu centre in a protein (Fig. 1.1). For example; CCs (‘‘copper chaperone to superoxide dismutase”) is a Cu carrier that apparently transfers Cu to cytosolic superoxide dismutase, and other peptides (AtX1 homologus) transfer Cu to the ATPase in the Golgi network (Culotta *et al.*, 1997; Lu *et al.*, 1999; Lutsenko *et al.*, 2008). Another Cu carrier called Cox17 (an oligomer with three Cu binding domains) transfers Cu to the inner mitochondrial membrane, whereas other types of carriers (Sco1, Cox 1 and Cox 2) could facilitate loading of cytochromes with Cu (Paret *et al.*, 1999; Heaton *et al.*, 2000; and 2001, Horn and Barrientos, 2008).

Generally, the transport of Cu in the blood and cellular metabolism of Cu depends on a series of membrane proteins and smaller soluble peptides that include a

functionally integrated system for maintaining cellular Cu homeostasis. The liver is the major organ for Cu homeostasis (Grosell *et al.*, 1997; 2000), and accumulates Cu absorbed from diet or water, as well as being a site for synthesis of ceruloplasmin. Ceruloplasmin is a Cu- containing protein secreted into the blood and plays a role as a source of Cu to other organs (Harris, 2000; Bury *et al.*, 2003). Cu may also bind to albumin and other low-molecular mass protein circulating in the body (Harris, 2000). The essential route for metal (Cu) excretion in teleost fish is hepatobillary (Grosell *et al.*, 1997; 2000), with renal losses usually being very low (Grosell *et al.*, 1998b). Grosell *et al.* (1998b) explained the reason for reduced renal Cu excretion as likely due to increased plasma ceruloplasmin levels that lead to reduced accessibility of total Cu to the kidney (by tight Cu binding). Additionally, the gills of fish may also be able to excrete some metals ions from the systemic circulation by active efflux on the branchial ion transport pathway, although this is not likely for Cu.

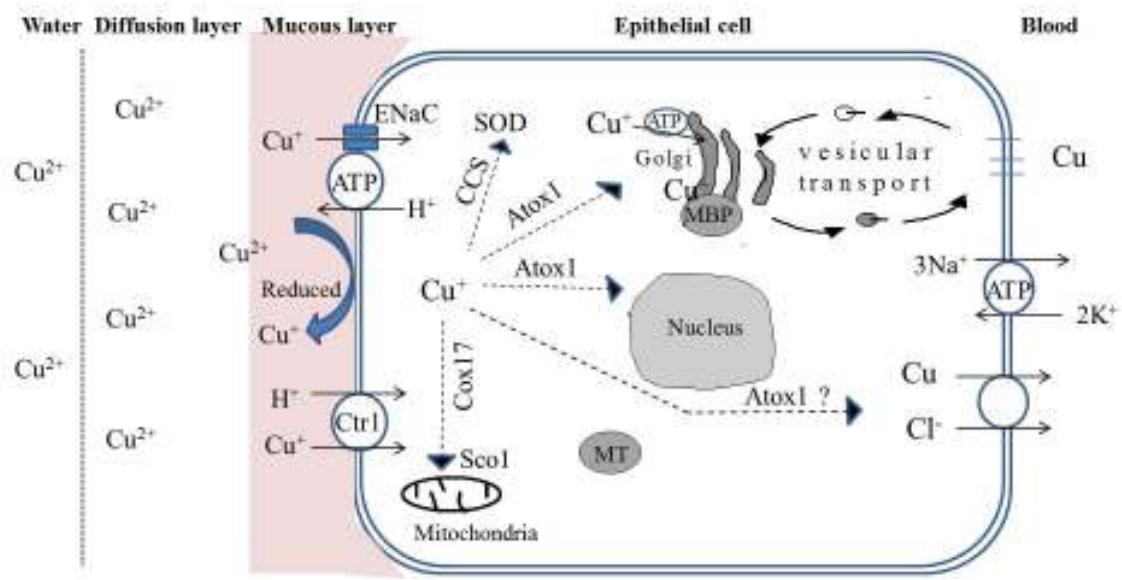

Figure 1.1 Basic scheme of apical cellular copper uptake pathways in fish, combining data from gill and intestine. Briefly, cupric ions (Cu^{2+}) are reduced to cuprous ions (Cu^{+}) before import to the cell via either copper transporter 1 (Ctr1) or putative epithelium Na channels (ENaC). Metallochaperones (such as Atox1) bind Cu^{+} and drive it to the golgi apparatus via Cu^{+}-ATPase. Then Cu^{+} is incorporated into metal binding protein (MBP) within the golgi apparatus. Golgi vesicles then move the Cu to the basolateral membrane for releasing into the blood via exocytosis. Another chaperone inside the cell involves Atox1 (delivered Cu to the nucleus), CCs (delivered Cu to cytosolic superoxide dismutase), Cox17 (delivered Cu to the inner mitochondrial membrane), Sco1, Cox 1 and Cox 2 (facilitated loading of cytochromes with Cu). In the intestine and gill, apical entry is assumed to be a passive pathway. Excess Cu could bind to low molecular mass protein such as metallothionein (MT).

Table 1.2 The accumulation of different metals in target organs of fish

Metals & routes of exposure	Concentration / time	Species	Target organs	Authors
Waterborne Cd	0.06 mg l^{-1} for 60 days	Silver barb *Puntius gonionotus*	Kidney recorded higher concentration than gill and liver tissue.	Wangsongsak *et al.* (2007)
Dietary Cd	0, 0.5, 5, 25, 125, or 250 mg Cd kg^{-1} for 4 months	Atlantic salmon *Salmo salar L.*	5 mg l^{-1} Cd caused accumulation in the gut, kidney, and muscle. After 1 month Cd accumulated first in the gut, followed by the kidney (2 months) and after 4 months in the muscle.	Berntssen *et al.* (2001)
Waterborne Cd	0, 0.8, 4, and 20 µM for 29 days	Common carp *Cyprinus carpio L.*	Cd accumulated in the kidney > liver > gills.	De Smet and Blust (2001)
Waterborne Cd	0.5 mg Cd/dm^3 water for 24 hours	Common carp *Cyprinus carpio L.*	The greatest accumulation of Cd was seen in the gills, kidneys, alimentary canal and hepatopancreas while the least accumulation was in the spleen, skin and muscles.	Morsy and Protasowicki (1990)

Continued

Table 1.2

Dietary Cu	2000 mg kg^{-1} dry weight or control diet 3 mg kg^{-1} dry weight for 42 days	Nile tilapia *Oreochromis niloticus*	Dietary Cu exposure showed an increase in Cu concentration in the intestine (30 fold), liver (3 fold) and gill (2.7 fold) after 42 days of exposure fish to Cu compared to the control.	Shaw and Handy (2006)
Dietary Cu	250 and 500 mg kg dry wet.$^{-1}$ for 30 days	Common carp *Cyprinus carpio*	The Cu accumulation in tissues were in the order liver > intestine > gill.	Mustafa *et al.* (2012)
Waterborne Cu	12.8 or 65.2 µM for 30 days	Gulf Toadfish *Opsanus beta*	Drinking Cu caused to Cu accumulation in the intestinal fluids at levels three to five times higher than the ambient Cu concentration. Gill appeared more rapid accumulation of Cu than the intestine. Muscle, spleen and plasma appeared little accumulation if any disturbance of Cu homeostasis while renal Cu accumulation were evident at both ambient Cu concentrations. Liver recorded the highest Cu concentration during exposure to 55.2 µM.	Grosell *et al.* (2004)

Continued

Table 1.2

Waterborne $CdCl_2$	7 ppm after 60 days	Walking catfish *Clarias batrachus*	The rate accumulation of Cd was in the order gill > kidney > liver > skin > muscle.	Jayakumar and Paul (2006)
Waterborne Pb	0.05, 0.10, 0.50, and 1.00 mg l^{-1}	Blue tilapia *Oreochromis aureus*	Pb accumulated in the highest concentration in the trunk kidney. The concentration of Pb in the kidney was decreased by coexposure to Hg or Cd, but increased in the muscle and liver.	Allen (1995)
Pb + Hg	(0.50 or 0.05 mg l^{-1}) + (0.05 mg l^{-1})			
Pb + Cd	(0.50 or 0.05 mg l^{-1}) + (0.05 mg l^{-1}) for 140 days			
Waterborne Zn	0.5 and 5.0 mg l^{-1}	Nile tilapia *Oreochromis niloticus*	Increased accumulation of metals in tissue with elevated metal concentrations and duration of exposure. Accumulation of Zn + Cd combination lower than in fish exposed to the single metal. The highest accumulation of metal observed in the liver.	Özgür *et al.* (2009)
Cd	0.1 and 1.0 mg l^{-1}			
Zn + Cd	0.5 + 0.1 mg l^{-1} 5.0 + 1.0 mg l^{-1} for 7 and 28 days			

Continued

Table 1.2

Waterborne combined metal solution (Cr, Ni, Cd, Pb)	5 ppm for 32 days	Common carp *Cyprinus carpio*	In the gill and liver, the order of heavy metal accumulation was Cd > Pb > Ni > Cr and Pb > Cd > Ni > Cr, respectively. In the kidney and flesh tissues, the order was Pb > Cd > Cr > Ni and Pb > Cr > Cd > Ni, respectively. Generally, the Cd and Pb were increased in the tissue of carp.	Vinodhini and Narayanan (2008)

1.2.3 Sub-lethal effects of metals on fish tissues

Sub-lethal effects are defined as impairment of an animal`s development or its capacity to perform and adapt, which can reduce the chances of survival, the potential for growth and reproduction. The sub-lethal effects of metals include effects on ionic regulation, enzyme activity, oxidative stress, immunotoxicity, neurotoxicity and renal toxicity (Table 1.3, Fig. 1.4). The effects of metals on hydromineral and acid-base balance can be attributed to damage of the gills and alteration in branchial function in fish (Wendelaar Bonga and Lock, 2008). The action of trace metals on the intestine are comparable to those on the gill as they include inhibition of ion-transporting mechanisms and increase the permeability of the intestinal epithelium (Wendelaar Bonga and Lock, 2008). Reduction of intestinal and branchial Na^+/K^+-ATPase activity and ionoregulatory impairment, has been reported for several metals including, copper (Handy *et al.*, 2000; Handy *et al.*, 2002b; Burke and Handy, 2005; De Boeck *et al.*, 2007; Nadella *et al.*, 2007), silver (Bianchini *et al.,* 2005), mercury (Klinck *et al.,* 2005), cadmium (Matsuo *et al.*, 2005; Cooper *et al.*, 2006), and iron (Cooper *et al.,* 2006). Additions of electrolytes can decrease the effect of some metals. Kamunde *et al.* (2003) showed that juvenile rainbow trout fed with a high Na^+ diet before expose to Cu, caused a slight decrease in Cu concentration in the liver, gill and other internal organs. Similar observations were found by Niyogi *et al.* (2006) when rainbow trout were fed with dietary sodium chloride and waterborne Cu.

Trace metals also have haematological and immunological effects on fish. For example dietary exposure to copper for 30 days in *Cyprinus carpio* L. showed significant differences in the haematological parameters such as increased red and white

blood cells, haematocrit value and haemoglobin concentration (Mustafa *et al.*, 2012). In teleost fish (Rowley *et al.*, 1988), as in mammals, the T- and B- lymphocytes are responsible for cellular defence against antigen entering the body (Carlson and Zelikoff, 2008), and there is a suggestion that the immune system may view metals as antigen. There are many examples of immunotoxic of metals in fish. A study by Zelikoff *et al.* (1995) found that exposure of rainbow trout to waterborne cadmium at 2 ppb led to altered macrophage–mediated immune functions including phagocytosis and free radical production by macrophages.

Table 1.3 Sub-lethal effects of metals on fish tissues

Types of Metal	Concentration/ Time	Type of exposure	Species	Sub-lethal effects or Responses	Authors
Cd	0.01, 0.05, and 0.1 ppm for 3, 7, 11 days	Water	Pacific oyster *Crassostrea gigas*	Cd caused significantly increase in an antioxidant enzymes mRNA expression in the gill in a time and dose dependent. The mRNA expression at 0.1 ppm Cd concentration increased up to 3 days (CAT, GPX) or 7 days (SOD) and then decreased by 7 days (CAT, GPX) or 11 days (SOD). Aspartate aminotransferase, alanine aminotransferase and hydrogen peroxide levels were increased with exposure to 0.05 or 0.1 ppm Cd for 7 days.	Jo *et al.* (2008)
Cd	5, 50, and 500 µg l^{-1} for 4 and 9 weeks	Water	European flounder *Pleuronectes flesus*	Cd caused a reduction in the haematocrit, haemoglobin, and red blood cell count in the low Cd concentration for 96 h. Cd exposure caused significant increase of the number of lymphocytes, which indicating an action on the immunological defense of the fish.	Johansson-Sjöbeck and Larsson (1978)

Continued

Table 1.3

Cd	0.06 mg l^{-1} for 60 days	Water	Silver barb *Puntius gonionotus*	Cd caused increase MT mRNA in both liver and kidney. Hepatic MT levels remained high after fish were removed to Cd-Free water. MT in the kidney was peaked after 28 days of exposure and dropped when fish were removed to Cd-Free water.	Wangsongsak *et al.* (2007)
Pb	0, 100, 400, and 800 µg g^{-1} concentration for 60 days	Food	Nile tilapia *Oreochromis niloticus*	Pd caused a decrease in the (ALT), (AST) and (LDH) activities in the kidney, while these enzyme activities in the liver were stimulated. Demonstrate the inhibitory effects of dietary Pb on alkaline phosphatase, Na^+/K^+-ATPase, Ca, and Mg-ATPase activities in both liver and kidney were Pb concentration–dependent. Decreased the content of Fe, Cu and Zn in the liver and kidney with the increasing dietary Pb concentration.	Dai *et al.* (2009)
Cu	1500 mg Cu kg^{-1} dw feed	Food	African catfish *Clarias ariepinus*	Copper caused only transient disturbances to tissue electrolytes and Na^+/K^+-ATPase activity at day 20. Cu exposed showed an increase in TBARS in the gill	Hoyle *et al.* (2007)

Continued

Table 1.3

	Control 15 mg kg^{-1} dw feed for 30 days			(1.5 fold) and intestine (2 fold) compared to controls. Total glutathione content in the intestine doubled by the end of the experiment compared to controls.	
Cu	12.8 or 65.2 µM for 30 days	Water	Gulf Toadfish *Opsanus beta*	No inhibition of Na^+/K^+-ATPase activity in either gills or intestine was seen despite the Cu accumulation in these organs.	Grosell *et al.* (2004)
Cu	40 and 400 µg l^{-1} for 0, 3, 7, 14, 21 days	Water	Nile tilapia *Oreochromis niloticus*	Inhibited gill Na^+/K^+-ATPase activity after 3 days. Decreased plasma Cl^- values with time of exposure but only at 400 µg l^{-1} of Cu after 21 days. Decreased plasma Na^+, protein and osmolality with exposure time at the highest concentration after 21 days. Increased plasma glucose and cortisol levels.	Monteiro *et al.* (2005)
Ni	215.3 and 606.1 µM in sea water	Water or infused	Gulf Toadfish *Opsanus beta*	Plasma Ca^{2+} was decreased at both concentrations. Hydromineral balance in the intestinal fluid was	Pane *et al.* (2006)

Continued

Table 1.3

	after 72 hours, (salinity of 34)			impacted, with Na^+, Cl^-, SO_4^{2-}, K^+ and Mg^{2+} concentrations increased after 72 h of exposure to waterborne Ni. Following arterial Ni infusion (0.40 μmol kg^{-1} h^{-1}), perturbation of hydromineral balance of the intestinal fluid was specific only to Na^+ (significantly increased) and Mg^{2+} (significantly decreased) by Ni infusion. Nitrogen excretion was not significantly impacted by Ni infusion. Ni excretion, dominated at 24 h by extra renal routes, was primarily a function of renal excretion by 72 h of infusion.	
Pb^{2+}	10, 75, and 300 μg l^{-1} for 30 days	Water	Rainbow trout *Salmo gairdnerii*	The activity of the enzyme delta-aminolevulinic acid dehydratase (ALA-D) was depressed in erythrocytes, spleen and renal tissue of *Salmo gairdnerii*. The highest lead concentration showed an anaemic response and basophilic stippling of erythrocytes. Lead exposure did not cause any changes of the white blood cell picture.	Johansson-Sjöbeck and Larsson (1979)

Continued

Table 1.3

Ag ($AgNO_3$)	0, 0.1, 1, 3, and 5 µg l^{-1} for 23 days	Water	Rainbow trout *Oncorhynchus mykiss*	Ag caused reductions of specific growth rate, cumulative food consumption, food-conversion efficiency and critical swimming speed during 5 µg l^{-1} Ag exposures. On day 5 and 10 exposures to 5 µg l^{-1} caused significant lowered plasma Na^+ and Cl^-. Unidirectional Na^+ uptake and gill Na^+/K^+-ATPase activity was significantly inhibited by 3 and 5 µg l^{-1} Ag exposure. Gill Na^+/K^+-ATPase was inhibited on day 5 in both the 3 and 5 µg l^{-1} Ag but increased to approximately 1.5 times of control levels by day 23.	Galvez and Wood (2002)
Combined-factors Hypoxia + Cu	0.4 mg l^{-1} Cu^{2+}, hypoxia 50 mm Hg (HPx) and 0.4 mg l^{-1} Cu^{2+} + hypoxia 50 mm Hg	Water	Pacu *Piaractus mesopotamicus*	The exposure to 0.4 Cu caused an increase (ROS) in the liver, accompanied by increases in (SOD) and decreases in (CAT) activity. The exposure to hypoxia caused a decrease in the activity of GSH-PX and CAT. Exposure to combined-factor caused an increase in the ROS production followed by an increase in SOD and a	Garcia Sampaio *et al.* (2008)

Continued

Table 1.3

	(0.4 CuHpx) for 48 hours			decrease in GSH-Px and CAT. In the gill, caused decrease in Na^{+}/K^{+}-ATPase activity in 0.4 Cu and 0.4 CuHpx, and increased in Hpx. Increased metallothionein concentration in the gills under combined- factors caused significant disturbances in the antioxidant defense and biochemical parameters than single-factors.	
Zn and Cu	2.3 - 4.6 mg Zn l^{-1} and 0.02 – 0.04 mg Cu l^{-1} for 30 and 45 days	Water	Boga *Leporinus obtusidens*	Exposure to Zn^{2+} and Cu^{2+} caused a decrease in the haematological parameters and also (ALA-D) activity in the liver and kidney at all concentration. Increased liver CAT activity after Zn and Cu exposure at all concentration and exposure time. Increase (TBARS) in the brain and liver of the fish exposed to Zn^{+2} for 45 days at both metal concentrations while in muscle increased TBARS at both exposure times and concentration. Cu^{+2} exposure caused reduced to TBARS	Gioda *et al.* (2007)

Continued

Table 1.3

				levels in the liver at both concentration and times while in the brain caused a decrease after 45 days whereas in muscles caused a decrease after 30 days.	
Ag^{+}, Cd^{2+}, Cr^{6+}, Cu^{2+} and Zn^{2+}	Different concentration of metals for 96 hours	Water	Nile tilapia *Oreochromis niloticus*	*In vivo*, all metals except Ag^{+} in the liver were stimulated CAT and the highest increase in CAT activity (183%) result from 1.0 mg Cd^{2+} l^{-1} whilst 0.5 mg Ag^{+} l^{-1} exposure resulted in a sharp decrease (44 %). In the kidney, Cd^{2+} and Zn^{2+} had no significant effect on CAT activity, while 0.1 mg Cr^{6+} l^{-1} exposure caused a decrease (44 %). In the gill, Cd^{2+} and Zn^{2+} did not significantly affect on the CAT activity while 0.5 mg Ag^{+} l^{-1} caused an increase (66 %) and 1.5 mg Cr^{6+} l^{-1} caused a decrease (97 %) in CAT activity. In the brain, 1.0 mg Zn^{2+} l^{-1} caused an increase in CAT activity (126 %) while 1.5 mg Ag^{+} l^{-1} caused a decrease (54 %).	Atli *et al.* (2006)
Cd, Cu, Zn, Pb	5, 10, 20 µM	Water	Nile tilapia	Liver (CAT) activity was affected by Cd and Pb exposure	Atli and Canli

Continued

Table 1.3

	for 14 days		*Oreochromis niloticus*	while it was inhibited by Zn exposure. Copper did not cause significant alteration in CAT activity. Liver (AP) activity was stimulated at lowest 5 µM exposure and caused inhibitions at higher 10 µM. At highest 20 µM, AP activity was compensated coming to control level except Pb exposure. Intestine and serum AP activities stimulated by all Zn exposures and 10 µM Cu exposure, while other exposure did not cause alteration in AP activity. Na^+/K^+-ATPase activity in the gill and intestine was inhibited by all metal exposures except 20 µM Pb that caused an increase in the activity in the gill. Similarity, muscle Ca-ATPase activity was inhibited by all metal exposures except Cu.	(2007)
Zn, Cd and mixture of Zn + Cd	0.5 - 5.0 mg l^{-1} Zn 0.1 – 1.0 mg l^{-1} Cd 0.5 mg l^{-1} Zn + 0.1 mg l^{-1} Cd	Water	Nile tilapia *Oreochromis niloticus*	Glutathione (GSH) level and glucose 6- phosphate dehydrogenase (G6PD) activity in the gill and liver were increased under Zn, Cd and Zn + Cd exposure especially in the higher concentration. High increase	Özgür *et al.* (2009)

Continued

Table 1.3

	5.0 mg l^{-1} Zn + 1.0 mg l^{-1} Cd for 7 and 28 days			with Cd alone and in combination with Zn than with Zn alone. Increase (GSH) level and (G6PD) activity with increasing exposure period only for Cd alone and Cd + Zn.	
$AlCl_3$	2.5 mg Al l^{-1} for 48 hours	water	Zebrafish *Danio rerio*	Caused thickening of interlamellar regions and swelling of lamellae and more decreased in the activity of Na^+/K^+-ATPase in the gill.	Griffitt *et al.* (2011)

Metalothioneins = (MT), catalase = (CAT), glutathione peroxidase = (GPX)) superoxide dismutase = (SOD), dalta- aminolevulinic acid dehydratase = (ALA-D), thiobarbituric acid reactive substance = (TBARS), alkaline phosphatase = (AP), glutathione = (GSH), glucose 6- phosphate dehydrogenase (G6PD), alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehydrogenase = (LDH), reactive oxygen species = (ROS).

1.2.4 Histopathological studies of metal on fishes

Histopathological changes associated with trace metals in fish have been studied by many authors. These changes are summarized in Table 1.4. The gill is the main target organ that gets affected easily when contact with contaminated water. The common lesions observed in the gill following exposure to $CuSO_4$ for long-term (60 days) include a fusion of secondary lamellae, oedema, lifting of lamellar epithelia, hyperplasia of gill epithelium, hypertrophy of the lamellar epithelial cells and aneurism (Wani *et al.*, 2011), and these lesions could lead to respiratory abnormalities and ionoregulatory disturbance. The gastrointestinal tract is the other route of exposure, and the main changes involved fusion of intestinal villi, necrosis of the villi epithelial cells as well as hypertrophy following exposure to 0.8 mg lead acetate l^{-1} for 96 h in silver mollies (Mobarak and Sharaf, 2011).

Metabolism and detoxification are the main liver functions and observed lesion in the liver as a response to the metal toxicity effects. Vacuole formation and necrosis in the hepatocytes were observed following exposure to either copper (Figueiredo-Fernandes *et al.*, 2007; Mustafa *et al.*, 2012) or chromium (Parvathi *et al.*, 2011). Athikesavan *et al.* (2006) showed also degeneration of blood vessels, hypertrophy, pyknotic nuclei in the liver of freshwater fish (*Hypophthalmichthys molitrix*) following exposure to nickel.

There are fewer pathological studies about the effects of trace metal on the haematopoietic system such as the spleen and kidney of fishes. The spleen is one of the important organs in removing damaged blood cells from the circulatory system and could be affected by the presence of metals in the circulation. Alteration in the

proportion of red and white pulp in the spleen is one of the typical responses following exposure to environmental pollution. A study by Handy *et al.* (2002c) showed that exposure of three-spined sticklebacks *Gasterosteus aculeatus* (Linnaeus 1758) to sewage treatment effluents caused a decrease in the proportion of sinusoid space. The kidney plays a main role in the accumulation, detoxification and excretion of metal, and are regarded as a target organ of metal toxicity. The degeneration of renal tubule cells and glomerular damage were observed in the fish following exposure to Cd (Wangsongsak *et al.*, 2007) or $CuSO_4$ treatments (Al-Bairuty *et al.*, 2013). The fish kidney treated with Ni also showed vacuole formation, pyknotic nuclei with necrosis of the tubular epithelium, as well as disrupture of glomerulus (Athikesavan *et al.*, 2006). Alteration in the number and size of the melanomacrophage centre in the liver, spleen, and kidney could be related to important organ injuries and immunological response (Agius and Roberts, 2003) as well as a possible indicator of stress induced by toxicants that are present in the aquatic environment (Suresh, 2009). A study by Suresh (2009) found that exposure of *Tilapia mossambicus* to cadmium chloride for 5 days caused an increase in the average number and size of melanomacrophage centres in the liver, spleen and kidney.

In the brain, little is known about the precise regional brain pathology in fish following exposure to trace metals. The pathological changes associated with neural dysfunction are normally observed in the meninges and blood vessels as well as the microglia (Roberts, 1989). For example, the dietary exposure to either Cu (Handy, 2003) or Hg (Berntssen *et al.,* 2003) in fish showed occasional vacuole formation and the appearance of necrotic cell bodies throughout the brain. Recently, a study by Al-Bairuty *et al.* (2013) found that exposure to $CuSO_4$ caused brain injuries that include

occasional necrotic nerve cell bodies, occasional cells with pyknotic nuclei, vacuole formation in the cell body, and apparently enlarged nerve cells in the telencephalon region; apparent enlargement of blood vessels in the mesencephalon; and increases in blood vessel diameter on the surface of the cerebellum and occasional necrotic cells between the molecular and the granular layer of the cerebellum cortex.

So far there have been few studies of the pathological effect of trace metals on the skeletal muscle in fish. One old study by Vogel (1959) showed that exposure gold fish to copper caused a decrease in the size of skeletal muscle fibres with an associated increase in spaces between the muscles. A recent study by Al-Bairuty *et al.* (2013) showed that exposure to $CuSO_4$ in trout was consistent with the observations of Vogel (1959).

The reproductive system of fish is also affected by metal. In the testis, the lesion includes degeneration and necrotic changes in the cellular elements of the seminiferous tubules after exposure to Fe, Zn, Cu, Pb, Cd and Co (Mohamed, 2008). Ovaries showed injuries that include degeneration and necrosis of the follicles with low concentration of lead nitrate while high concentration caused severe degeneration of ovarian follicles (Adeyemo, 2008). Overall, the morphological changes in organs of animals that contact with contaminated water could occur due to metal accumulation in organs which leads to generation of free radicals that causes these changes or duo to systemic hypoxia (respiratory distress).

Table 1.4 Histopathological changes in fish tissues by metal exposure

Types of Metal	Dose/ Time	Species	Histopathological damage	Authors
Cd	0.06 mg l^{-1} for 60 days	Silver barb *Puntius gonionotus*	Exposure to Cd caused lesions in the gill (hypertrophy and hyperplasia of primary and secondary gill lamellae); liver (vacuolisation in hepatocytes) and kidney (prominent tubular and glomerular damage).	Wangsongsak *et al.* (2007)
Cu	25 and 29 µg l^{-1} for 96 hours	Grumata *Prochilodus scrofa*	Cu exposure caused gill lesions that include lifting, rupture, peeling of lamellar fusion, and hyperplasia. Cell degeneration by necrosis and apoptosis were also observed. Pavement cells showed a microridge reduction on their surface. Chloride cells proliferated in the lamellar epithelia close to the onset of the lamellae.	Mazon *et al.* (2002)
Cu	Sub-lethal 0.056, 0.1 and 0.32 mg l^{-1} for 7 and 28 days	Catfish *Saccobranchus fossilis* (Bloch)	Fish exposed to Cu showed lower antibody values, declined of a number of splenic and kidney plaque-forming cells, increased counts of splenic lymphocytes compared to controls. Exposure to 0.32 mg l^{-1} of Cu for 7 days showed abnormalities in the arrangement of microvilli on the surface	Khangarot and Tripathi (1991)

Continued

Table 1.4

			of superficial epidermal cells of the skin. Accumulation of mucous; loss of shape, size and structural arrangement of epidermal cells, and mucous goblet cells as well as gill injury (oedema, fusion of secondary gill lamellae at many places, and degeneration of epithelial cells) were observed at day 7 of Cu exposure. After 28 days of exposure to 0.32 mg l^{-1} of Cu, fish showed 2-3 day delay in the eye-allograft rejection.	
$CuSO_4$	46 mg l^{-1} for 96 hours	Nile tilapia *Oreochromis niloticus*	Exposure to Cu showed lesions in the gill (hyperplasia in the primary filament, lifting epithelium, oedema, fusion in the secondary lamella and aneurism), liver (swollen hepatocytes with vacuoles and congestion in sinusoids and necrosis) and kidney (glomerulus atrophy, tubular swelling and necrosis) at the end of the exposure.	Kosai *et al.* (2009)
$CuSO_4$	0.5,1.0 and 2.5 mg l^{-1} for 21 days	Nile tilapia *Oreochromis niloticus*	The highest concentration caused oedema, lifting of lamellar epithelial, fusion and aneurisms in the lamellae of gill region. Vacuole formation and necrosis were observed in the liver.	Figueiredo-Fernandes *et al.* (2007)

Continued

Table 1.4

Pb	24 and 71 mg l^{-1} for 96 hours	Ray-finned fish *Prochilodus lineatus*	Lead exposure caused gill lesions that include lifting epithelium, hyperplasia, and lamellar aneurism. The haematocrit value did not show alteration during exposure to both lead concentrations.	Martinez *et al.* (2004)
Cr	Lethal concentration for 96 hours	Common carp *Cyprinus carpio L.*	Chromium caused lesions in the gill (clubbed tips of the secondary lamella, fusion, epithelial lifting and necrosis), liver (fatty changes, degeneration of parenchyma cells and necrosis of liver cell), and kidney (hypertrophy of epithelial cells, contraction of glomerulus, increased space inside the tubule, and glomerular oedema).	Parvathi *et al.* (2011)
$Pb(NO_3)_2$	0.0, 0.05, 0.1, 0.5 and 1 mg l^{-1} for 8 weeks	African catfish *Clarias gariepinus*	Exposure caused lesions in the gill (epithelial hyperplasia, atrophy and fusion of gill filaments, degeneration and necrosis of the epithelial cells, haemorrhage and rupture in the secondary lamellae) and ovaries (degeneration and necrosis of the ovarian follicles) with low and high concentration of lead nitrate.	Adeyemo (2008)

Continued

Table 1.4

$HgCl_2$ CH_3HgCl	0, 10, and 100 mg kg^{-1} DW 0,5 and 10 mg kg^{-1} DW for 0, 1, 2 and 4 months	Atlantic salmon *Salmo salar*	In the brain, the lesion includes vacuole formation in the medulla which was widespread and necrotic cell bodies that diffuse into the white matter. In one fish induced oedemous separation of the grey and white matter.	Berntssen *et al.* (2003)
$NiCl_2$	5.7 mg l^{-1} for 10, 20, and 30 days	Silver carp *Hypophthalmichthys molitrix*	Histopathological changes in the gill include mucus proliferation, fusion of the gill lamellae and hypertrophy. Necrosis in hepatocytes, degeneration of blood vessels, vacuole formation, hypertrophy and pyknotic nuclei were shown in the liver.	Athikesavan *et al.* (2006)
Cu, Cd, Fe, and Ni	Mixture of metals containing 50 µg l^{-1} $CuCl_2$, 80 µg l^{-1} $CdCl_2$, 750 µg l^{-1} $FeCl_2$ and 150 µg l^{-1} $NiCl_2$ for 7, 15, 30 days	Spotted snakehead *Channa punctata* (Bloch)	The light microscopy studies showed gill injuries that include fusion of the secondary lamellae, formation of club–shaped filaments, vacuolisation and necrosis of filament epithelium in the inter lamellar region. The scanning electron microscopy showed gradual increase of the density and apical surface area of the chloride cells and transformation of the surface structures of the pavement cells.	Pandey *et al.* (2008)

Continued

Table 1.4

Fe, Zn, Cu, Pb, Cd, Co	nil	Nile tilapia *Oreochromis niloticus* and Nile perch *Lates niloticus*	Exposure showed lesions in the liver (vascular degeneration in the hepatocytes, necrosis, haemorrhage and haemolysis), gill (proliferation in the epithelium of gill filaments and secondary lamellae, degenerative and necrotic changes in the epithelium), intestine (degenerative and necrotic changes in the intestinal mucosa with necrotized cell aggregate in the intestinal lumen), testis (severs degenerative and necrotic changes in the cellular elements of the seminiferous tubules with necrosis and degeneration in the wall of seminiferous tubules), heart (degeneration and atrophy in myocardial muscle fibres that accompanied with haemorrhage in cells) and muscle (degeneration in muscle bundles, necrosis, and atrophy).	Mohamed (2008)
Mixed Cd^{+} Pb^{+} Cr^{+} Ni	5 mg l^{-1} of combined metal solution for 32 days	Common carp *Cyprinus carpio L.*	Heavy metals caused lesions in the gill (oedema and lifting of lamellar epithelium), liver (vacuole formation, presence of hemosiderin and fibrosis) and kidney (the presence of macrophages with lipofuscin granules accumulated in the affected cells).	Vinodhini and Narayanan (2009)

1.3 Nanomaterials

A particulate nanomaterial is a specific term suggested by Lövestam *et al.* (2010), and defined as materials at the nanoscale with external dimensions and/or an internal structure within the size 1-100 nanometers (nm) range. Some materials revealed additional or different properties and behaviour when compared to bulk materials with similar chemical composition (Lövestam *et al.*, 2010). However, this definition is arbitrary because toxicological studies should also include work on aggregates of particles with dimensions of a few hundred nanometer (Handy and Shaw, 2007; Handy *et al.*, 2008b). The size of aggregates and the distribution of particle sizes in the material also need to be considered. Particulate matter (PM) in mammalian studies has historically used a range of PM classes. These include coarse particles with a diameter range between 10 μm and 2.5 μm ($PM_{10-2.5}$), fine particles that diameter is between 2.5 μm or less ($PM_{2.5}$), and ultrafine particles that the diameter is < 0.1 μm ($PM_{0.1}$), thus nanoparticles could be regarded as ultrafine particles or smaller (Handy *et al.,* 2008b).

1.3.1 Product and chemical classification of Nanomaterials

Nanomaterials are generally used in a wide variety of consumer products such as paint and coatings (TiO_2, SiO_2, Ag and quantum dots), fuel catalysts (CeO_2), cosmetics and personal care products such as sunscreen formulations (TiO_2, ZnO, fullerene (C_{60}), Fe_2O_3 and Ag), water treatment and environmental remediation (Fe, Fe-Pd and polyurethane), food packaging (Ag, nanoclay, and TiO_2), electronics (C_{60} fullerene) (Aitken *et al.,* 2007). Copper nanoparticles (Cu-NPs) is used in a variety of industrial applications as a reduce friction and act as catalysis on activated carbons to lower levels of nitrate in water (Barrabés *et al.*, 2006; Tarasov *et al.*, 2002).

Metal oxide NPs such as titanium dioxide (TiO_2) and zinc oxide (ZnO) are made by hydrolysis of the transition of metal ions (Masala and Seshadri, 2004) and the current studies are investigating their catalytic properties (Bertelli and Selli, 2006) and their potential use within the solar cell (Quintana *et al.,* 2007) and fuel cells for alternative forms of energy production. Cai *et al.* (2006) found that photocatalytic properties of TiO_2 may be used for solar driven self-cleaning coatings.

Nanoparticles of zero-valent iron have been used for the remediation of ground waters by removing nitrates, organochlorine pesticides and poly chlorinated biphenyls (Zhang, 2003). Copper oxide NPs have the potential to replace noble metal catalysts for carbon monoxide oxidation (Zhou *et al.,* 2006) and has excellent thermal conductivity for it to be used as a heat transfer fluid in machine tools (Chang *et al.,* 2005). Metal and metal oxide nanomaterials involve a large segment of the growing nanotechnology market. Nanoparticles can take many different shapes such as wires, rods and spherical particles (Shaw and Handy, 2011). Nanotube and nanowires, with other different shapes such as helices, zigzags, and belts were described by Buzea *et al.* (2007) (Fig. 1.2A). Some substances may have several different crystal structures. For example, Jolivet *et al.* (2010) showed the typical morphologies of titanium dioxide in accordance to the crystalline symmetry which were octahedral macrocrystals and spherical NPs of the anatase form, platelets of brookite, needles or rods of rutile TiO_2 (Fig. 1.2B). Nanoparticles can present in fused, aggregated or agglomerated forms (Nowack and Bucheli, 2007).

Classification of nanomaterials is done for different purposes, but the main classification is by chemical structure which involved consideration of the main

chemical component of the materials (Handy *et al.,* 2008b). The chemicals include: carbon-based materials [single- walled carbon nanotubes (SWCNTs), multi-walled carbon nanotubes (MWCNTs) and fullerenes (*e.g.*, C_{60})]; metal-based materials [metals and metal oxides (TiO_2 and ZnO)]; quantum dots [contains more than one chemical]. Otherwise, Handy and Shaw (2007) classified nanomaterials by material types into three groups that include: materials where bulk is made of nanostructure; coating materials (materials with nanostructure on the surface); and materials containing nanoparticles.

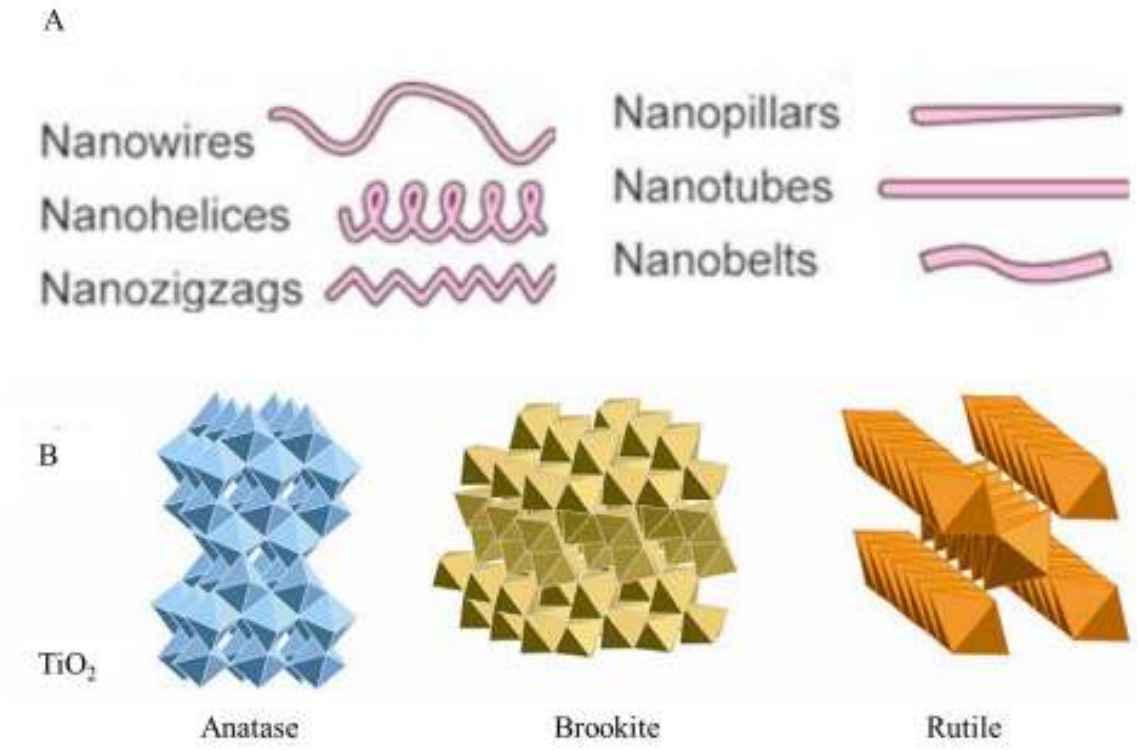

Figure 1.2 (A) Morphological characteristic of nanoparticles redrawn from Buzea *et al.* (2007). (B) Crystalline structures and morphologies of natural crystals and nanoparticles of the different polymorphs of titanium dioxide redrawn from Jolivet *et al.* (2010).

1.3.2 Properties of metal nanoparticles

Concern about nanoparticle toxicity originally focused on the idea that NPs will be much more reactive than large particle (The Royal Society and The Royal Academy of Engineering, 2004). However, the physico- chemistry issues are much more complex than simply a matter of particle size. Biological activity of nanoparticles may depend on physical and chemical properties not considered previously in toxicity studies (Oberdorster *et al.*, 2005). These properties include particle size, size distribution, number, concentration, agglomeration state, shape, crystal structure, chemical composition, surface area, surface chemistry, surface charge, porosity and method of synthesis (Oberdorster *et al.*, 2005; Baker *et al.*, 2005; Murdock *et al.*, 2008). Some authors suggest that cytotoxicity of nanoparticles is dependent on the particle size (Pan *et al.*, 2007), whilst other authors disagree and showed that particle size does not always alter the toxicity (Warheit *et al.,* 2006). In mice, Grassian *et al.* (2007) explained that larger instilled TiO_2 nanparticles were more toxic, while Inoue *et al.* (2009) showed inhalation of a smaller size of NPs generated a greater inflammatory response than larger particles. However, the size may not be an accurate dose metric because size and surface area are related. In mammalian models (mice and rat), instillation studies showed that although smaller sized NPs caused a greater inflammatory response than larger sized particles on a mass basis, the level of inflammatory response is dependent on the total surface area of particles instilled (Oberdörster *et al.,* 2000). Oberdörster *et al.* (2007) mentioned that specific surface area (SSA) sometimes may be more important to the toxicity of nanoparticles and is considered to be a better way to describe the dose- effect of nanomaterials when surface reactivity is a key characteristic. The large surface area of some metal nanoparticles may facilitate transport of other toxic pollutants in the environment. For example, Sun *et al.* (2007) showed the TiO_2

NPs had stronger adsorption for Arsenic (As) and the potential of TiO_2 NPs to facilitate the transport and accumulation of As into carp after 25 days of exposure increased.

Abiotic factor of water chemistry could influence the biological response or toxicity of nanoparticles. These factors include pH, salinity, water hardness, temperature, and the presence of dissolved or natural organic matter in the water (Handy *et al.*, 2008b; Handy *et al.*, 2008a). Alternatively, there may be indirect effects associated with aggregation chemistry. Elevation of hardness can increase aggregation of the nanoparticles, but the toxicity of these effects is unclear. Additionally, the ecotoxicity of nanoparticles in seawater is unclear compared to freshwater, but the experimental studies of colloid chemistry in saline conditions show that any small increases in salinity above that of freshwater such as 2.5 parts per thousand (ppt) can cause decrease colloid concentration by aggregation processes (Stolpe and Hassellöv, 2007). The high ionic strength of seawater will tend to cause aggregation of NPs and some NPs can aggregate in the presence of other naturally occurring particles (heteroaggregation), as well as with other NPs that may be present in liquid phase. However, in fresh water, the presence of organic matter such as humic and fulvic acid substances can stabilize nanoparticles in the water column (Lead and Wilkinson, 2006; Handy *et al.*, 2008b). Nanoparticle aggregation chemistry is complex, but partly depends on the large surface area proportional to the volume of nanoparticles, and how they react physically and chemically with other particles, or colloid materials present. Nanoparticle aggregation is found to depend on pH and surface charge. Gilbert *et al.* (2007) showed a pH-driven aggregation and disaggregation with a larger aggregate radius at higher pH, and vice versa in the case of 6 nm iron oxyhydroxide (FeOOH) particles. Aggregation may happen by interactions between the same types of particles,

or different types of particles in the medium. The interactions may be by weak forces, such as Borne repulsion, diffuse double layer potential and Vander Waals attraction (Handy *et al.,* 2008b). These forces are described in the DLVO theory which refers to the names of the original authors of the theory (Derjaguin and Landau, 1941; Verwey and Overbeck, 1948) that devised the basic features of particle interactions for spheroid particles (Handy *et al.,* 2008b).

The chemistry of metal nanoparticles suggests that NPs can aggregate in many types of hard freshwater or seawater, and there are possible methods that act to disperse the aggregations of NPs. Ecotoxicologists have used three basic approaches to alter the dispersion of nanoparticles namely solvents, sonication, or prolonged stirring (Shaw *et al.*, 2012). These approaches are worth considering in the context of the fundamental physico-chemical behaviour (Handy *et al.,* 2008b). For example solvents such as tetrahydrofuran (THF) which has been used to disperse C_{60} fullerenes (Oberdörster, 2004). THF can provide fullerene dispersions in water, but the THF is toxic to fish (Henry *et al.,* 2007). The stirring or sonication approaches provide energy to the nanoparticles and may break up aggregates (Federici *et al.,* 2007).

1.3.3 Nanoparticles in the environment

Natural sources of nanoparticles have existed in the environment since the beginning of earth`s history, for example, volcanic dust in the atmosphere (Ammann *et al.,* 1990), colloids in most natural waters, soils and sediments. In addition to these sources, there is an anthropogenic source which has been incidentally generating nanoscale pollutants such as airborne particles (Handy and Shaw, 2007). In water, a colloid is particulate matter in the 1 nm to 1 μm size range which comprises of

macromolecular of organic materials (*e.g*., humic and fulvic acids, peptides, proteins) as well as colloidal inorganic species (hydrous iron and manganese oxides; Buffle, 2006). In soil and sediments, natural nanoparticles such as clays, organic matter, iron oxides and other minerals played an important role in biogeochemical processes (Klaine *et al.,* 2008). Manufactured nanomaterials enter the environment through atmospheric emissions and solid or liquid waste streams from production facilities. In addition, nanoparticles in paints, fabrics, and personal health care products (sunscreens and cosmetic) can enter the environment according to proportional to their use (review, Biswas and Wu, 2005). The concentrations of nanoparticles in the environment have not been measured but the models suggest concentrations between ng l^{-1} to low µg l^{-1} range, whereas typical dissolved and colloidal organic matter in fresh water may be found at 1 - 10 mg l^{-1} concentration. In marine and estuarine conditions, nanoparticle concentrations would be much lower than fresh water because of increased aggregation and sedimentation at higher ionic strengths (Klaine *et al.,* 2008).

1.3.4 Lethal concentration of metal nanoparticles on fish and invertebrate

A few tests of lethal toxicity (LC_{50}) have been conducted using metal nanoparticles in fish (Table 1.5). Handy *et al.* (2008b) suggested that the reason for a lack of LC_{50} values for fish was technical issues related to achieving the high concentration (mg l^{-1}) needed to give acute lethal toxicity. The exposure modelling on fish and invertebrates predicted that acute lethal concentrations are in the mg l^{-1}, rather than µg l^{-1} range. For example, the LC_{50} of several metal nanoparticles (Ag, Cu and Zn) in zebrafish (*Danio rerio*) is approximately between 1.5 - 1.79 mg l^{-1} after 96 h of exposure (Griffitt *et al.*, 2007; Zhu *et al.*, 2008a). The value of LC_{50} for fathead minnow

(*Pimephales promelas*) exposed to titanium dioxide was 500 mg l^{-1} (Hall *et al.,* 2009), and these values are significantly higher than the typical µg l^{-1} ranges reported for ordinary metal salts (Spry and Wiener, 1991). This could suggest that metal NPs may have low toxicity. Nevertheless, metal NPs (Cu and Ag) may dissolve in solution to release free metal ions (Griffitt *et al.*, 2007; Kittler *et al.*, 2010). Shaw *et al.* (2012) explained that exposure of juvenile rainbow trout to 100 µg l^{-1} of either dissolved copper or copper nanoparticles for 96 h caused 80 % and 15 % mortality respectively, which suggests that dissolved Cu is more toxic than Cu-NPs.

Studies on fish and invertebrates showed that carbon-based NMs (C_{60} fullerenes) are toxic in mg l^{-1} range, but the LC_{50} values obtained are very dependent on the method of preparation of the material and the addition of dispersants (THF, sonication, SDS) (Lovern and Klaper, 2006; Zhu *et al.*, 2006; Smith *et al.*, 2007). In *Daphnia magna*, the exposure to 350 and 440 ppb of C_{60} fullerenes dispersed in tetrahydrofuran (THF) caused the highest mortality (52 and 60 %, respectively) after 48 h while the 7.9 ppm of sonication C_{60} caused high mortality (50 %) (Lovern and Klaper, 2006). Zhu *et al.* (2008a) described that toxicological endpoints for zebrafish embryos or larvae including hatching rate and malformation within 96 h of exposure to nanoscale zinc oxide and found the LC_{50} of nanoscale zinc oxide was 1.79 mg l^{-1} for zebrafish.

Table 1.5 Lethal concentration (LC_{50}) values for metal nanoparticles in fish and *Daphnia*. Modified from Handy *et al.* (2011).

Metal nanoparticles	LC_{50}	Species	Toxic effects	Authors
Nanoscale zinc oxide (nZnO) in aqueous suspensions.	1.793 mg l^{-1}	Zebrafish *Danio rerio*	Both nZnO and ZnO bulk aqueous suspensions caused delay zebrafish embryo and larva development, declined their survival and hatching rates, and occurred tissue damage.	Zhu *et al.* (2008a)
ZnO/bulk	1.550 mg l^{-1} for 96 hours			
Carbon NP (C_{60}). Dispersed by tetrahydrofuran (THF). Purity was 99.5 %. Particle size was 100 nm.	1.56 mg l^{-1} for 96 hours	Zebrafish embryos *Danio rerio*	The survival of embryos was 45 % and caused delay embryo and larval development, decreased survival and hatching rates, and induced pericardial edema.	Zhu *et al.* (2007)
Carbon NP (C_{60} fullerene). Dispersed by THF or stirring for weeks, suspended in ultrapure water. 99.5 % pure, 0.5 % impurities not quantified.	0.5 mg l^{-1} of either water-stirred or THF-prepared C_{60} for 48 hours	Fathead minnow *Pimephales promelas*	Tetrahydrofuran solubilised nC_{60} caused 100 % mortality within 18 hours. nC_{60} stirred in water was less toxic and small, but showed significantly increased in CYP-like genes in the liver.	Zhu *et al.* (2006)
Carbon NP (C_{60} fullerenes). Dispersed	350 - 880 ppb	*Daphnia magna*	C_{60} caused 52 % mortality in concentration of	Lovern and

Continued

Table 1.5

in THF and filtered, particles size was 0.72 nm diameter which formed aggregates of 10-20 nm, uncoated.	for 48 hours		350 ppb and 100 % mortality in the concentration 880 ppb.	Klaper (2006)
Carbon NP (C_{60} fullerenes). Dispersed by sanction instead of THF and filtered, formed aggregates are 20-100 nm.	7.2 – 9 ppm for 48 hours	*Daphnia magna*	Highest mortality rate at 9 ppm (80%).	Lovern and Klaper (2006)
Nanocopper. Dispersed by sonication for six seconds. (80 nm particles in agglomerates > 1 μm in diameter).	1.56 mg l^{-1} for 48 hours	zebrafish *Danio rerio*	Nanocopper was moderately toxic (95 % CI= 0.79-3.08 mg l^{-1}) to fish and caused histological and biochemical changes in the gills.	Griffitt *et al.* (2007)
Nanocopper (mean primary particle size of dry powder was 26.7 ± 7.1 nm) sonicated with a probe sonicator for 0.5 s pulses prior to dosing.	0.71 mg l^{-1} 0.94 mg l^{-1} for 48 hours	Zebrafish (juvenile) Zebrafish (adult) *Danio rerio*	Copper nanoparticles were more toxic than soluble copper which had a 48 h LC_{50} value of 1.78 mg l^{-1} for larvae	Griffitt *et al.* (2008)

Continued

Table 1.5

Aluminium nanoparticles (mean primary particle size of dry powder was 41.7 ± 8.1 nm), sonicated with a probe sonicator for 0.5 s pulses prior to dosing.	0-10 mg l^{-1} for 48 hours	Zebrafish *Danio rerio*	High concentration (10 mg l^{-1}) of AL-NPs not elevated enough to establish an LC_{50} for adults or larvae; dissolved AL gave LC_{50} concentration of 7.92 mg l^{-1} and over 10 mg l^{-1} in adults and larvae respectively.	Griffitt *et al.* (2008)
Nanosilver (mean primary particle size of dry powder was 26.6 ± 8.8 nm) stabilised with, 5 % sodium citrate solution sonicated with a probe sonicator for 0.5 s pulses prior to dosing.	7.20 mg l^{-1} 7.07 mg l^{-1} for 48 hours	Zebrafish (larvae) Zebrafish (adult) *Danio rerio*	Silver NPs caused toxicity in zebrafish with less toxic than soluble silver.	Griffitt *et al.* (2008)
Nanosilver (two commercial Ag NPs (NanoAmor and sigma) with a nominal particle size of 35 nm and < 100 nm, purity for both is 99.99 %) sonicated at 15 KHz for 1.5 h.	9.4 g l^{-1} (NanoAmor) 10.6 g l^{-1} (sigma) for 96 hours	Fathead minnow *Pimephales promelas* embryos	Concentration dependent increase in larval abnormalities. Analysis TEM images showed that both types of Ag NPs were presented within embryos after 24 h of exposure.	Laban *et al.* (2010)

Continued

Table 1.5

Nanosilver (commertial name of Nanocid, particle size of 4.5 nm, data taken from manufacturers website).	3.5 µg l^{-1} for 48 hours 3.0 µg l^{-1} for 72 hours 2.3 µg l^{-1} for 96 hours	Rainbow trout *Oncorhynchus mykiss*	The toxicity of Nanocid is moderate in rainbow trout.	Shahbazzadeh *et al.* (2009)
Nanosilver (25 nm spherical particles).	0-8 mg l^{-1} for 48 h (adults) 0-4 mg l^{-1} for 168 h (embryos), followed by 0-1000 µg l^{-1} for 60 d from embryo stage 10	Japanese medaka (adults & embryos) *Oryzias latipes*	In adult fish, the 48 h LC_{50} was 1.03 mg l^{-1}, whilst in the embryo was 2 mg l^{-1} that caused 100 % mortality. Retarded development and reduced pigmentation were seen in embryos exposed to ≥ 400 µg l^{-1} Ag-NPs with morphological malformations (oedema, spinal abnormalities, finfold abnormalities, heart malformations and eye defects) that seen in all Ag-NPs treatments.	Wu *et al.* (2010)
Ag NPs (30-40 nm particle size) coated with 0.2 % polyvinyl	84 µg l^{-1} for 48 hours	zebrafish	Exposure to Ag NPs showed an increase rate of operculum movement and surface	Bilberg *et al.*

Continued

Table 1.5

pyrrolidone (PVP). Dispersed in Milli-Q water, followed by sonication for 15 min (4 times) over a 2-hour time span. After sonication, the suspension was centrifuged at1000 g for 2 hours, and finally the supernatant was filtered through a 0.2 μm mixed cellulose ester filter.		*Danio rerio*	respiration.	(2012)
Ag NPs (nano products from Iranian nanotechnology company, Nanosil®). Dispersed in deionized water by sonication (particle size < 100 nm).	66.4 ppm 83.9 ppm for 96 hours	silver carp *Hypophthalmichthys molitrix* Gold fish *Carassius auratus*	A significant increase in mortality was seen in silver carp and goldfish exposed to acute doses of Nanosil®. Nevertheless, this acute effect was higher in silver carp than goldfish.	Jahanbakhshi *et al.* (2012)

Continued

Table 1.5

TiO_2 NPs. Dispersed by stirring for 30 minutes. Purity was 99 % with a primary particle size 10 nm.	500 mg l^{-1} for 96 hours	Fathead minnow *Pimephales promelas*	The study also involved LC_{50} measurement on invertebrates. TiO_2 NPs were more toxic to the invertebrate species tested. The LC_{50} value for *Ceriodaphnia dubia* and *Daphnia pulex* were 7.6 and 9.2 mg l^{-1}, respectively.	Hall *et al.* (2009)
TiO_2 NPs. Dispersed by sonication.100-500 nm aggregates was formed.	50 - 500 ppm for 48 h	*Daphnia magna*	No significant mortality reported.	Lovern and Klaper (2006)
TiO_2 NPs. Dispersed in THF and filtered. 30 nm aggregates were formed.	6-10 ppm for 48 hours	*Daphnia magna*	Concentration 6 ppm caused 50 % mortality while concentration 10 ppm caused 100 % mortality.	Lovern and Klaper (2006)

1.3.5 The accumulation of metal nanoparticles in target organs

Information about the accumulation of metal nanoparticles in target organs is very limited. The exposure route and the toxico-kinetics of metal nanoparticles are very important to explain the ADME (Handy *et al.,* 2008a). Adsorption of nanoparticles is the first step in the biological uptake that is via the gill surface, or gut epithelium by endocytosis because manufacture NPs would be too big to cross the cell membrane by membrane transporter or paracellular diffusion (Handy *et al.,* 2008a). However, Handy *et al.* (2008a) have reviewed the biological uptake and unstirred layer chemistry of NPs in fish gills, and the main differences between metal ions or nanometals in fish are summarised in Fig. 1.3. For example, the chemistry and behaviour of nanomatels such as aggregation properties and the ability of some nanometal (Cu-NPs) to release of free metal ions from the surface of the metal (Cu) when compared to the traditional metal form used for free metal ions. Additionally, the biological uptake of metal ions across the membrane involves carrier transporter (Bury and Handy, 2010) whereas NPs enters via the endocytosis pathway.

The accumulation of NPs is dependent on the route of exposure. For example, waterborne exposure of *Japanese medaka* to 39.4 nm fluorescent particles showed the highest accumulation in the gills then shortly followed by the intestine. This result suggests that NPs can attach the gill surface and then may enter the epithelial cell (Kashiwada, 2006). Dietary exposure to TiO_2 NPs in rainbow trout showed Ti accumulation in the gill, gut, liver, brain and spleen (Ramsden *et al.*, 2009). Whereas, intravenously injected with 100 μg TiO_2 NP in trout showed higher accumulation in the kidney then followed by the liver (15 times lower than kidney) (Scown *et al.*, 2009). In

mammals, Jani *et al.* (1990) showed that oral administration of polystyrene latex can be absorbed across the gastrointestinal tract, and pass through the mesentery lymph supply and lymph node to liver and spleen. Whilst, Wang *et al.* (2007) found that Ti was accumulated in the spleen, kidney and lung tissue after 2 weeks from oral uptake of 5 g kg^{-1} TiO_2 NPs in mice.

Distribution of NPs within the body and the target organs through the circulatory system are unknown. The behaviour of metal NPs to aggregate or agglomerate in saline condition (*e.g*., blood plasma) could suggest that they adhere to blood cells (Handy *et al.*, 2011). Actually, very little data is known about the kinetic of NPs in the circulation of fish. One study by Scown *et al.* (2009) found 15 times more Ti accumulated in the kidney of trout than the liver after 6 h from giving a single injection of 1.3 mg kg^{-1} TiO_2 NPs in physiological saline with the kidney measuring for 10 -19 % of the total burden of the injected dose. However, very little of the NPs material was measured in the blood, suggesting that NPs could be either rapidly removed from the circulation or deposit on the blood vessel as well as they might not withdraw via a syringe during blood sampling (Handy *et al.*, 2011). Information about the metabolism and excretion of nanoparticles in fish is very limited, but the hepatic excretion via the bile duct seems more typical mechanism than through the renal or branchial excretion (Handy *et al.*, 2008a). Metal granules are known to deposit in the liver of rainbow trout (Cu, Lanno *et al.*, 1987), and it would seem logical that nanometal could also deposits in the hepatic cell. In fish, it remains unclear if nanometal deposits could stay as inert storage granules in the liver, or be excreted via the bile.

Some organs in fish may be considered as target organs for toxic effects of some nanoparticles such as the gills, gut, liver and brain (Handy *et al.*, 2011). Exposure to different metal NPs showed differences in the biological response. Griffitt *et al.* (2009) found that acute exposure to nanocopper, nanosilver, and nanotitanium in zebrafish do not produce similar responses in the gill and also the effects of nano Cu or nano Ag were not due to release of soluble metals into the water column. For example, this researcher showed an increase in the gill filament width with nano Cu, while nano Ag and nano TiO_2 failed to do alteration in the gill filament.

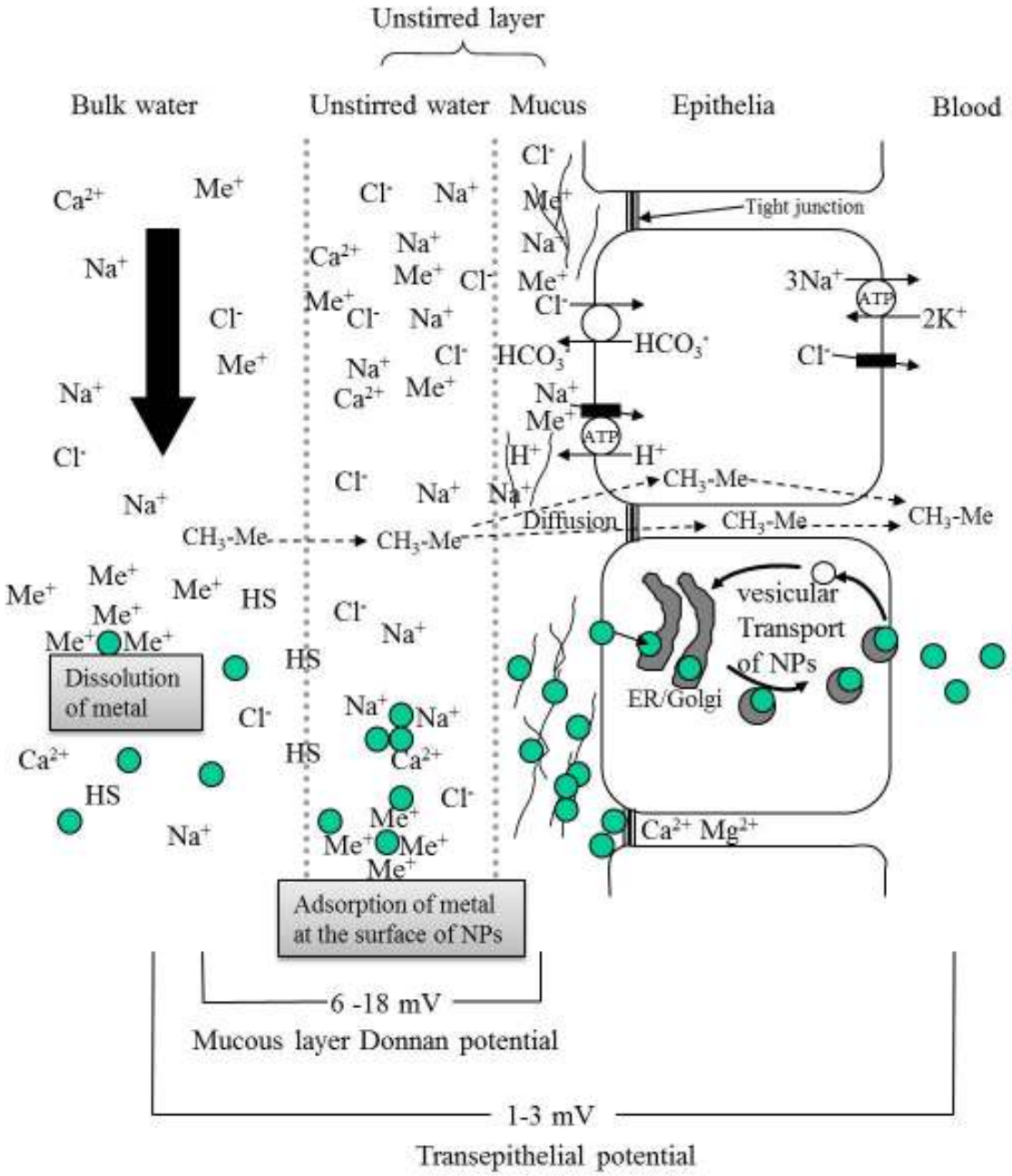

Figure 1.3 An idealised diagram of the freshwater fish gill showing the mechanisms of uptake for electrolytes, toxic metal ions (Me^+), and electroneutral diffusion of some small organo-metals (CH_3-Me), compared to nanoparticles (NPs, filled circles). Modified form Shaw *et al.* (2012), Handy *et al.* (2008a) and (Handy and Eddy, 2004). The substances in the bulk solution (the freshwater) must diffuse into an unstirred layer (USL) comprising of water/mucus secretions, prior to transfer across the gill epithelium. The upper portion of the diagram shows electrolytes and toxic metals ions which diffuse into the USL, and may bind to strands of mucus (mostly polyanionic) where the exclusion of free anions like Cl^- from the mucus layer contributes to the Donnan potential at the apical surface. Electrolytes and toxic metal ions usually move through

the cell using ion transport pathways (Na^+ transporters are illustrated here). In contrast, small lipophilic organic chemicals can diffuse into the USL and then through the cells (transcellular diffusion), or between the cells via the tight junctions (paracellular diffusion). The situation for NP uptake across the gill will be a little different. NPs will diffuse into the USL, albeit at a slower rate than smaller molecules or solutes, and may be influenced by humic substances (HS). Cationic NPs will bind to strands of mucus (electrostatic attraction, fundamentally similar to other cations), but regardless of surface charge, may also become entangled in the mucoproteins (steric hindrance) to prevent uptake by the epithelial cells. NPs are too large to be taken up by ion or other transporters on the cell membranes, and although diffusion cannot be excluded for lipophilic NPs. The Ca^{2+} and Mg^{2+} rich environment in the tight junctions suggest that NPs would aggregate rather than diffuse through the paracellular route. Diffusion of charged NPs into the USL will be affected by the Donnan and transepithelial potentials, in a similar way to other charged substances. NP uptake through vesicular transport seems likely.

1.3.6 Effects of sub-lethal exposure to metal nanoparticles on tissues of organisms

The effects of sub-lethal concentration of metal nanoparticles can be seen in Table 1.6 and Fig 1.4. The studies about the effect of sub-lethal metal nanoparticles on wildlife are fewer than on metals. Some studies used *Daphnia magna* and fish to identify mechanisms of toxicity for NPs (Smith *et al.*, 2007; Griffitt *et al.*, 2008). These mechanisms include oxidative stress, ionic disturbances and enzymatic activity changes (Oberdörster, 2004; Zhu *et al*., 2006; Federici *et al.*, 2007; Handy *et al.*, 2008a). Oxidative stress may be a key route in causing the cytotoxicity of nanoparticles (Yang *et al*., 2009), who also observed that carbon nanotubes were moderately cytotoxic but caused more DNA damage compared with zinc dioxide nanoparticles. Generation of reactive oxygen species have been shown to damage cellular lipids, carbohydrates, proteins and DNA (Kelly *et al.,* 1998) and then lead to inflammation and oxidative stress responses associated with particle exposures (Singh *et al.,* 2006). Hao *et al.* (2009) found that exposure of carp to 100 mg l^{-1} of TiO_2 NPs for 8 days cause oxidative

stress that resulting in lipid peroxidation and then affecting cellular enzymatic activities involved in redox defense. The exposure of 0.1 - 1 mg l^{-1} TiO_2 NPs to rainbow trout for less than 14 days caused an increased in TBARS levels in the gill, intestine, and brain which indicated the fish suffered from oxidative stress (Federici *et al.,* 2007). Zhu *et al.* (2008b) mention that waterborne exposure of juvenile carp (*Carassins auratus*) to 0.04 mg l^{-1} C_{60} for 32 days caused an increase in superoxide dismutase and catalase in the gills and liver, a decrease in the level of glutathione and lipid peroxidation in the gills and brain, while exposure to 1.0 mg l^{-1} of C_{60} led to a significant ($P < 0.01$) increase in lipid peroxidation level in the liver and significantly ($P < 0.05$) decrease in the body weight and total length of carp.

The effects of nanoparticles on fish haematology, immune cells, and plasma biochemistry remain to be investigated. However, for the effects of TiO_2 NPs in rainbow trout, there seems to be no important disturbances to blood cell counts or plasma electrolytes following waterborne exposure (Federici *et al.*, 2007) and no indication of lipid peroxidation (TBARS assay) in blood plasma following intravenous administration (Scown *et al.*, 2009).

Table 1.6 The sublethal effects of nanoparticles on fishes.

Metal nanoparticles/ chemical characteristic	Concentration/ Time	Species	Sublethal effects	Authors
C_{60} fullerenes. Dispersed by stirring in water. 99.5 % pure.	0.5 mg l^{-1} for 48 hours	Male fathead minnow *Pimephales promelas*	C_{60} fullerenes caused increased lipid peroxidation in the gill and possibly in the brain.	Zhu *et al.* (2006)
C_{60} aggregates suspended in water.	0-1.5 mg l^{-1} for 96 hours	Zebrafish (embryos) *Danio rerio*	C_{60} caused delay embryonic and larva development. Declined hatching rates and occurred pericardial oedema.	Zhu *et al.* (2007)
C_{60} fullerenes (aggregates between 50 and 300 nm), pure 99.5 % dispersed in water by either stirring for 7 days with sonication or by use of THF.	0-25 % (vol/vol) for 72 hours	Zebrafish (larval) *Danio rerio*	Survival of larvae zebrafish reduced in THF-C_{60} and THF control treatments, but not in the water stirred treatment. The latter treatment showed minimal changes in the gene expression compared to controls, whilst changes observed in THF C_{60} and THF control fish deemed to be linked to a THF degradation product (γ-butyrolactone).	Henry *et al.* (2007)

Continued

Table 1.6

C_{60} fullerenes (aggregates of 30-100 nm), dispersed in tetrahydrofuran (THF) with overnight stirring, uncoated 99.5 % pure carbon.	0.5 or 1.0 mg l^{-1} for 48 hours	Juvenile largemouth bass *Micropterus salmoides*	0.5 mg l^{-1} of C_{60} showed reduced the lipid peroxidation in the liver and gill whereas the level of lipid peroxidation were increased in the brain tissue in both concentrations. Reduced glutathione activity in the gill tissue.	Oberdörster (2004)
TiO_2 NPs (mean particle size of 24.1 ± 2.8 nm), dispersed by sonication.	0.1- 1.0 mg l^{-1} for 14 days	Rainbow trout *Oncorhynchus mykiss*	No major haematological disturbances were seen. Decrease in Na^+/K^+-ATPase activity in the gill and intestine. TBARS was increased in the gill, intestine and brain which lead to oxidative stress. Pathologies are reported in the gill and other internal organ such as the brain.	Federici *et al.* (2007)
TiO_2 NPs (50 nm primary particle size), surface area of 30 ± 10 m^2 g^{-1}, rutile form crystal structure, with a purity of > 98 %, dispersed by sonication for 30 min.	5, 10, 25, 50, 100, 200 and 400 mg l^{-1} for up 8 days	Juvenile Common carp *Cyprinus carpio*	100 and 200 mg l^{-1} of TiO_2 NPs caused a decrease in SOD, CAT and POD activities and increase in LPO levels in the gill, liver and brain.	Hao *et al.* (2009)

Continued

Table 1.6

TiO_2 NPs (mean particles size 24.1 ± 2.8 nm), dispersed by sonication for 6 h without the use of solvents and applied to a commercial trout diet.	0-100 mg TiO_2 NPs kg^{-1} diet for 8 weeks followed by 2 weeks recovery on the control diet	Juvenile rainbow trout *Oncorhynchus mykiss*	No impact on growth or nutritional performance, and no major changes were seen in haematological parameter. The brain appeared disturbances to Cu and Zn levels and 50 % inhibition of Na^+/K^+-ATPase activity.	Ramsden *et al.* (2009)
SWCNT. Dispersed in sodium dodecylsulphate (SDS) and sonication. Size 1.1 nm mean outside diameter, 5-30 µm length, maximum impurities were: Al 0.08, Cl 0.41, Co 2.91 and S 0.29 %.	0.05, 0.1, or 0.5 mg l^{-1} for up 10 days	Rainbow trout *Oncorhynchus mykiss*	SWCNT caused an increase in the ventilation rate, gill pathologies. Increased Na^+/K^+-ATPase activity in the gill and intestine but not in brain. Decreased TBARS in gill, brain and liver. Increased in total glutathione in the gill and liver, and reported pathological changes in the liver and brain.	Smith *et al.* (2007)
Al-NPs. Dispersed by sonication for 1 min, the diameter of particle size 40-60 nm.	0.5 or 2.5 mg Al l^{-1} for 48 hours	Zebrafish *Danio rerio*	Al-NP caused a decrease in Na^+/K^+-ATPase activity in the gill, although it did not observe alteration in the morphology of gill.	Griffitt *et al.* (2011)

Continued

Table 1.6

Ag-NPs (mean particle size was 49, 114, and 137 nm (bulk), dispersed with sonication for 30 min prior to exposure.	10-100 µg l^{-1} for 10 days	Juvenile rainbow trout *Oncorhynchus mykiss*	Low uptake with the smallest particles concentrated in the gill and liver tissues (the latter along with the bulk silver particles). No effects on LPO amongst any treatment (TBARS assay), although potential evidence of oxidative metabolism in gills of fish exposed to 49 nm Ag NPs.	Scown *et al.* (2010)
Cu-NPs (average particle size < 50 nm ; purity 99.9 %) dispersed in ultrapure water with stirred.	20 and 100 µg l^{-1} for 4 and 10 days	Rainbow trout *Oncorhynchus mykiss*	Cu-NPs caused depletion in Na^+/K^+-ATPase activity in the gill and brain. Decreased TBARS level in the brain was seen with both Cu-NPs concentration.	Shaw *et al.* (2012)

THF = tetrahydrofuran; TBARS = thiobarbituric acid reactive substances; SOD = superoxide dismutase; CAT = catalase; POD = peroxidase; LPO = lipid peroxidation; TiO_2 NPs = titanium dioxide; SWCNT = single -wall carbon nanotube; Ag NPs = silver nanoparticles; Al NPs = aluminium nnanoparticles; Cu-NPs = copper nanoparticles.

1.3.7 Histopathological studies of nanoparticles on fishes

There are very few histopathological studies of metal nanoparticles on fish. Possible pathological changes in various target organs following exposure to NPs are summarised in Fig 1.4. Gills are the major organs for respiratory gas exchange and observed injuries in the gill structure following waterborne exposure to NPs could lead to hypoxia (Federici *et al.*, 2007; Griffitt *et al.*, 2007; Al-Bairuty *et al.*, 2013). Proliferation of the epithelial cells and oedema of both the primary and secondary filaments were observed in *D. rario* exposed to 1.5 mg l^{-1} nano-Cu for 48 h. Hao *et al.* (2009) showed that exposure carp to TiO_2 NPs suspension caused an increase in the incidence of thickening, oedema, fusion and hyperplasia in gill lamellae and filaments after 20 days of exposure. Nano-related gill injury may be observed. For example, waterborne exposure to SWCNTs in trout causes unusual hyperplasia of the epithelial cells in the primary filament and at the base of the secondary lamellae (Smith *et al.*, 2007). This uncommon form of hyperplasia was also observed in the gills of *O. mykiss* after 14 days of waterborne exposure to TiO_2 NPs (Federici *et al.,* 2007). Similarly, Griffitt *et al.* (2009) found nanocopper caused increased filament width in zebrafish gills by three to four folds between 24 and 48 h of exposure, whilst nanosilver did not alter the gill filament width at either time point, and they suggest that some pathologies are material specific. Exposure of medaka *Oryzias latipes* to nano-iron also caused cell swelling and hyperplasia in the gill filament (Li *et al.*, 2009). Generally, waterborne exposure to NPs could lead to cause respiratory toxicity in fish, with increases in ventilation rate and mucus secretion as well as enlarged of mucocytes on the gills. A study by Smith *et al.* (2007) showed that waterborne exposure to SWCNT in trout

causes an increase in the ventilation rate and they suggest that some alteration in respiratory gases might occur.

There are some data on the effect of NPs on the internal organs. The gut epithelium can be damaged by nanomaterials, and this damage is probably related to the route of exposure. Stress- induced drinking of NP-contaminated water in rainbow trout has previously observed following waterborne exposure to TiO_2 NPs (Federici *et al.*, 2007), SWCNT (Smith *et al.*, 2007), and Cu-NPs (Shaw *et al.*, 2012). Work by Federici *et al.* (2007) suggested that drinking by rainbow trout exposed to 1 mg l^{-1} suspension of TiO_2 NPs for 14 days caused erosion and a fusion of the intestinal villi, as well as the appearance of vacuoles in the layer of mucosa. More severe erosion and inflammation were observed with rainbow trout ingesting SWCNT in suspension after 10 days of exposure (Smith *et al.*, 2007), whereas for the same SWCNTs added in the food such severe lesion was not seen (Fraser *et al.*, 2010). Recently, waterborne exposure to $CuSO_4$ or Cu-NPs in trout showed similar types of gut injury, but these injuries were more severe in the Cu-NPs treatment after 10 days of exposure (Al-Bairuty *et al.*, 2013). However, the matrix of the food in the gut lumen may affect both the bioavailability and toxicity of NPs to the gut (Handy *et al.*, 2005). There are many more information gaps about the physiological effects of NPs on gut function such as; specific nutritional deficiencies caused by NPs, as well as effects on digestive enzymes, gut microbial communities, gut motility and blood flow.

The liver functioning as an excretory organ for NPs in fish is discussed in Handy *et al.* (2008a). Alterations in the liver structure were observed in trout after 10 days of exposure to SWCNT and these alterations include changes in nuclear morphology with condensed nuclear bodies that look like apoptotic bodies, nuclear division with

condensed nuclear material and early stages of cellular necrosis (Smith *et al.,* 2007). Similar types of liver pathologies were observed in rainbow trout following exposure to $CuSO_4$ or Cu-NPs (Al-Bairuty *et al.*, 2013) and bulk or TiO_2 NPs (Boyle *et al.*, 2013), but the severity was different. Therefore, the liver function may be affected in a material-specific way.

Little is known about the kidney and spleen pathologies in fish following exposure to nanoparticles. A recent study by Al-Bairuty *et al.* (2013) showed that exposure of *O. mykiss* to $CuSO_4$ or Cu-NPs caused similar types of pathologies in the kidney including some damage to the epithelial cell of the renal tubules, changes in the Bowman's space, and an increase in the presence of melanomacrophage deposits by the end of the experiment. The lesions observed in the kidney could have effects on renal function.

Less detail is known about the immunological effects of NMs in fishes. The spleen is considered one of the important tissues of the haematopoietic system in fish, which is important in removing damaged blood cells from the circulation and could be affected by the presence of NMs in the circulatory system. Therefore, it might be expected to see increase the activity of the spleen during NMs exposure, or even pathological changes in the cells contained in the spleen. For example, the spleen prints of fish used in the TiO_2 study of Federici *et al.* (2007) showed alteration in the morphology of red blood cells and small alteration in the proportion of immune cells (review paper, Handy *et al.*, 2011). Recently, a study by Boyle *et al.* (2013) showed that waterborne exposure of trout to bulk or TiO_2 NPs caused similar types of changes in the spleen structure which included a decrease in the fractional volume of red pulp with a

concomitant increase in sinusoid space as well as elevating the number of melanomacrophage deposits. The same author also showed the number of melanomacrophage infiltrate was greater with the NP-exposed fish than control and bulk form treatment, and they suggest that these deposits could reflect the normal activity of the spleen to remove damaged blood cell and foreign material from the circulating blood (Boyle *et al.*, 2013).

The brain is also a potential target organ for nanomaterials. The brain shows regional histopathological changes that included injury to the cerebral vasculature (suspected aneurisms) and individual necrotic bodies and small foci of vacuolisation in parts of the brain after exposure of rainbow trout to 0.5 mg l^{-1} CNT for 10 days (Smith *et al.,* 2007). Treatment with $CuSO_4$ or Cu-NPs showed similar types of brain injuries in rainbow trout after 10 days of exposure (Al-Bairuty *et al*., 2013) and these injuries included necrosis of some nerve cells and the occasional vacuole formation in the telencephalon; a minor alteration in the thickness of mesencephalon with the preventriculare layer being damaged; as well as increases of the size of blood vessels in the cerebellum (Al-Bairuty *et al.*, 2013). Given these potential brain injuries, there is an emerging concern that animal behaviour and locomotor activity could affect by NPs.

There are a few data about the pathological effects of nanoparticles on fish muscle. A recent study by Al-Bairuty *et al.* (2013) found that exposure of *O. mykiss* to Cu-NPs caused similar types of injuries to $CuSO_4$ including an increase in the relative proportion of the extracellular space in the muscle. A similar observation was found in the muscle of trout after 14 days of exposure to bulk or TiO_2 NPs (Boyle *et al.*, 2013). Boyle *et al.* (2013) also found that *O. mykiss* exposed to 1 mg l^{-1} TiO_2 NPs spend proportionally less time at high swimming speed. Therefore, the muscle injuries could

affect on the muscle function that lead to alteration in the swimming speed and muscle biochemistry.

The information about the effects of nanoparticles on the reproductive system in fish is rare. One study showed that using 0 - 1.5 mg l^{-1} C_{60} (aggregation in suspension) for 96 h on zebrafish caused delay embryonic development (Zhu *et al.*, 2008a). In mice, the intratracheally administration of three sizes (14, 58 and 95 nm) of carbon black NPs (0.1 mg $mouse^{-1}$ 10 times every week) to ICR male mice caused partial vacuolation of the seminiferous tubules (Yoshida *et al.*, 2009). *In vitro* study, Komatsu *et al.* (2008) found that diesel exhausted particles (DEP), TiO_2 and carbon black (CB) were taken up by mouse Leydig cells, and caused changes in the viability, proliferation and gene expression.

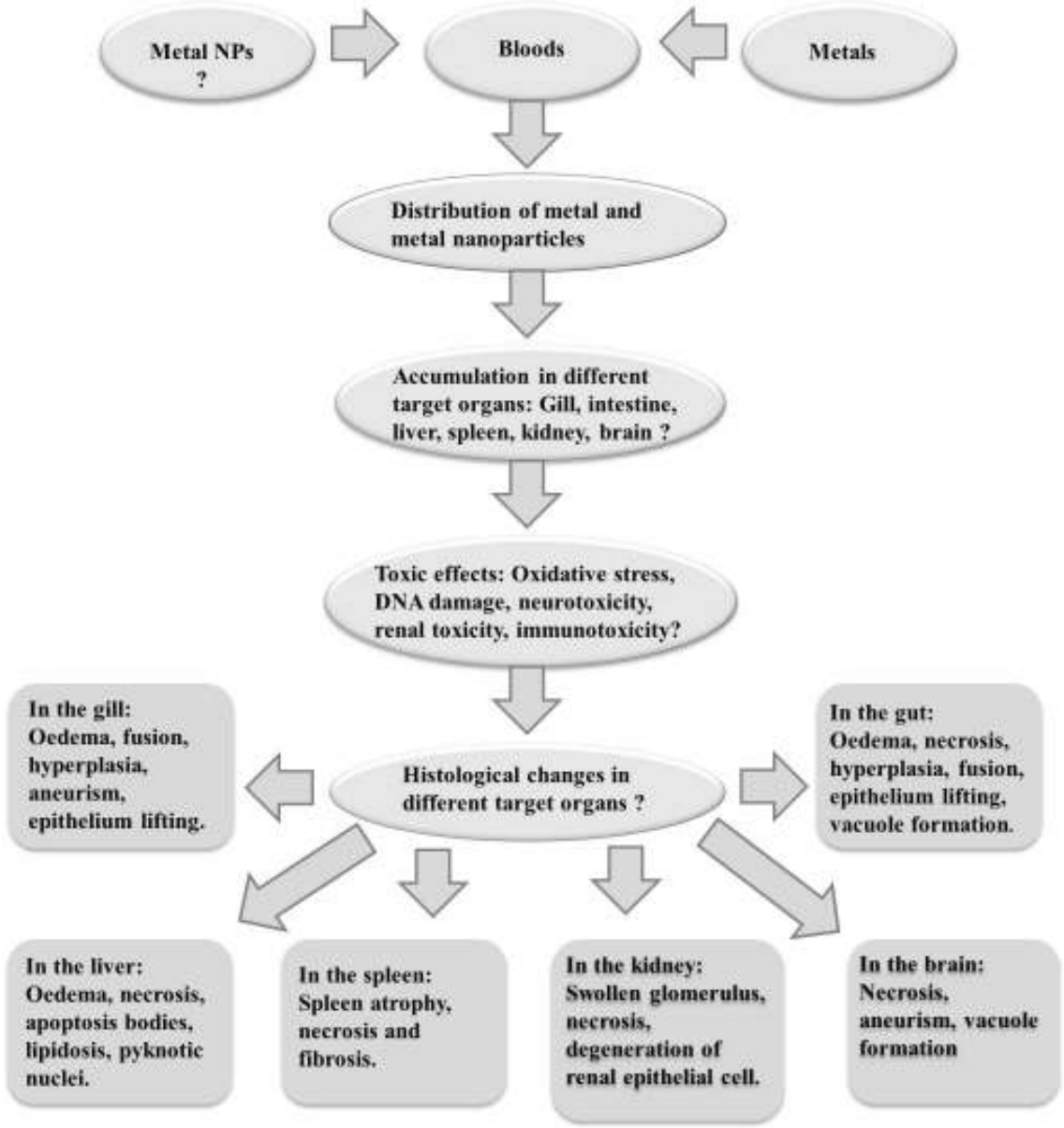

Figure 1.4 Possible differences between metals and metal nanoparticles. Metal ions can reach the blood and then distribute to the target organs while metal NPs when it reaches the blood may aggregate at the surface of blood cell or be distributed to different target organs. Metal ions and metal NPs may cause different toxic effect on the target organ and then cause different pathological effects on those organs.

1.4 Hypothesis

Increasing use of metal and metal nanoparticles could lead to release of these materials into the aquatic environment and cause risk to the aquatic organism. The main hypothesis is that metal nanoparticles behave differently to metal ions and may therefore have different target organs. It is also possible that the novel behaviour of NPs will lead to novel pathological effects in those organs. Therefore, this study will try to compare the effects of metal and metal nanoparticles on target organs in terms of the extent of toxic effects and histological changes that caused by these pollutants on target organs and identifying if the histopathological effects lead to loss of tissue/organ function.

Overall, this thesis aimed to investigate the following hypotheses:

(a) Waterborne exposure to $CuSO_4$ compared to Cu-NPs induces different pathological effects on different target organs of rainbow trout, *Oncorhynchus mykiss*, as well as immunological effects in the spleen (Chapter 3).

(b) Low water pH alters the physico-chemical properties of Cu-NPs in a different way to the effects of speciation chemistry on $CuSO_4$; leading to different biological effects of each form of the Cu during response to pH change in rainbow trout (Chapter 4).

(c) Waterborne exposure to bulk TiO_2 and TiO_2 NPs induce different pathological effects in different target organs of trout as well as immunological effects due to differences in particle size or crystallinity (Chapter 5).

(d) Intravenous injection of either bulk or TiO_2 NPs in trout leads to different pathological effects on different target organs due to particle size or crystallinity (Chapter 6). The effects of injection may be more severe than waterborne exposure as

protective epithelial barriers are by-passed with direct systemic administration of particles.

1.5 Aim and objectives

The overall aim of this study will be to test these hypotheses and to investigate the organ pathologies of a range of commercially available NPs. The study will use rainbow trout as a model organism. The current work on metals focuses on two metals in particular; Cu and Ti. Copper and titanium dioxide were chosen as the main study compounds, because both are widely used in industrial applications in their bulk or nanoparticulate forms, and both forms are already used in a wide range in consumer products and industry.

The specific experimental objectives of the study will be to:-

- Investigate the histopathological effects and major target organs for Cu-NPs and TiO_2 NP in fish compared to Cu and Ti following waterborne exposure.
- Investigate the histopathological effects and major target organs for TiO_2 NP in fish compared to bulk TiO_2 after injection exposure.
- Investigate the effects of varying water quality parameters such as pH on the bioavailability, uptake and target organs of dissolved copper as $CuSO_4$ compared to copper nanoparticles.
- More detailed investigation on the precise effects of metal oxide NPs in tissues and cells by using high resolution techniques such as electron microscopy.

- Identify whether the histopathological effects lead to loss of tissue or organ function. This can include traditional biochemical and physiological measurement on the tissue of interest as well as spleen prints to measure the alterations in the proportion of haematopoietic cells and the proportion of red and white pulp in the spleen.

Chapter 2

General methodology

General methodology

The general methodology aimed to prove protocols for waterborne exposure and intravenous injection of titanium dioxide (as bulk TiO_2 or TiO_2 NPs) and copper (as $CuSO_4$ or Cu-NPs, waterborne exposure only) in rainbow trout by using semi-static test regime. Tissues from these experiments were subsequently used to examine sublethal effects with a focus on histopathology and spleen prints. Therefore, the end points include histological examinations of organs (gill, gut, liver, spleen, kidney, brain and sometimes muscles) to assess the general injury in the target organs. The spleen print method was used to assess any immunological response by counting the proportion of haematopoietic cells (Peters and Schwarzer, 1985). All the chemicals and reagents used throughout this thesis, except where cited, were obtained from the Sigma Chemical Company, UK.

2.1 Fish husbandry and water quality

Before the start of each experiment, a fish husbandry plan was devised, and these plans set out the lower and upper limits of acceptable water quality parameters for maintaining fish health during experiments. These included pH, temperature, water hardness, and dissolved oxygen levels. The considerations of water quality included meeting the husbandry requirements of the fish species, and for experiments, balancing this with the need to maintain the NP exposure concentrations required to confirm exposures. Water quality parameters were observed throughout the experiment in order to keep it within the limits set out in the fish husbandry plan so to reduce any possible environmental effects upon the fish health.

Rainbow trout were used as study species (Fig. 2.1) as it is easy to keep under laboratory condition and their general physiology and behaviour are well studied. Also, it is sensitive to a variety of contaminant and environmental stressors and are used for determining the health of aquatic systems and therefore served as a good model organism for investigating the ecotoxicology of many nanoparticles. Briefly, stock rainbow trout (*Oncorhynchus mykiss*) were obtained from a commercial supplier (supplier and size as detailed in each chapter) and held in recirculating dechlorinated Plymouth tap water. The dechlorinated Plymouth tap water used to do each experiment was left vigorously aerating in a big container (about 800 l) for 24 h before use in order to remove chlorine. Samples of water were daily checked for pH, temperature and oxygen saturation as detailed in each chapter by using HACH HZ40d multi meter. Prior to feeding, samples of water were also collected from the waste outlet in the recirculation systems or mid water column from semi-static tanks for measuring total ammonia using LCK kit 304 read on a HACH LANGE GmbH DR 2800 spectrophotometer. The stock fish were fed twice daily with a commercial trout diet (EWOS, Westfield, UK) until at least 24 hours prior to start each experiment.

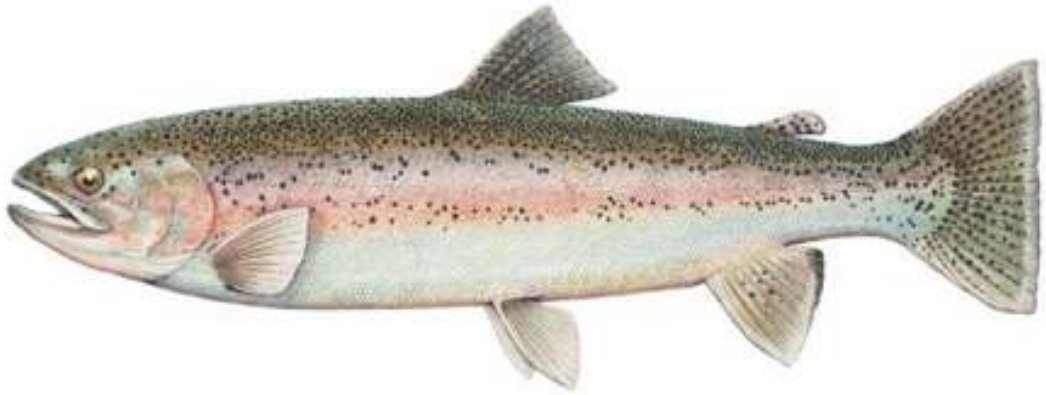

Figure 2.1 The rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792).

2.2 Incidental observations of the fish behaviour in the tanks

Some incidental observations on fish behaviour were made during experiments. These included ventilator behaviours such as laboured breathing, the loss of position holding, swimming fatigue and the appearance of increased mucus secretion into the water.

2.3 Routine anaesthesia and morphometric

Fish were anaesthetised to prevent causing pain or stress. For collecting blood from fishes, 25 mg l^{-1} of MS222 (Tricaine methane sulphonate, MS222/ 1-V1, PHARMAQ, buffered to neutral pH with 200 mg l^{-1} $NaHCO_3$) was added to a bucket containing about 2-3 litres of tank water. Then, fish were immersed into the buffered anaesthetic solution until the fish lost equilibrium and did not respond to the touch on the flank. After collecting blood samples and prior to dissection, fish were euthanized with an overdose of buffered MS222 (100 mg l^{-1}). Then, fish were weighed, total length recorded and any observation on fish health noted. Acid washed instruments were used throughout the animal dissection to minimise any-cross contamination between samples. Dissection was done carefully to conserve organ integrity for histological examination. All experimental work on animals was ethically approved and conducted with the 1986 Animals Scientific Procedures Act under the Home Office project licence 30/2313 and personal licence number 30/9099.

2.4 Tissue ion analysis

Following blood sampling, fish were terminally anaesthetised with MS222 and dissected for tissue metal analysis. Samples of tissue (*e.g.*, gill, intestine, liver, spleen,

kidney, and brain) were harvested and washed with Milli-Q water and processed for ion analysis according to Handy *et al.* (2000) with modification. All glassware was acid–washed (5 % Aristar HNO_3 for at least 2 h) to ensure that the minimal concentration occurs, and then triple rinsed in deionised water. Samples were placed on clean individual slides and dried to constant weight in an oven (Gallenkamp Oven BS Model OV-160) at 100 ^{o}C for 48 h and then tissue removed into plastic polypropylene (with polyethylene cap) scintillation vials (VWR International Ltd, Poole, UK). Samples (typically 0.1- 0.5 g dried tissue) were digested in 4 ml of concentrated nitric acid (HNO_3, 69 % analytical grade, Fisher Scientific, UK) at 70 ^{o}C for 3 h in a water bath and allowed to cool then diluted to 16 ml using Milli-Q water. For very small tissue samples (less than 0.1 g dry weight), the volumes of reagents were reduced to 1 ml of nitric acid and then diluted to a final volume of 4 ml Milli-Q water. Samples were vortexed while being drawn into the instrument and analysed in triplicate for trace elements (*e.g.*, Cu, Ca^{2+}, Na^{+}, K^{+}, Mn and Zn) by using inductively coupled plasma optical emission spectrometry (ICP-OES, Varian 725-ES). Calibration of the instrument was achieved using mixed, matrix-matched standards between 0-500 mg l^{-1}, prepared from Aristar ® plasma emission grade solutions, with accuracy checked after every 10-15 samples during the analysis by running a blank or standard as a sample. The calibration blank of the instrument contained 25 % nitric acid with no metals added. Certified fish tissues were used where possible, along with tissue spike recovery tests (Shaw *et al.*, 2012). Briefly, recovery of metals from tissue digests were determined from stock trout (non-experimental) and digested as above and spiked with element standards as either metal or metal nanoparticles. The recovery value ≥ 90 % of the nominal concentrations was considered acceptable. Water samples taken from the

exposure tanks were also analysed by ICP-OES for Ca^{2+}, K^{+}, Mg^{2+}, Na^{+} and metal as Cu or Ti. The values are described in the relevant chapters.

2.5 Histopathology

Histopathology is the study of lesions or abnormalities in tissue structure and is used here to detect for any harmful effects following exposure to metal and metal nanoparticles. Histological examinations were performed as described in Smith *et al.* (2007) with minor modifications. Fish were anaesthetised in buffered MS222 (as above) and organs were dissected in the following order: the second and third gill arch, the hind part of the intestine, whole liver, spleen, trunk kidney (posterior end of the body cavity), skeletal muscle (from the flank of the fish), and the whole brain. Then, tissues were immediately fixed in 10 % buffered formaldehyde solution [ratio of the tissue to the fixative solution was 1:100; 250 ml of 36.6 % formaldehyde, 10 g $NaH_2PO_4.1H_2O$, 16.5 g NaH_2PO_4 (anhydrous), diluted to 2.25 l with distilled water] for at least 48 h. The conserved tissues were then dehydrated through an ascending alcohols series to remove excess water (70 % for 24 h, 90 % industrial methylated spirit and IMS for 2 h each, 100 % absolute IMS for 2 h and left in another bath of 100 % absolute for 24 h). Then, three changes of xylene (100 %) were made for gill, gut, liver (1 h in each change), spleen and brain (two times for 30 min and one time for 15 min), to remove alcohol and also leaving tissue hydrophobic ready for paraffin infiltration. Sample of tissues was immersed in liquid paraffin for two steps of change. The period of each step was 2 h and then 90 min for gill, gut and liver, whereas, for the spleen and brain the period was 30 min and then 20 min, respectively. After that samples were transferred to the paraffin oven (58-60 °C) for 30 min to ensure the tissue was completely permeated with

paraffin. The paraffin blocks were made manually and then left to cool with the adjacent cold plate. The gills were decalcified for 30 min prior to processing for walks (Rapid Decalicifier, CellPathPLc, UK) and sectioning. The paraffin blocks for most tissues (gill, gut, liver, spleen, kidney and muscle) were cut as transverse sections at 7 µm thickness and then mounted on slides. The brain tissues were just cut as serial sagittal sections (7 µm thickness) to show general architecture. Major steps in histology processing that mentioned above were described in Fig. 2.2.

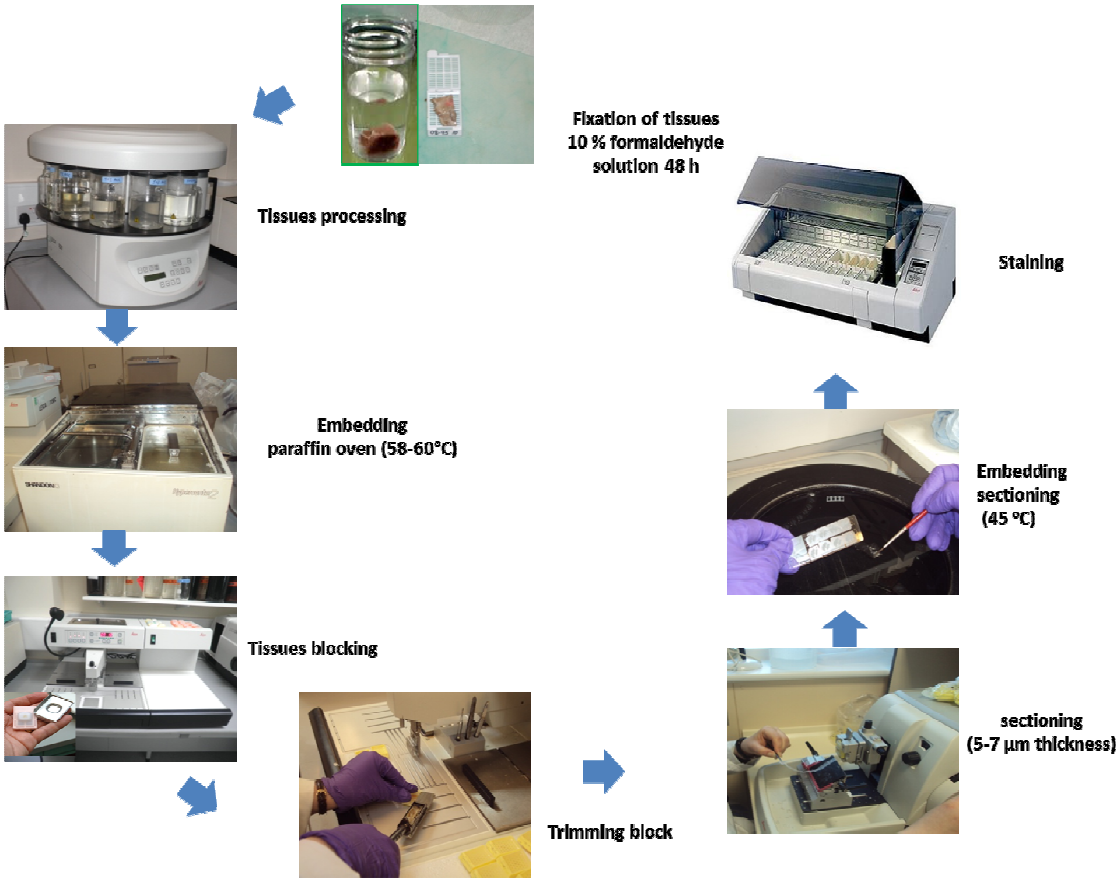

Figure 2.2 The main steps for the sectioning of organs tissue for histopathological studies using different instruments.

2.6 Slide staining

Gill slides were stained manually with Mallory's trichrome following a standard protocol with some modifications in staining times to obtain the best cellular details. Mallory's trichrome is often used to differentiate acidophilic extracellular fibers from acidophilic cytoplasm. The Mallory's stain is a combined of three different dyes: aniline blue, orange G and acid fuchsin (Chieffi *et al.*, 1992a; Chieffi *et al.*, 1992b). This multiple dye staining has the ability to differentiate a number of tissue structures in organs. Collagen fibres appear in a dark blue, collagen-containing connective tissue in blue, erythrocytes in orange, and chromatin, nuclei, basophilic cytoplasm, and muscle cell are shown in red colour. Briefly, slides were first cleared through 3 changes of xylene (100 %) to deparaffinise the sections (2 min each), then dehydrated in 2 changes of absolute alcohol (2 min each) and then one change each in a descending alcohol series (90 %, 70 %, 50 % and 30 %) for 2 min each. Gill sections were stained with haematoxylin (from BDH) for 40 min, followed by a wash in running tap water until the water was no longer coloured (~ 5 min). Slides were immersed in lithium carbonate (1 %, for blued), then in 1 % of acid alcohol (for differentiating) and again in $LiCO_3$ for a few second in each step. All slides were rinsed well in distilled water and put into 1 % of acid fuchsin for 8 sec (distinguished muscle in red colour from collagen in green or blue colour), then rinsed three times again with distilled water. After that, all slides were put into 1 % of the phosphomolybdic acid solution for 1:30 min, rinsed in distilled water and then stained with Mallory's (from BDH) for 25 sec. Again slides were washed with distilled water and dehydrated in 90 % alcohol and 100 % two times for 2 min in each step. Finally, slides were cleared in 3 changes of xylene (2 min each) and

anointed with DPX followed by covering with clean cover slips (making sure that no air bubbles were trapped underneath the cover).

Haematoxylin and eosin (H&E) staining was used to stain hind gut, liver, spleen, kidney, muscle and the brain sections following standard protocol by using the auto staining machine. H&E staining is one of the most commonly used techniques in histology and histopathology (Gamble, 2008). Haematoxylin itself is not a dye and must first be oxidised to haematein when it used for staining (Cook, 2001). The haematein has two less hydrogen atoms and the rearrangement of bonds introduces the quininoid ring structure and hence colour as can be described by the formula in Fig. 2 3 A. Generally, haematein is a weak acid dye, giving a yellowish colour to the tissues, but if it is combined with a suitable mordant haematein becomes probably the most widely used nuclear dye. The nucleic acid rich nucleus has an affinity for basic dyes. Therefore, the nucleus is an acid nature and is so described as a basophil, and when treated with a basic dye the acid is thought to combine with the coloured basic part of the dye to produce an insoluble salt. Therefore nuclear stains are basic in nature that is stained nucleus blue or purple (Gamble, 2008). The mechanism of eosin staining involved an attracted between an anionic dye (negatively charged) and protein groups that are positively charged (cations) such as amino groups. For example, the amino groups in the protein must become ionised by binding to a hydrogen ion and then this charged group will attract the eosinic ion as explained in Fig. 2.3 B. Eosin is also called acidic dye in histology because it is derived from coloured acid (eosinic acid) and not because of the pH of the solution (Cook, 2001). This dye stains the cytoplasm because the cytoplasm is more basic and has an affinity for acid stains. Therefore the eosin is

described as acidophil. Thus cytoplasmic stains are acid in nature that stains collagen, red blood cells, and the cytoplasm of many cells pink or orange (Gamble, 2008).

Briefly, all section slides was cleared, dehydrated, stained with haematoxylin, washed with tap water, blued in $LiCO_3$, differentiated in 1 % acid alcohol, blued again in $LiCO_3$, and rinsed with distilled water by using the same steps and time that was mentioned above with Mallory's staining. All slides after rinsed well with distilled water and were stained with acidic eosin (1 %) for 30 sec, then again washed with distilled water. After that slides were dehydrated in 90 % and 100 % alcohol two times for 2 min in each step. Finally, slides were cleared in 3 changes of xylene (2 min each) and mounted with DPX followed by covering with clean cover slips. All organ tissues from control and treated animals were processed together in batches for histology to eliminate artefacts between treatments. Slides were then examined by light microscopy using an Olympus Vanox-T microscope and photographed using an Olympus digital camera (C-2020 Z) at total magnifications of x 100, 200, and 400 to determine the normal and abnormal structure of organs (pathological effects). The normal structure of major organs is outlined below, and a description of the injuries is summarised in Table 2.1.

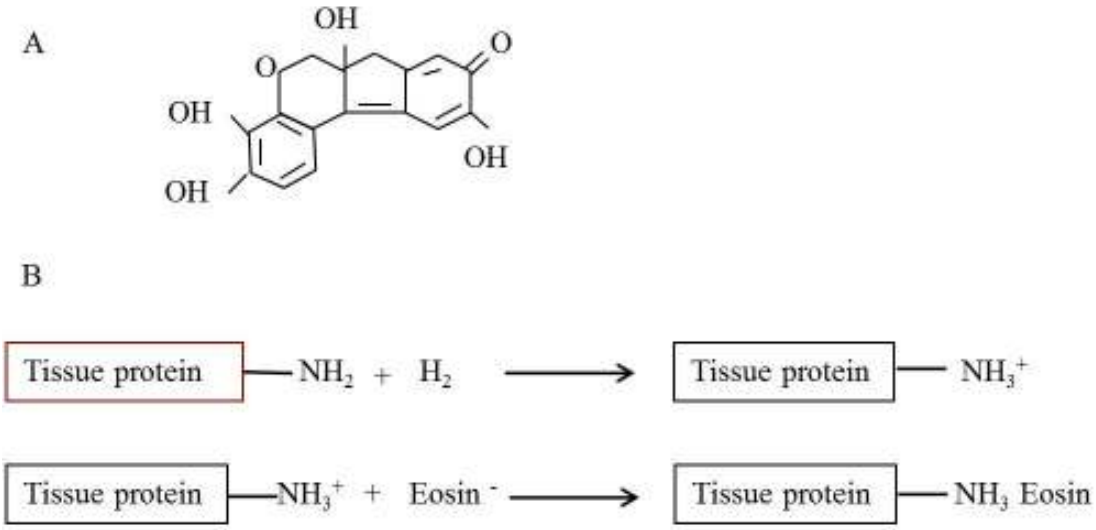

Figure 2.3 (A) The formula of haematein structure (B) The ionisation of tissue amine and subsequent binding of eosin. Re-drawn from Cook (2001).

2.7 Normal histological observations

2.7.1 *Gills*

Trout has four pairs of gill arches in each side of the body, and each gill arch has double rolls of primary lamellae which consist of a stratified squamous epithelium tissue. The secondary lamellae which are located anteriorly and posteriorly, perpendicular to primary lamellae are covered with a simple squamous epithelium. The secondary lamellae are supported and separated by the pillar cell. Typically, the gill epithelium is one of three cell layers and composed of squamous pavement cells and basal as well as intermediate non differentiated cells. Mucous cells are located on the surface of lamellae, seeming as granule- filled domes or vacuolated cells. The chloride cell is another type of cells seems to be more spherical than other epithelial cells, and located on the basement membrane of the secondary lamellae. The entire branchial

epithelium contains different types of cells such as lymphocytes, macrophasges, eosinophilic granular (Fig. 2.4 a) (Yasutake and Wales, 1983).

2.7.2 Intestine

The structure of the intestine composes of the mucosal lining epithelium, lamina propria (loose connective tissue), muscularis mucosa (thin layer of longitudinal smooth muscle), stratum compactum (dense collagen fibers), stratum granulosum (eosinophilic granular cells), muscularis layer (inner circular and outer longitudinal smooth muscle), and serosa (Bruno and Poppe, 1996). The surface of the mucosa has numerous villi that are lined by a single layer of columnar cells. Each of these cells typically have basally located nucleus containing a nucleolus, and brush border that covers the apical surface of the cell. Goblet cells lie between the columnar cells which are characterised by an enlarged upper region that contains a translucent cytoplasm and a basal region with associated nuclei (Fig. 2.4 c).

2.7.3 Liver

The liver in trout is not arranged in distinct lobes, as in mammals. Hepatocytes are polygonal cells containing a clear spherical nucleus usually with one nucleolus, which located among sinusoids. Normally these cells contain large quantities of lipid and glycogen. Lattice fibers and some connective tissue extend inward into the parenchyma which supports the hepatocytes (Fig. 2.4 e) (Bruno and Poppe, 1996). The liver of trout contains a few deposits of melanomacrophage centres which consist of aggregates of macrophages containing various pigments such as haemosiderin, lipofucin, and melanin (Ferguson, 2006).

2.7.4 Spleen

The spleen is dark red in colour and discrete organ with sharply defined edges situated near the greater curvature of the stomach. The trout spleen is covered by a thin capsule layer of connective tissue. The spleen is formed of two networks (connective tissue and capillary), and of the cells that fill up the spaces in this network. Red (rich in erythrocytes) and white (rich in lymphocytes) pulp are recognised in the spleen structure of trout (Bruno and Poppe, 1996). Melanomacrophage centre deposits present in the spleen as a small amount (Fig. 2.4 g). Measuring the proportion of red and white pulp as well as the number of melanomacrophage deposit in the spleen sections was used as an indicator to immunological responses. Additionally, the preparation of spleen prints are another way to detect the immunological effect as described in section 2.10.

2.7.5 Kidney

The kidney occupies a dorsal position along most of the length of the body cavity and lies ventral to the vertebral column. The anterior part of the kidney which is called the head kidney is composed of haematopoietic tissue; whereas the posterior part (trunk kidney) contains neurons that are embedded in the haematopoietic tissues. The main function of the trunk kidney is the maintenance of a stable internal environment with respect to water and salts; therefore we chose to examine this part of the kidney. The structure of the functional unit of the kidney, the glomerular nephron, consists of the following regions: a renal corpuscle which made up of Bowman's capsule and the glomerulus; a short neck segment; a proximal tubule; an intermediate segment; a distal segment; and a collecting duct system that terminating in the mesonephric duct. In trout the glomeruli are large and nearly fill the Bowman's capsule. Inside the glomerulus,

nucleated red blood cells can be recognised in the capillary lumen, as well as the nuclei of the mesangial cells, capillary endothelial cells and the podocytes of the visceral epithelium of the Bowman's capsule. The Bowman's capsule is composed of two layers; the parietal layer consists of thin squamous cells; and the visceral layer consists of podocytes. The cortex of the kidney contains numerous proximal tubules that are characterized by taller columnar cells which have rounded or oval, centrally or basally-located nuclei; an apical brush border and a small tubule lumen. Distal tubules are fewer in number and recognised by shorter columnar cells with oval, basally-located nuclei and no brush border, but with a larger tubule lumen (Fig. 2.4 i) (Yasutake and Wales, 1983).

2.7.6 Brain

The trout brain is composed of telencephalon, diencephalon, mesencephalon, metencephalon and mylencephalon regions. Three regions (telencephalon, mesencephalon, metencephalon) were examined throughout this thesis. The telencephalon is located anteriorly and represented by olfactory lobes and the cerebrum. The basic cell types in the telencephalon are neurons which conducts the nervous impulses and the neuroglial cells that have supporting role (Ferguson, 2006). The telencephalon neurons consist of a cell body containing large central nuclei with a prominent nucleolus, several cell processes called dendrites, and a single long process called an axon. The nuclei of a neuron stain lightly due to the chromatin dispersal, whereas, the nucleolus stains deeply due to the density of chromatin. Neuroglia nuclei have rounded or elongated shape, which stained deeply (Fig. 2.5 a).

The mesencephalon (also called the optic lobe) is the largest region that consists of six principal layers. The first layer called the stratum marginale lies beneath the pia and dura matter at the surface of optic lobe, which composed of unmyelinated fibers and lacks of the cell body. The second layer called the stratum opticum that contains numerous myelinated axons of medium and large caliber; the tissue appears to be distinctly fibrous with scattered neuroglial cell nuclei. The third layer called the stratum fibrosum et griseum superficiale, is consisted of myelinated axon dispersed in all directions, and is interlaced horizontally and vertically by dendrites. The fourth layer called the stratum griseum centrale is the broadest of the several layers that contained several kinds of neuron. The fifth layer called the stratum album centrale which is prominent for its heavy fibre bundle. The sixth layer called the stratum periventriculare which is another wide layer contained a band of closely packed neuronal cell nuclei with the nuclei of the ependymal cells that line the optic ventricle (Fig. 2.5 c) (Yasutake and Wales, 1983).

The cerebellum region is the major component of the metencephalon that lies along the midline, dorsal to the medulla oblongata. The cerebellum histology is consisting of an outer molecular layer, central Purkinje cell, and inner granule layer that overlie centrally located white matter. The molecular layers contains fibres directed horizontally with relatively few cells. The Purkinje cell is pyriform in shape and has ramified dendrites that extend into the molecular layer. The granular layer contains numerous small cells with deeply stained nuclei. Between the capsule layer that covers the surface of cerebellum and the molecular layer, there is a normal size of blood vessels (Fig. 2.5 e) (Ferguson, 2006).

2.7.7 Muscle

The skeletal muscle contains multiple nuclei that lie beneath to the membranous sarcolemma. Between muscle fibres, the endomysium connective tissue contains nuclei of fibroblast and a blood capillary (Fig. 2.5 g).

2.8 Abnormal histological observations in organs

The lesions observed in the gill, gut, liver, kidney, spleen, brain and muscle are summarised in Table 2.1.

Table 2.1 Types of lesion observed throughout this thesis.

Organs	Pathologic types observed in each organ	Pathologic description	References
Gills	Oedema	Increased interstitial space between the epithelial cells, often associated with water.	Roberts (1989)
	Hyperplasia of lamellar epithelium	An increase in the number of cells or the rate of cell division which appear as an area contains a more cells.	Ferguson (2006)
	Swollen mucocytes	Increase in the number and size of mucocytes on the gill epithelium.	Ferguson (2006)
	Fusion of the secondary lamellae	More than two secondary lamellae bind together and this leads to produce a solid fusion of many or all of the lamellar capillaries within a mass of hyperplastic epithelium.	Roberts (1989)
	Aneurism of	Recognised as an area of swollen or	Ferguson

Continue

Table 2.1

	lamellae	damaged vasculature contains a lot of blood cell.	(2006)
	Clubbed tips of the secondary lamellae	The thickness of lamellae tips is increased compared to the mean thickness of the individual filament.	Roberts (1989)
Gut	Erosion of villi	Recognise as a villi tip sloughing into the lumen.	Roberts (1989)
	Swelling goblet cell	The size of the cell is increased compared to the normal individual cell.	Al-Bairuty *et al.* (2013)
	Epithelial necrosis	An epithelial containing cells with sometimes condensed chromatin and showing disintegrated of the nucleus. In some cases the erosion of the contents of the cell is more complete.	Ziegler and Groscurth (2004)
	Epithelial lifting	Separation of epithelial cell from the basement membrane.	Athikesavan *et al.* (2006)
Liver	Hepatitis-like inflammation	Damage of the liver cells which appears as a cluster of dark spots or as hepatocellular necrosis preceding the infiltration of inflammatory cells.	Ferguson (2006)
	Vacuole formation	Appearance of small or large spherical vacuole in the cytoplasm of hepatic cells.	Roberts (1989)
	Melano-macrophage	An increase in the number and size of MMC deposits and these deposits appears	Roberts (1989)

Continue

Table 2.1

	centre deposits (MMC)	as light or dark brown deposits.	
	Necrosis in the hepatocytes	The cell morphology is changed to include for example, condensation of the nuclear chromatin associated with shrinkage of the nuclei. Sometimes the cells have ghost outlines and the cytoplasm stains well with eosin.	Roberts (1989)
	Pyknotic nuclei	Shrinkage of nuclei with condensed chromatin.	Fernandez *et al.* (2011)
Kidney	Renal tubule separation	Separation of the epithelial cells from the membrane.	Altinok and Capkin (2007)
	Bowman's space enlargement	The Bowman's space is increased with condensation of the Bowman's capsule (in some cases) when compared to the normal space.	Ferguson (2006)
	Degeneration of the renal tubules	The structure of renal tubule is damaged. For example, degeneration of epithelial cell, sometimes with loss of integrity of the epithelium in the renal tubules.	Roberts (1989)
Spleen	MMC deposits	The same description as above.	Altinok and Capkin (2007)
	Necrosis	The same description as above.	Altinok and Capkin (2007)

Continue

Table 2.1

Brain	Pyknotic nuclei	The same description as above.	Al-Bairuty *et al.* (2013)
	Vacuole formation	Appearances of spherical vacuole in the structure of the brain.	Handy (2003)
	Swollen nerve cells	The size of nerve cell is enlarged, may be associated with hydropic change in the tissue.	Handy (2003)
	Enlarged blood vessel	The diameter of the blood vessel is increased.	Smith *et al.* (2007)
	Necrotic nerve cell	The same description as above.	Handy (2003)
Muscle	Enlarged extracellular space	The space among the muscle fibres is increased.	Vogel (1959)

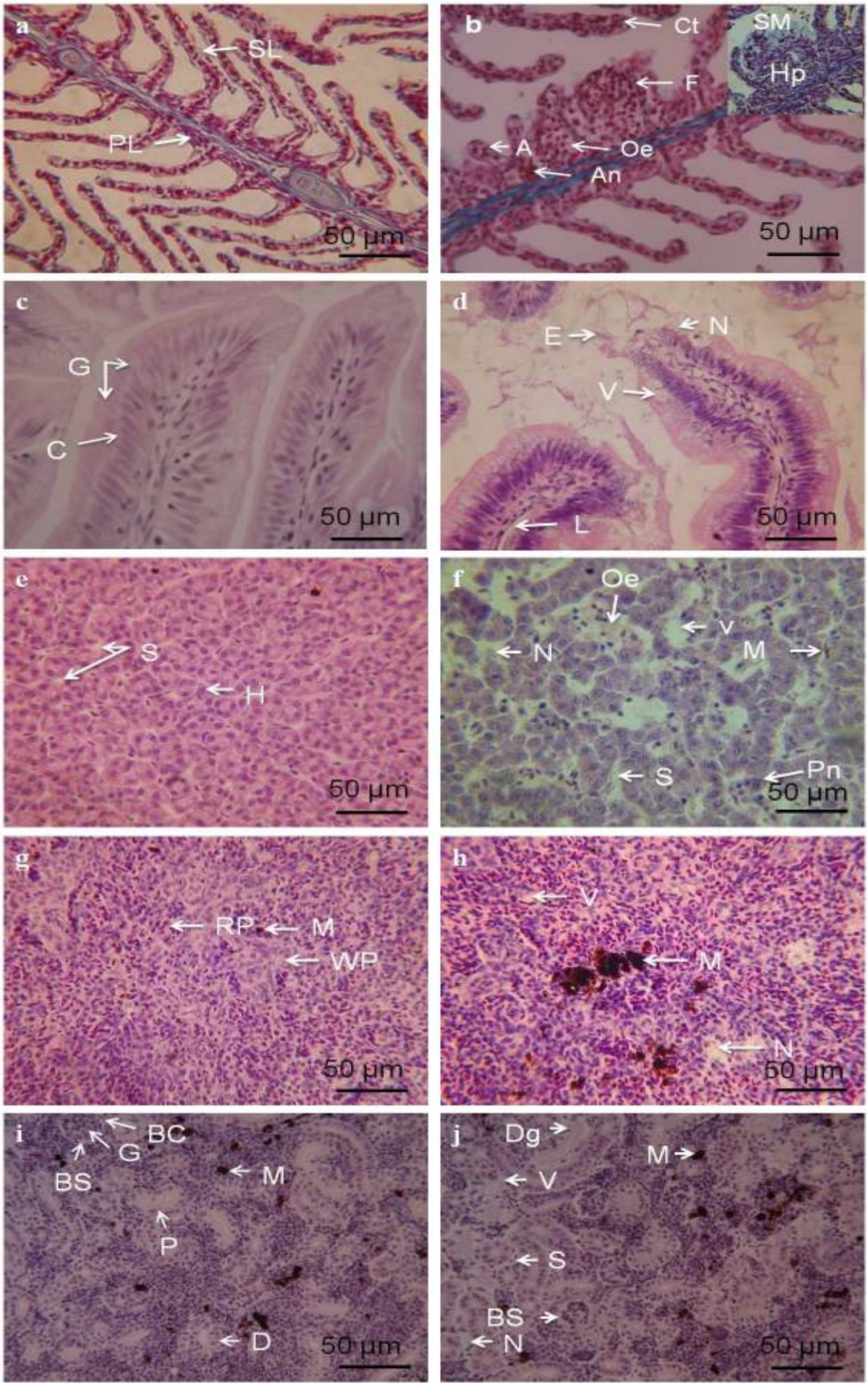
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Figure 2.4 Normal (from the stock control) and abnormal organ structure of gill, gut, liver, spleen, and kidney of trout following exposure to metal or metal nanoparticles observed throughout this thesis. For normal organs structure panels (a) gill, (c) gut, (e) liver, (g) spleen, (i) kidney. For abnormal structural panels (b) gill, (d) gut, (f) liver, (h) spleen, (j) kidney. The gill region shows secondary lamellae (SL). primary lamella (PL), clubbed tips (Ct), fusion (F), oedema (Oe), atrophy (A), aneurism (An) hyperplasia (Hp), and swollen mucocytes (SM). In gut region shows columnar cell (C), goblet cell (G), erosion (E), necrosis (N), vacuole formation (V), and lifting epithelium (L). In liver region shows hepatocytes (H), sinusoid space (S), necrosis (N), oedema (Oe), vacuole formation (v), melanomacrophage deposits (M) and pyknotic nuclei. In the spleen shows red pulp (RP), white pulp (WP), melanomacrophage deposits (M), vacuole formation (V), necrosis (N). Kidney shows Bowman's capsule (BC), glomerulus (G), Bowman's space (BS), melanomacrophage deposits (M), proximal tubule (P), distal tubule (D), degeneration of renal tubules (Dg), vacuole formation (V), necrosis (N).

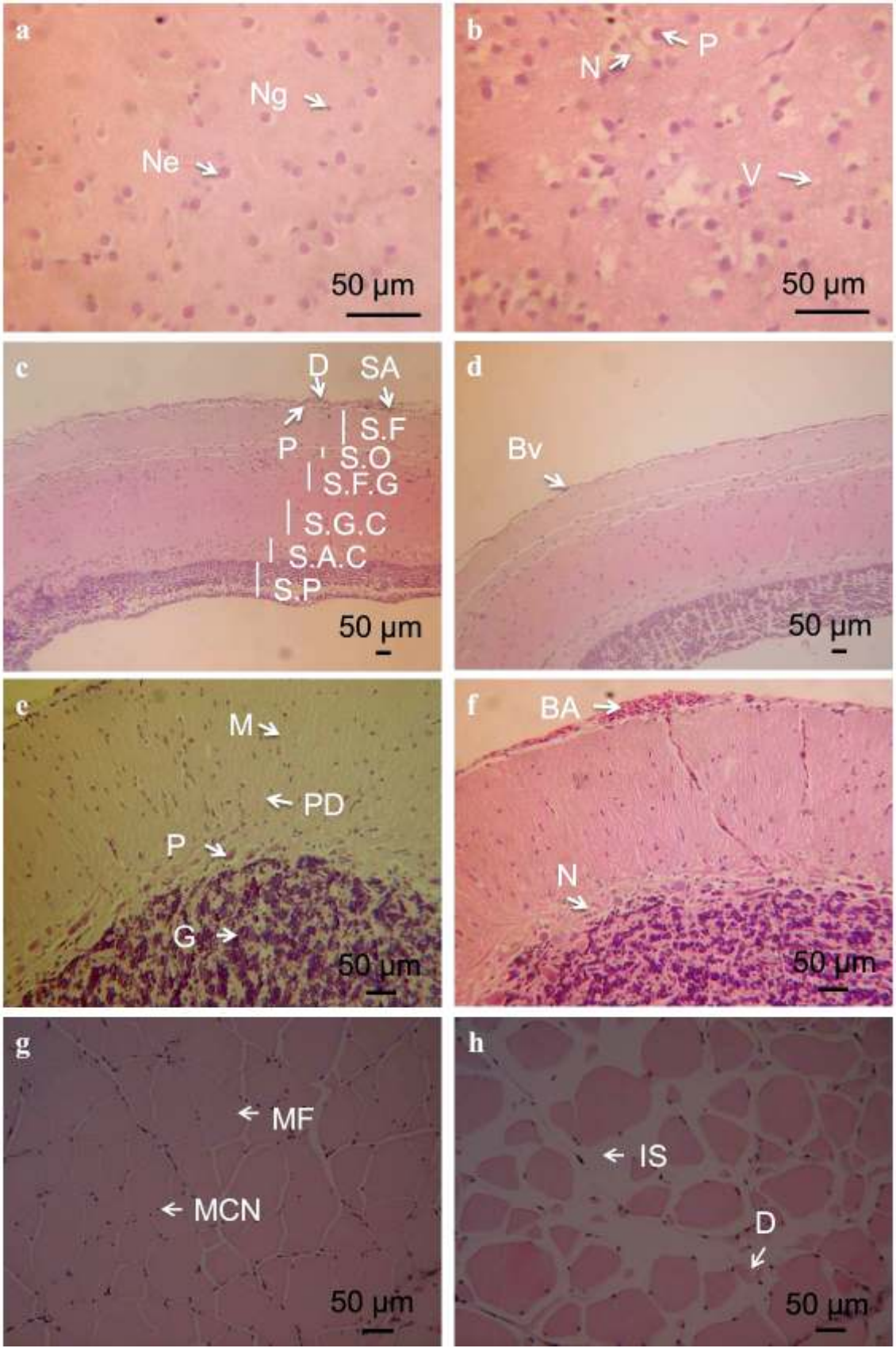
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Figure 2.5 Normal (from the stock control) and abnormal organ structure of brain and muscles of trout following exposure to metal or metal nanoparticles observed throughout this thesis. For normal organs structure panels (a) telencephalon, (c) mesencephalon, and (e) cerebellum as the main brain region, (g) skeletal muscle. For abnormal structural panels (b) telencephalon, (d) mesencephalon, (f) cerebellum, (h) skeletal muscle. Telencephalon shows nerve cell nuclei (Ne), neuroglia cell nuclei (Ng), pyknotic neucli (P), necrosis (N), vacuole formation (V). Mesencephalon shows dura mater (D), subarachnoid space (SA), pia mater (P), stratum fibrosum marginale (S.F), stratum opticum (S.O), stratum fibrosum et griseum superficiale (S.F.G), stratum griseum centrale (S.G.C), stratum album centrale (S.A.C), stratum periventriculare (S.P), blood vessels (Bv). Cerebellum regions show molecular layer (M), Purkinje layer (P) and granular layer (G), Purkinje dendrites (PD), enlarged blood vessel (BA), necrosis (N). Muscle tissue shows muscle cell nucleus (MCN), muscle fibres (MF), the increased extracellular space between muscle bundles (IS), degeneration of muscle bundle (D).

2.9 Stereological methods for quantitative histology

The pathological changes that were induced in organs were quantified by stereological methods. In gill, the incidence of lesions was performed exactly as described in Hoyle *et al.* (2007) with minor modification. Briefly, gill lesions were quantified by manually counting the number of lesions observed on each gill arch. Two primary filaments were randomly selected from the middle of the second gill arch, and at least 80-100 secondary lamellae were counted in each specimen. The total number of secondary lamellae with lesions, and the percentage incidence of each type of lesion (hyperplasia, fusion, aneurism, clubbed tips, atrophy and swollen mucocyte) were counted. For the gut epithelium, morphometrics included manual measurements of the length of the villi, the width of columnar cell and columnar cell nuclei. Similarly, dimensions of hepatocytes and hepatocytes nuclei in the liver, nerve cells in the brain, columnar epithelial cells in the kidney tubules (total width of renal tubule) and muscle fibres in the muscle were also measured. At least ten cells on each image per specimen

(3 image/specimen) were measured. In some organs the dimension of the tissues was also measured manually, in triplicate from three random images from each specimen, including the total length and thickness of each layer in the gut (starting from mucosal until the serosal layer), mesencephalon and cerebellum regions of the brain (that explained in the normal histology) and the total dimension of renal corpuscles (glomerulus and Bowman's capsule). Fractional areas of tissues in organs were counted manually from a randomly selected area on a section from each fish using the point counting method of Weibel *et al.* (1966) where the fractional volume (area) $Vi = Pi / P_T$; and Pi is the number of points counted, P_T is the total number of points on the counting grid. This method was used to calculate the proportion of red and white pulp and sinusoid space in the spleen, hepatic area and sinusoid space in the liver; the proportion of renal corpuscles, renal tubule and haematopoeitic tissue in the kidney; and the fractional fibre areas in skeletal muscle. Mean number of melanomacrophages in the spleen and liver were counted in 53,200 μm^2 of tissue (ie. per field of view) at x 400 magnification whereas, in the kidney was counted in 212,400 μm^2 of tissue (ie. per field of view) at x 200 magnification. Generally, all the numeric values observed in each organ have been summarised in the respective chapter.

2.10 Spleen prints

Spleen samples were collected from juvenile rainbow trout at the end of exposure from each experiment. Spleen prints were prepared as described in Peters and Schwarzer (1985). Briefly, spleen prints were prepared onto glass slides from one half of the spleen from each fish, whereas the other half of the spleen was used for histology. The slides left at room temperature for drying and then fixed in 100 % methanol for 10-

20 minutes. Immersed the slides in a buffer (tablets from BDH, 362242D) (pH = 6.8) for 5 minutes. Then the slides transferred to May-Grünwald stain (Sigma, 32856) for approximately 5 minutes and continue until the nuclei were stained deep blue, then the slides were rinsed in buffer solution. Slides were then immersed in freshly prepared buffered Giemsa solution (BDH, ratio 1:9; 1 from stain diluted to 9 from buffer) for roughly 15 minutes and continue until the nuclei were stained a rich purple then the slide rinsed in buffer. Finally, the slides allowed to air dry and then mounted with DPX.

2.10.1. Scoring spleen prints

Scoring of abnormal cells and differential cell counts in the spleen prints were performed under using an oil immersion lens (100 x objectives) with a total magnification of x 1000 on an Olympus Vanox-T microscope and an Olympus digital camera (C-2020 Z). Cells were identified by using the morphological criteria of Peters and Schwarzer (1985) (Table 2.2). All blood cells within randomly selected fields of view were counted on the slide. A total of at least 200 cells per slide (2 slides/fish) were counted (Peters and Schwarzer, 1985) and the percentage of each cell type was calculated from the number counted divided by the total number of cells (200 cells). The total number of erythrocytes with lesions, and the percentage of lesion that include: the presence of micronuclei, cells with a dividing nucleus (erythrocytes in the process of cell division), membrane abnormalities, and cell swelling (enlarged, rounded red cells) were counted. The types of haematopoietic cells that were observed in these experiments included haemocytoblast, erythroblast, progranulocyte, erythrocyte, lymphocyte, neutrophil, macrophage, monocyte and thrombocyte (Fig. 2.6). Basophilic and eosinophilic granulocyte as well as plasma cells were not counted because are not

usually clearly apparent in sufficient numbers to score accurately (Peters and Schwarzer 1985).

Table 2.2 Morphological description that used to identify and count the cells in the spleen of rainbow trout.

Cells types	Descriptions
Haemocytoblast	Large round cells in which the basophilic cytoplasm forms a narrow band around the large, spherical nucleus. Sometimes the nucleus is placed symmetrically and sometimes it has an eccentric position. The chromatin forms a loose irregular network and the nucleus fill about 80-90 % of the cell volume.
Erythroblast	Round or slightly ovoid cell with basophilic cytoplasm and round or slightly ovoid nucleus. The nucleus contains diffuse chromatin of varying density and fills 40-60 % of the cell volume.
Progranulocytes	Round to oval cell shaped with slightly basophilic cytoplasm and large bean shaped nucleus situated eccentrically. The nucleus occupies 50-70 % of the cell volume.
Erythrocytes	Oval cell with oval nucleus shapes. The chromatin structure varies according to the maturation of the cell.
Neutrophil	Round cell with curving 'bean' shaped, indented or segmented nucleus clearly delimited from the cytoplasm. The nucleus occupies 30-40 % of the cell volume. The homogeneous cytoplasm is neutrophilic or slightly basophilic that contains no visible granules.
Lymphocytes	Small round cell with the strongly basophilic cytoplasm. The large

Continued

Table 2.2

	round nucleus is centrally located and slightly indented and occupies approximately 85 % of the cell volume. Chromatin structure of the nucleus is dense and lumpy**.**
Macrophages	Large, polymorphic cell with polymorphic nuclei shape. Both the cytoplasm and the nucleus stain weakly and contain many vacuoles. In some of them phagocytised inclusions or pigments are evident.

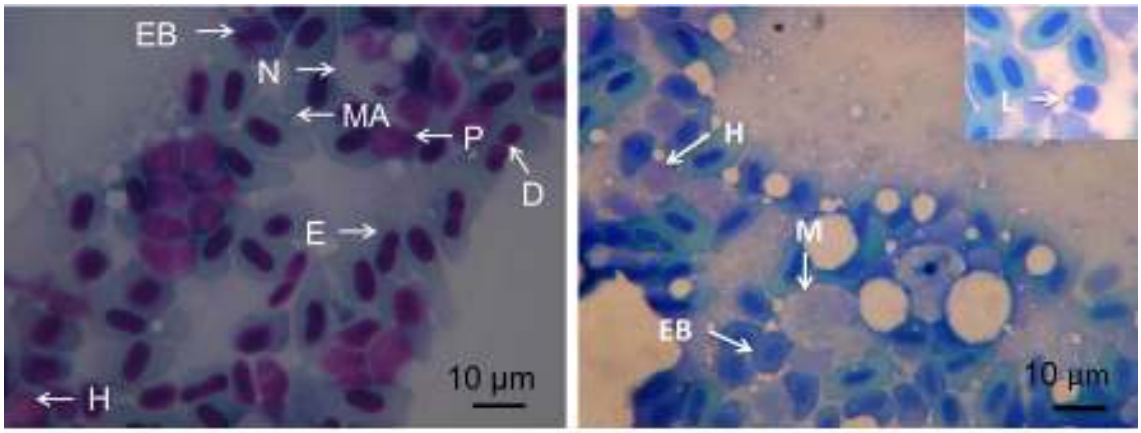

Figure 2.6 The morphology of haematopoietic cells in the spleen prints of trout after exposure to metal and metal nanoparticles that is observed throughout this thesis. Erythroblast (EB), erythrocyte (E), membrane abnormalities (MA), neutrophil (N), a dividing nucleus cell (D), macrophage (M), haemocytoblast (H), progranulocyte (P), and lymphocytes (L). Scale bar indicates magnification. Smear stained with May-Grunwald Giemsa stain.

2.11 Routine statistical analyses

The resulting data from the experiment were analysed by StatGraghics Plus version 5.1, using a rejection level of $P = 0.05$. Most figures were drawn using an Excel spreadsheet. Data were checked for inter-tank variability and where no tank effects

observed within triplicates (*i.e.*, no significant difference observed within the three tanks of the triplicate in the general water quality parameters that were measured in each treatment). The data were pooled by treatment for statistical analysis. The resulting data were analysed using two-way ANOVA looking for combined treatment and time effects. If this model showed statistically significant differences between time and treatment, then one-way ANOVA followed by the least squares difference multiple range test was applied to identify the additional resolution in some data and to locate specific treatment and time effects within ANOVAS. Bartlett`s test was used initially for variance checking. For non-parametric data, the Kruskal-Wallis test (analysis by ranks) was used for data that could not be transformed and differences located by notched box and whisker plots. The student's *t*-test (two tailed, unpaired) was also used to investigate the differences between pairs of data, or the Mann-Whitney W test where suitable for non- parametric data. The confidence limit for all statistical analysis used was 95 %.

Chapter 3

Histopathological and haematopoietic changes following exposure to copper or copper nanoparticles in rainbow trout (Oncorhynchus mykiss)

Hypotheses: Waterborne exposure to $CuSO_4$ compared to Cu-NPs induces different pathological effects on different target organs of rainbow trout, Oncorhynchus mykiss, as well as immunological effects in the spleen.

Abstract

It is unclear whether copper nanoparticles are more toxic than traditional forms of dissolved copper. This study aimed to describe the pathologies in gill, gut, liver, spleen, kidney, brain and muscle as well as the immunology response by using spleen prints from juvenile rainbow trout, *Oncorhynchus mykiss* (n = 210), that were exposed in triplicate (14 fish/tank) to either a control (no added Cu), 20 or 100 μg l^{-1} of either dissolved Cu (as $CuSO_4$) or Cu-NPs (mean primary particle size of 87 ± 27 nm) in a semi-static waterborne exposure regime. Fish were sampled at day 0, 4, and 10 for histology (6 fish/treatment, 3 slides/each organ of fish). All treatments caused organ injuries, and the kinds of pathologies observed with Cu-NPs were broadly of the same type as $CuSO_4$ including: hyperplasia, aneurisms, and necrosis in the secondary lamellae of the gills; swelling of goblet cells, necrosis in the mucosa layer and vacuole formation in the gut; hepatitis-like injury and cells with pyknotic nuclei in the liver; damage to the epithelium of some renal tubules, increased Bowman's space and renal tubular separation in the kidney; aggregation of melanomacrophage deposits and necrosis in the spleen. The spleen also showed a decrease in the proportion of red pulp and an increase in the proportion of white pulp with all Cu treatments compared to the control (all statistically significant, ANOVA, $P < 0.05$). Spleen prints (6 fish/treatment, 2 slides/fish) showed both Cu treatments ($CuSO_4$ or Cu-NPs) caused a significant (ANOVA, $P < 0.05$) increases in the proportion of erythrocytes abnormalities and

alteration in the proportion of some types of mature and immature erythrocytes, as well as immune cells compared to controls. In the brain, some mild changes were observed in the nerve cell bodies in the telencephalon, alteration in the thickness of the mesencephalon layers, and enlargement of blood vessels on the surface of the cerebellum. Changes in the proportional area of muscle fibres were observed in the skeletal muscle. Overall the data showed that pathology from $CuSO_4$ and Cu-NPs were of similar types, but there were some material-type effects in the severity or incidence of injuries with Cu-NPs causing more injury in the intestine, liver and brain than the equivalent concentration of $CuSO_4$ by the end of the experiment, but in the gill and muscle $CuSO_4$ caused more pathology.

3.1 Introduction

The exposure to Cu can stimulate the formation of ROS (superoxide anion and hydroxyl radicals) through redox cycling or Fenton reaction (Sevcikova *et al.*, 2011), and this could lead to producing oxidative damage to membrane lipids, proteins of enzymes, DNA and changes to antioxidant enzymes,. apoptosis as well as cell death. The pathological effects of waterborne copper, and target organs for Cu toxicity are reasonably well known in fish (Wilson and Taylor, 1993; Handy, 2003; Grosell *et al*., 2007; Mustafa *et al*., 2012). The gill is considered the main route for waterborne Cu uptake, and injuries include oedema, epithelial lifting and fusion of the lamellae during acute toxicity (Sola *et al.*, 1995), and changes in the proportions of epithelial cells and mucocytes are noted in chronic exposure (Dang *et al.*, 1999). For dietary Cu exposure, the gill does not show acute pathology, but instead, changes can occur in the intestinal mucosa including intestinal cell proliferation and apoptosis (Berntssen *et al.*, 1999). The

liver is the central compartment for handling Cu in teleost fish (Grosell *et al.*, 1996), and Cu is excreted in the bile. However, waterborne (Baker, 1969; Figueiredo-Fernandes *et al.*, 2007) or dietary (Handy *et al.*, 1999) exposure to Cu can produce liver pathology in fishes. There are concerns that Cu may be immunotoxic, although studies on the haematopoietic system of trout with Cu are limited, melanomacrophage aggregates are noted in the kidney of trout during Cu exposure (Handy, 2003). Studies on the haematopoietic system of Nile tilapia (*Oreochromis niloticus*) have shown spleen pathologies including congestion of the splenic vessels, depletion of lymphoid tissue, and hyperactivation of melanomacrophage centres following exposure to $CuSO_4$ (Osman *et al.*, 2009). The spleen is considered one of the main haematopoietic organs in fishes (Press and Evensen, 1999; Lange *et al.*, 2000) and has a role in removing foreign material and damaged blood cell from the circulation. The proportions of red and white pulp in the spleen are known to change following exposure to environmental pollutants (*e.g.*, in sticklebacks exposed to sewage treatment effluents, Handy *et al.*, 2002c).

Copper is also known to effect excitable tissue such as nerve and muscle. Additions of Cu alter the electrical properties of epithelia (*e.g.*, Na channels in frog skin, Flonta *et al.*, 1998), and while excess Cu in the CNS results in brain pathology in mammals (*e.g.*, Wilsons disease, review Menkes, 1999), and vacuole formation in the brain of trout has been observed from dietary Cu exposure (Handy, 2003). Fish do show altered behaviour and locomotor activity during Cu exposure (Handy *et al.*, 1999; Campbell *et al.*, 2002), but the relative contributions of CNS and skeletal muscle pathology to such events is less clear.

Relatively recently, a new form of Cu metal has been engineered comprising of Cu nanoparticles (Cu-NPs), which are one type of metal-containing nanomaterials

(review on " nano metals", Shaw and Handy, 2011). However, despite an emerging body of literature on the ecotoxicity of nanomaterials (Moore, 2006; Handy *et al.*, 2008b; Klaine *et al.*, 2008; Kahru and Savolainen, 2010; Handy *et al.*, 2011), less attention has been given to organ pathologies in aquatic species. Some evidence is emerging on Cu-NPs in fish and waterborne exposure to high concentrations (1.5 mg l^{-1} Cu-NPs for 48 h) caused oedema in the gills of zebrafish (Griffitt *et al.*, 2007). Other nanomaterials also cause gill pathology (TiO_2 NPs, Federici *et al.*, 2007; Single- walled carbon nanotube, Smith *et al.*, 2007; Nano-Fe, Li *et al.*, 2009). Data on internal organ pathologies from Cu-NPs in fish are generally lacking, but other metal NPs had been reported to cause pathologies. For example, liver tissue shows fatty change with lipidosis, and abnormal nuclei in the hepatocytes from trout following waterborne exposure to TiO_2 (Federici *et al.*, 2007), and apoptosis in liver tissue is reported in zebrafish with Ag-NPs (Choi *et al.*, 2010). There are fewer details about the spleen pathology and immune responses of fishes exposed to NPs. The spleen prints of trout used in the TiO_2 study of Federici *et al.* (2007) showed minor alteration in the blood cell morphology (*e.g*,. Swollen red blood cells, a dividing nucleus cell or cell membrane abnormalities) and the percentage of immune cells (*e.g*., small increases in monocytes, a mixed response in lymphocytes that depending on the TiO_2 concentration) following waterborne exposure to TiO_2 (review, Handy *et al*., 2011). The brains of rainbow trout exposed to 0.1 mg l^{-1} TiO_2 NPs for 14 days also exhibited some necrotic cells in the cerebrum (Federici *et al.*, 2007).

Overall, the relative hazard of injury to tissues from nano forms of Cu compared to traditional metal salts is unknown. Therefore, the main goal of the current study was to determine the effects of dissolved Cu as $CuSO_4$ and Cu-NPs on the organ integrity

and histology of the gill, gut, liver, kidney, spleen, brain and muscle as well as haematopoietic responses measured from spleen prints of rainbow trout following waterborne exposure to these materials. The second objective was to compare and contrast the effects of Cu metal with Cu-NPs, to identify any nano-specific pathologies.

3.2 Materials and methods

3.2.1 Experimental design

The experimental design and water quality are based on the method used by Federici *et al.* (2007) for TiO_2 NPs. Briefly, juvenile rainbow trout (mean ± S.E.M., n = 210; 29.4 ± 1.0 g) were exposed, in triplicate tanks (14 fish/tank), to 20 µg l^{-1} and 100 µg l^{-1} of Cu metal either as $CuSO_4.5H_2O$ or nano copper (Cu-NPs) for 0, 4, 10 days using a semi- static waterborne exposure regime (80% water change every 12 h with re-dosing after each change). Two concentrations of $CuSO_4$ and Cu-NPs were selected for the exposure. A high Cu concentration (100 µg l^{-1}) was selected as an approximation of the LC_{50} for waterborne Cu in salmonids (Shaw and Brown, 1974) so that the toxicity of Cu-NPs could be compared to a concentration of dissolved Cu that would be clearly toxic to trout, whilst the lower concentration of 20 µg l^{-1} was chosen in order to detect for any sublethal affects at a more environmentally relevant level. The exposure time of 4 days was selected to detect the LC_{50} values whereas 10 days was chosen to assess the physiological, biochemical as well as histopathological responses to the exposure. Water samples were taken before and after each water change for pH, temperature, saturated oxygen (HACH HQ40d multi reader), total ammonia (HACH LANGE GMBH LCK kit 304 read on a HACH LANGE GmbH DR 2800 spectrophotometer) and water hardness (Ca and Mg measured by inductively coupled plasma optical emission

spectroscopy (ICP-OES)). As there were no significant differences between any tanks in water quality, data were pooled and were (mean ± S.E.M., *n* = 240-528 samples); pH, 6.98 ± 0.004; temperature, 16.0 ± 0.01 °C; oxygen saturation, 90.9 ± 0.2 %; total ammonia, 0.85 ± 0.05 mg l^{-1} (equivalent to 0.049 ± 0.002 mmol l^{-1} of total ammonia, < 0.1 μmol l^{-1} as NH_3), total hardness (mg l^{-1} $CaCO_3$), 52.02 ± 1.2. Photoperiod was 12 h light: 12 h dark. The electrolyte composition of the dechlorinated Plymouth tap water used was 9.89, 1.56, and 18.05 mg l^{-1} Na^+, K^+ and Ca^{2+} respectively (0.43, 0.04, and 0.45 mmol l^{-1} respectively). Background Cu levels in the water were 6.04 ± 0.27 μg l^{-1} (0.095 ± 0.004 μmol l^{-1}). Fish were not fed 24 h prior to, or during the experiment, to avoid confounding the exposure with potential food particles in the water as well as to minimise the risk of the Cu-NPs absorbing to faecal material and to help maintain water quality. The entire experiment had ethical approval under the Animals (Scientific procedures) Act 1986 in the UK and fish were also subject to independent health checks. Fish were randomly sampled on day zero (initial stock fish), then days 4 and 10 for haematology, plasma analysis, tissue electrolytes, tissue biochemistry (presented in Shaw *et al.*, 2012) and histology as well as spleen prints (presented here).

3.2.2 Stock solutions and particle characterisation

Stock solutions of $CuSO_4$ and dispersions of nano Cu were prepared as well as characterised exactly as described in detail by Shaw *et al.* (2012) using the same stocks. Briefly, a powder form of nano Cu was obtained from Sigma Aldrich (manufacturer's information: 99.9% purity, mean particle size of 50 nm). A fresh 50 ml stock solution of 1.0 g l^{-1} Cu-NPs was made at 6 pm daily by dispersing the NPs in ultrapure water (Millipore, 18.2 MΩ-cm resistivity, ion free and unbuffered) without solvents and

stirring (magnetic stirrer IKA Werke RET basic C, at 300 rpm) for 4 h in a low-density polyethylene (LDPE) plastic container. This stock was then used to dose the fish at 10 pm following the evening water change and again at 10 am the following morning (the stock was stirred for a further hour prior to the morning dosing event). A 10 ml subsample was then taken from the stock for analysis of total Cu by ICP-OES, and NTA, the remaining stock discarded. The measured primary particle size in the stock solution was 87 ± 27 nm (mean ± S.D., *n* = 50, JEOL 1200EX II transmission electron microscope), and mean aggregate size by nanoparticle tracking analysis (NTA, Nanosight LM10) was 216 ± 122 nm (mean ± S.D., *n* = 6) with a mode of 48 nm. Particle size distribution measurements by NTA in the tank water during the experiments were not possible due to the low concentrations and interference from other natural colloids in the water (see Shaw *et al.*, 2012 for discussion). Inductively coupled plasma optical emission spectroscopy (Varian 725 ES) analysis revealed no metal impurities (data not shown).

A 1.0 g l^{-1} $CuSO_4$ stock solution was prepared by dissolving 3.929 g $CuSO_4.5H_2O$ (Sigma-Aldrich) in 1 l of ultrapure (Milli-Q) water with stirring for 30 mins (magnetic stirrer IKA Werke RET basic C, at 300 rpm). Nominal dosing concentrations in the experimental tanks were achieved by adding either 0.4 or 2.0 ml of the $CuSO_4$ stock for the 20 and 100 µg l^{-1} Cu (as $CuSO_4$) treatments respectively (with subsequent redosing following each 12 h water change).

3.2.3 Histopathology

Histological examinations were performed as described in chapter 2 (see 2.5, 2.6 and 2.9). Briefly, Fish was examined at the start (initial fish on day 0, *n* = 5) and at the

end of the exposure (day 10, n = 6 fish/treatment, 2 fish taken randomly from each of the triplicate tanks from each treatment, 3 slides/fish). However, for ethical and veterinary reasons during the experiment, some interim observations were also made at day 4 (n = 3 fish/treatment, one from each triplicate tank). Fish were terminally anaesthetised with buffered MS222 and tissue were collected and fixed into buffered formal saline for histology processing. Quantitative histological measurements were also made as described in Chapter 2 (see 2.9).

3.2.4 Spleen prints

Spleen prints were prepared as mentioned in chapter 2 (see 2.10). Briefly, spleen sample was collected from rainbow trout at day 4 and 10 of exposure to dissolved Cu as $CuSO_4$ and Cu-NPs treatments. Spleen print slides were fixed in methanol and stained with May-Grunwald-Giemsa. The cellular pathologies and the proportion of haematopoietic cells were counted as described in Chapter 2 (see 2.10.1).

3.2.5 Statistical analysis

All data were analysed by StatGraphics Plus version 5.1 as explained in Chapter 2 (see 2.11). Briefly, one way analysis of variance (ANOVA) was used to identify treatment effects at the end of the experiment (day 10), and where possible at day 4. The least squares difference (LSD) post hoc test was used to identify differences between treatment, or time-effects where appropriate. Bartlett's test was used to check the validity of each ANOVA. In addition, 2-way ANOVA was used to check for treatment × time effects in the data. The Student's t-test was sometimes used to investigate the differences between pairs of data, where appropriate. For non-parametric data, the

Kruskal–Wallis test was used for data that could not be transformed. Results are presented as mean or % mean ± S.E.M.

3.3 Results

Waterborne copper exposure was confirmed by ICP-OES analysis of water samples following the dosing of the experimental tanks. Copper concentrations were (mean ± SEM., n = 12 water samples per treatment) 3.01 ± 0.02, 22.3 ± 1.7, 102.3 ± 5.7, 19.7 ± 2.8, and 100.8 ± 6.9 μg l^{-1} (for the control, 20 and 100 μg l^{-1} Cu as $CuSO_4$ and Cu-NP treatments respectively), representing recoveries of 111.5, 102.3, 98.5, and 100.8 % of the nominal concentrations respectively. Waterborne exposure to both dissolved Cu and Cu-NPs caused some mortality in the experiment with the former being far more toxic. Full details are reported elsewhere (Shaw *et al.*, 2012). Briefly, exposure to 100 μg l^{-1} dissolved Cu caused 80 % mortality in 96 h and for ethical reasons this treatment was stopped at day 4. The other treatments continued for 10 days with cumulative total mortalities of 4.8, 16.7, 7.1, and 19.0 % for control, 20 μg l^{-1} Cu as $CuSO_4$ and 20 and 100 μg l^{-1} Cu as Cu-NPs respectively. Significant accumulation of Cu was observed on/in the gills in all Cu treatments, with both Cu-NP groups accumulating less Cu than the $CuSO_4$-exposed fish by the end of the experiment (Table 3.1). There was no statistical significant difference in branchial Cu accumulation between the high and low Cu-NP concentrations (Table 3.1). Elevated levels of Cu were observed in the intestine of fish exposed to the high concentration of Cu-NPs only. At the end of exposure to 20 μg l^{-1} $CuSO_4$, concentration of Cu increased in the liver, but not in any of the other internal organs compared to controls. Similarly, there were no

statistically significant increases of Cu in any of the internal organs from either Cu-NP treatment compared to controls at the end of the experiment (Table 3.1).

Table 3.1 Copper concentrations in the tissue of rainbow trout exposed to control (no added Cu), 20 μg l^{-1} Cu as $CuSO_4$, or 20 or 100 μg l^{-1} Cu as Cu-NPs at day 10 of the exposure.

Tissues	Treatments			
	Control	20 μg l^{-1} $CuSO_4$	20 μg l^{-1} Cu-NPs	100 μg l^{-1} Cu-NPs
Gill	0.03 ± 0.01 [a]	0.17 ± 0.02 [b]	0.091 ± 0.03 [c]	0.08 ± 0.01 [c]
Liver	1.79 ± 0.42 [a]	3.15 ± 0.63 [b]	1.52 ± 0.32 [a]	1.95 ± 0.31 [a]
Intestine	0.13 ± 0.04 [a]	0.05 ± 0.01 [a]	0.10 ± 0.03 [a]	0.69 ± 0.21 [b]
Spleen	0.07 ± 0.01 [a]	0.05 ± 0.01 [a]	0.13 ± 0.07 [a]	0.06 ± 0.01 [a]
Brain	0.13 ± 0.02 [a]	0.09 ± 0.01 [a]	0.10 ± 0.01 [a]	0.10 ± 0.01 [a]
Muscle	0.03 ± 0.01 [a]	0.14 ± 0.12 [a]	0.02 ± 0.01 [a]	0.03 ± 0.01 [a]

Data are mean ± S.E.M., μmol Cu g^{-1} dry weight tissue, n = 6 fish/treatment. Different letters denote a statistically significant difference between treatments at day 10, with identical letters indicating no significant difference (ANOVA or Kruskal–Wallis, $P < 0.05$). Note there were no 100 μg l^{-1} Cu (as $CuSO_4$) exposed fish present at day 10.

3.3.1 Gill histopathology

Gill morphology of trout was normal in all the unexposed control animals (Fig. 3.1). Exposure to waterborne copper sulphate caused gill injury. At day 4, trout from the 100 μg l^{-1} $CuSO_4$-treatment showed signs of acute Cu toxicity (laboured respiration,

disequilibrium), and, upon histological examination, all three samples of gill showed areas of hyperplasia at the base of the secondary lamellae, oedema of the gill epithelium, lamellar fusion, clubbed tips, atrophy of the secondary lamellae (shorten lamellae), the occasional aneurism in the secondary lamellae, swollen mucocytes and necrotic epithelial cells (Fig. 3.1). Exposure to the 100 μg l^{-1} Cu-NP treatment produced similar gill pathologies to those observed with $CuSO_4$, although the extent of the injuries appeared less severe at day 4 in the fish exposed to Cu-NPs (Fig. 3.1). No gills were examined from the 100 μg l^{-1} Cu as $CuSO_4$ treatment at day 10 because no fish survived. After 10 days of exposure to 20 μg l^{-1} of Cu as $CuSO_4$ or Cu-NPs and 100 μg l^{-1} of Cu-NPs, the lesions were of similar types, but generally worse than the same treatment at day 4 (Fig. 3.1). Typically, each lesion was observed in at least four fish out of six fish examined per treatment, with all Cu-exposed fish having more than one type of lesion.

A quantitative analysis of the gill injuries confirmed some material-type effects in incidence of some lesions. At day 4 of exposure to 100 μg l^{-1} of Cu as $CuSO_4$ or Cu-NPs, only the proportion of clubbed tips and atrophy showed a material-type effect (Table 3.2). Due to mortality in the highest $CuSO_4$ by day 10, only the lower concentrations could be compared for material-type effects on the gills at the end of the exposure. At day 10, the proportion of gill lamellae showing fusion and atrophy increased with 20 μg l^{-1} of either Cu as $CuSO_4$ or Cu as Cu-NPs compared to the control and were all statistically significant from each other (ANOVA, $P < 0.05$, Table 3.2). There was also a material-type effect in the incidence of atrophy of the secondary lamellae and swollen mucocytes after 10 days for the 20 μg l^{-1} of $CuSO_4$ and Cu-NP treatments (Student's *t*- test, $P < 0.05$, Table 3.2).

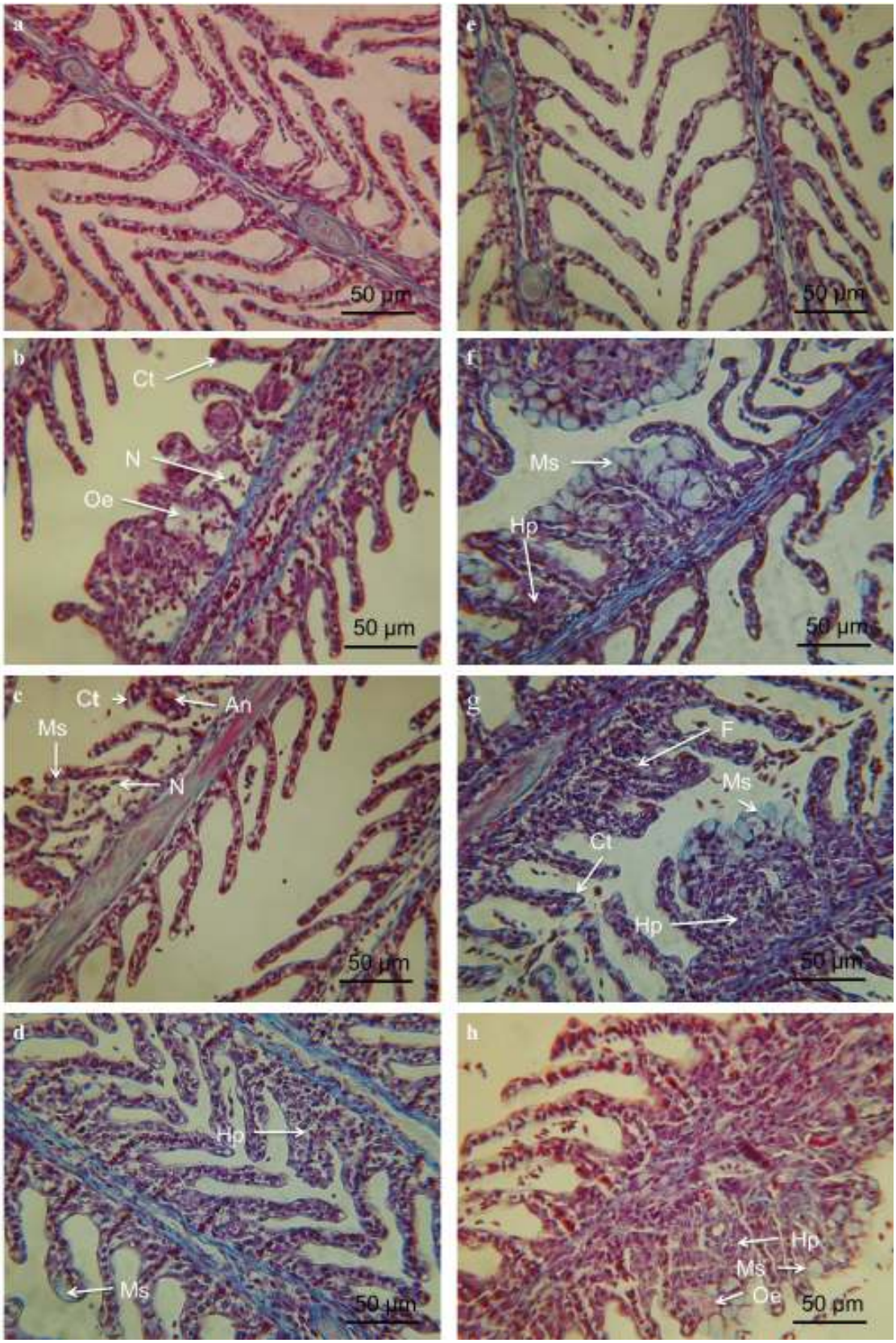
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Figure 3.1 Gill morphology in rainbow trout following waterborne exposure to $CuSO_4$ or Cu-NPs for 4 days (left column) and 10 days (right column). For day 4 the panels include (a) control, (b) 100 µg l^{-1} $CuSO_4$, (c and d) 100 µg l^{-1} Cu-NPs. For day 10 the panels include (e) control, (f) 20 µg l^{-1} $CuSO_4$ (g) 20 µg l^{-1} Cu-NPs and (h) 100 µg l^{-1} Cu-NPs. The gills of control fish showed normal histology, whilst all treatments showed injuries that include oedema (Oe), necrosis (N), clubbed tips (Ct), aneurism (An), mucocytes swollen (Ms), hyperplasia (Hp) and fusion (F). These injuries were greater with $CuSO_4$ than Cu-NPs at day 4 but the situation was reversed by day 10 with some types of lesion (see text for details). Scale bar indicates magnification; sections were 7 µm thick and stained with Mallory`s trichrome. Note the termination of the 100 µg l^{-1} $CuSO_4$ treatment after 4 days for ethical reasons.

Table 3.2 Quantitative analysis of gill lesions in rainbow trout following exposure to either 20 or 100 µg l^{-1} of dissolved Cu and Cu-NPs for 0, 4 and 10 days.

% of gill lesions	No. of fish	Time/ days	Treatments				
			Control	20 µg l^{-1} $CuSO_4$	20 µg l^{-1} Cu-NPs	100 µg l^{-1} $CuSO_4$	100 µg l^{-1} Cu-NPs
Hyperplasia	5	0	3.7 ± 0.4				
	3	4	3.3 ± 0.3			5.7± 0.8[a]	4.4 ± 0.9
	6	10	6.1 ± 0.3*[#]	6.8 ± 0.7*	8.2 ± 1.0*	Mortality	8.9 ± 0.7*[a#]
Fusion	5	0	Not observed				
	3	4	Not observed			1.8 ± 0.4*[a]	1.3 ± 0.1*[a]
	6	10	Not observed	7.1 ± 0.4*[a]	2.7 ± 0.4*[ab]	Mortality	Not observed [#+c]
Aneurism	5	0	Not observed				
	3	4	Not observed			1.4 ± 0.1*[a]	1.0 ± 0.5*
	6	10	Not observed	Not observed	1.6 ± 0.2*[ab]	Mortality	Not observed[+]
Clubbed tips	5	0	8.2 ± 0.6				
	3	4	11.6 ± 0.9*			21.4 ± 1.0*[a]	17.9 ± 0.1*[ac]
	6	10	16.3 ± 0.5*[#]	18.9 ± 1.2*	18.3 ± 0.7*	Mortality	30.7 ± 1.1*[a#+]

Continued

Table 3.2

Atrophy	5	0	Not observed				
	3	4	2.1 ± 1.3			4.6 ± 1.0 *[a]	1.63 ± 0.2 [c]
	6	10	1.9 ± 0.3	5.8 ± 1.7 *[a]	10.2 ± 1.5*[ab]	Mortality	10.5 ± 1.1*[a#]
Swollen mucocytes	5	0	Not observed				
	3	4	7.9 ± 0.7*			8.2 ± 0.8 *	8.2 ± 0.6*
	6	10	8.7 ± 0.7*[#]	10.2 ± 1.3*	14.2 ± 1.9*[ab]	Mortality	14.4 ± 1.3*[a#]

Data are % mean ± S.E.M., (*), significantly different from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). (a), significantly different from control within row at each time point (ANOVA, $P < 0.05$). (b), significantly different between low concentration of $CuSO_4$ and Cu-NPs at day 10 (nano-effect *t*-test, $P < 0.05$). (c), significant differences between high concentrations of $CuSO_4$ and Cu-NPs at day 4 (nano-effect *t*-test, $P < 0.05$). (+), significant differences from the previous Cu-NPs concentration within row at day10 (dose effect within time point, *t*-test, $P < 0.05$). (#), significant differences between day 4 and 10 (time effect within high Cu-NPs, *t*-test, $P < 0.05$). Note, there was no 100 µg l^{-1} $CuSO_4$ treatment at day 10 due to the termination of this treatment at day 4 following high mortality.

3.3.2 Gut histopathology

The gut of fish from the fresh water control showed normal histology (Fig. 3.2). Exposure to $CuSO_4$ caused mostly minor injuries to the gut mucosa. At day 4, trout from the 100 μg l^{-1} Cu as $CuSO_4$ or Cu-NPs showed the occasional areas of epithelial lifting associated with oedema, some swelling of goblet cells, occasional of hyperplasia in the epithelium, the occasional villi tip with some erosion of the surface and the tips of a few villi showing vacuole formation and/or evidence of few necrotic cells (in all three fish examined/treatment, Fig. 3.2). At day 10, fish exposed to 20 μg l^{-1} of Cu as $CuSO_4$ or Cu-NPs exhibited similar types of intestinal changes to those observed in four fish out of six fish examined from each treatment, with the injuries appearing slightly more severe in the equivalent Cu-NP treatment (Fig. 3.2). There was also a concentration-effect within the Cu-NP treatment at 10 days with the 100 μg l^{-1} of Cu as Cu-NPs causing more frequent erosion of the villi tips, numerous swollen goblet cells, more vacuole formation in the epithelium, and more necrotic cells (observed in all 6 fish examined) compared to the lower NP concentration.

A quantitative analysis of gut mucosa dimensions confirmed a small material-type effect for only the highest Cu concentrations at the end of the experiment. After 10 days of exposure, the width of intestinal villi was statistically significant increased for all Cu-treatments compared to controls but not between the 20 μg l^{-1} Cu as $CuSO_4$ or Cu-NPs treatments (ANOVA, $P < 0.05$). Values were (μm, mean ± S.E.M., $n = 6$); control, 118.7 ± 0.9; 20 μg l^{-1} Cu as $CuSO_4$, 149.4 ± 1.9; 20 μg l^{-1} Cu as Cu-NPs, 150.0 ± 0.9; 100 μg l^{-1} Cu as Cu-NPs, 153.9 ± 2.0 at day 10. The thickness of the intestinal mucosa was also measured. Only exposure to 100 μg l^{-1} Cu as Cu-NPs for 10 days

showed an increased in the thickness of mucosa layer (μm, mean ± S.E.M., n = 6; control, 66.7 ± 1.5; treated, 74.7 ± 0.9) compared to the control (ANOVA, $P < 0.05$).

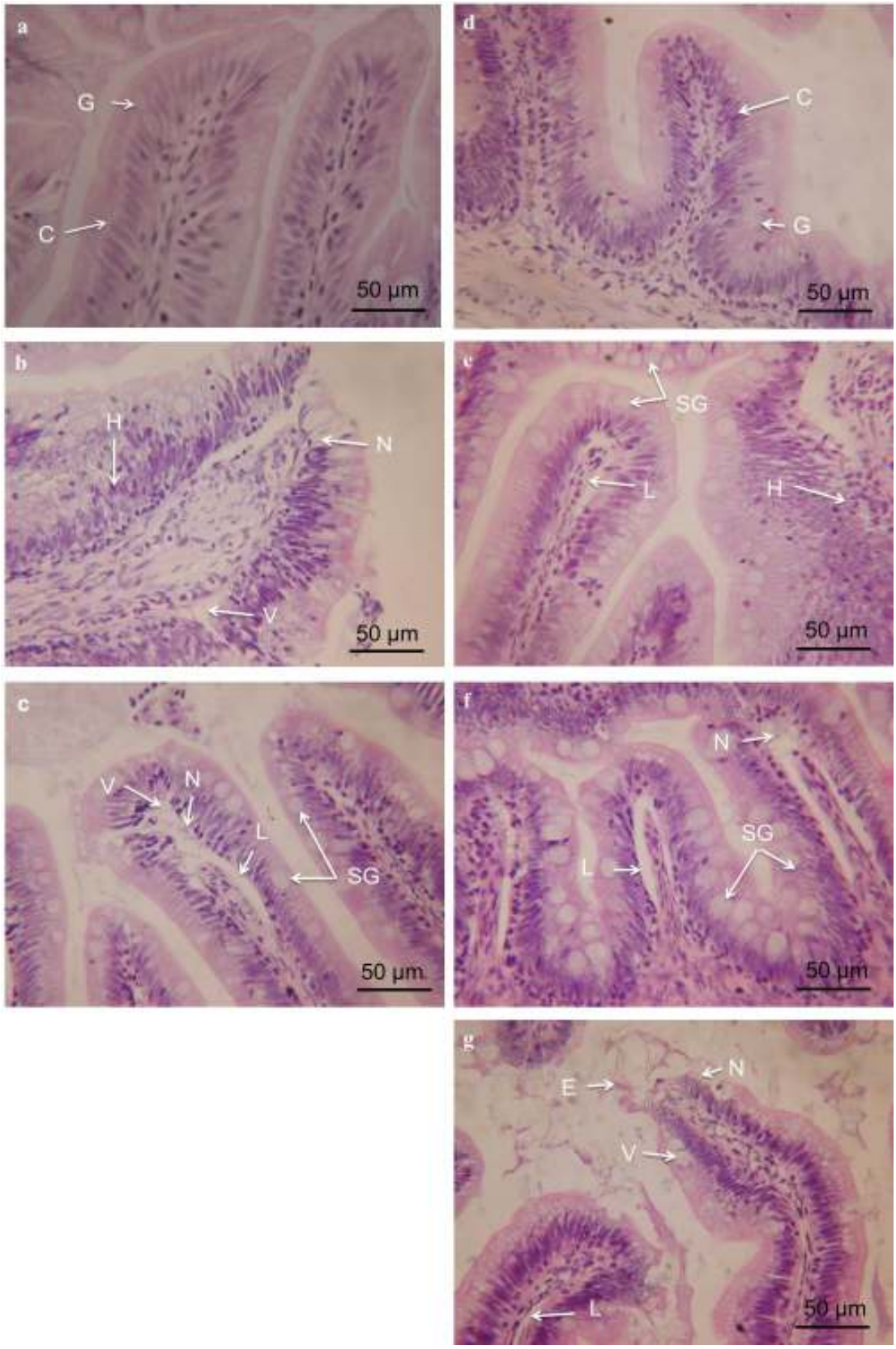
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Figure 3.2 Gut morphology in rainbow trout following waterborne exposure to $CuSO_4$ or Cu-NPs for 4 days (left column) and 10 days (right column). For day 4 the panels include (a) control, (b) 100 μg l^{-1} $CuSO_4$, (c) 100 μg l^{-1} Cu-NPs. For day 10 the panels include (d) control, (e) 20 μg l^{-1} $CuSO_4$ (f) 20 μg l^{-1} Cu-NPs and (g) 100 μg l^{-1} Cu-NPs. The gut of control fish showed normal histology with columnar cells (C) and goblet cells (G). At day 10, all Cu treatments showed similar type of injuries, but those from Cu-NPs were worse than $CuSO_4$. These injuries include the appearance of vacuoles in the lamina propria (V), hyperplasia (H), necrosis in the mucosal layer (N), swelling of goblet cells (SG), and erosion of villi (E). Scale bar indicates magnification; sections were 7 μm thick and stained with haematoxylin and eosin (H&E). Note the termination of the 100 μg l^{-1} $CuSO_4$ treatment after 4 days for ethical reasons.

3.3.3 Liver histopathology

The livers of control fish showed normal histology (Fig. 3.3). At day 4, fish exposed to 100 μg l^{-1} Cu as $CuSO_4$ or Cu-NPs showed similar types of lesion in all three fish examined per treatment, including the occasional cell with pyknotic nuclei or cytoplasmic vacuoles indicative of the early stages of necrosis in a few cells, small foci of hepatitis–like cell injury were observed as well as increase the number of melanomacrophage deposits in all three fish examined/treatment compared to controls (data not shown). After 10 days, the types of lesions in the Cu-exposed fish were similar to those observed in their respective treatments at day 4, although in addition the occasional separation of the endothelium from the walls of blood vessels was observed and changes in the sinusoid space (see measurements below). One or more of these lesions were observed in five fish out of six fish examined in each of the $CuSO_4$ or Cu-NP treatment (Fig. 3.3).

A quantitative analysis confirmed changes in liver sinusoid space (Table 3.3). At the end of the experiment (day 10), the exposure to 20 μg l^{-1} Cu as $CuSO_4$ caused a small but statistically significant decrease in the proportion of hepatic area and an

increase in the proportion of sinusoid space compared to the unexposed control (ANOVA, $P < 0.05$, Table 3.3). Exposure to 20 or 100 μg l^{-1} Cu as Cu-NPs had the opposite effect to $CuSO_4$ at day 10 with a small but statistically significant increase in the proportion of hepatic area and decrease in the proportion of sinusoid space compared to the unexposed control (ANOVA, $P < 0.05$, Table 3.3). The material-type effect was statistically significant for both the sinusoid space and hepatic area measurements. In the nano treatments the increase in hepatic area (decrease in sinusoid space) was accompanied by a fall in the ratio of the nucleus: hepatocyte diameter indicating cellular hypertrophy as the cause of the increased hepatic area in the livers with the concomitant decrease of sinusoid space (Table 3.3).

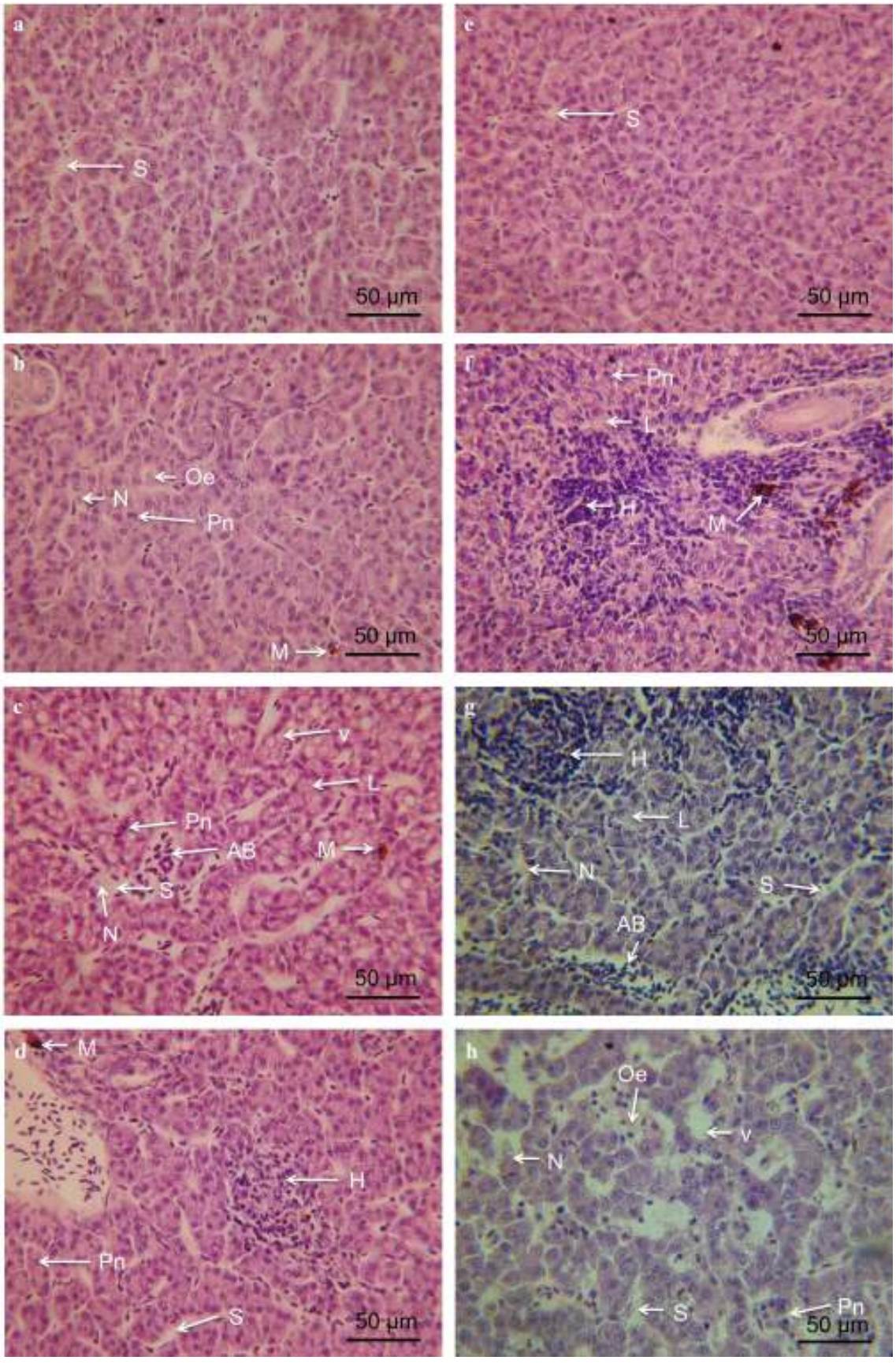
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Figure 3.3 Liver morphology in rainbow trout following waterborne exposure to $CuSO_4$ or Cu-NPs for 4 days (left column) and 10 days (right column). For day 4 the panels include (a) control, (b & c) 100 µg l^{-1} $CuSO_4$, (d) 100 µg l^{-1} Cu-NPs. For day 10 the panels include (e) control, (f) 20 µg l^{-1} $CuSO_4$ (g) 20 µg l^{-1} Cu-NPs and (h) 100 µg l^{-1} Cu-NPs. The livers of control fish showed normal histology with sinusoid space (S). Both materials caused similar types of injuries, although these were severe in the equivalent Cu-NP treatment by day 10. These injuries include cells with pyknotic nuclei (Pn), foci of hepatitis-like injury (H), foci of melanomacrophages (M), lipidosis (L), vacuole formation (V), necrosis (N), oedema in the tissue (Oe), and aggregation of blood cell (AB). Scale bar indicates magnification; sections were 7 µm thick and stained with haematoxylin and eosin. Note the termination of the 100 µg l^{-1} $CuSO_4$ treatment after 4 days for ethical reasons.

Table 3.3 The effect of dissolved copper and copper nanoparticles on the size of hepatocytes and hepatocytes nuclei as well as the proportion of hepatic cell area and sinusoids space in the liver of rainbow trout after 0, 4 and 10 days of exposure.

Parameters	No. of fish	Time/ days	Treatments				
			Control	20 µg l^{-1} $CuSO_4$	20 µg l^{-1} Cu-NPs	100 µg l^{-1} $CuSO_4$	100 µg l^{-1} Cu-NPs
Size of hepatocytes (µm)	5	0	15.6 ± 0.2				
	3	4				12.9 ± 0.4 *	14.8 ± 0.2 ^{c}
	6	10	18.3 ± 0.2 *	19.2 ± 0.3 *^{a}	17.3 ± 0.2 *^{ab}	Mortality	19.5 ± 0.3 *$^{a+\#}$
Size of hepatocyte nuclei (µm)	5	0	6.6 ± 0.1				
	3	4				4.9 ± 0.2*	5.1 ± 0.2 *
	6	10	6.7 ± 0.1	8.0 ± 0.1 *^{a}	5.8 ± 0.1 *^{ab}	Mortality	6.1 ± 0.1 *$^{a\#}$
Ratio of nucleus to hepatocytes diameter (µm)	5	0	0.43 ± 0.01				
	3	4				0.38 ± 0.01 *	0.37 ± 0.02 *^{c}
	6	10	0.36 ± 0.01 *	0.42 ± 0.01 *^{a}	0.34 ± 0.01 *^{ab}	Mortality	0.32 ± 0.01 *$^{a\#}$
% of hepatic cell area	5	0	80.2 ± 0.5				
	3	4				75.0 ± 0.8 *	85.1 ± 0.5
	6	10	86.4 ± 0.8	84.2 ± 0.5 *^{a}	90.7 ± 0.3 *^{a}	Mortality	90.5 ± 0.6 *^{a}

Continued

Table 3.3

% of sinusoids space	5	0	19.8 ± 0.5				
	3	4				25.0 ± 0.8 *	15.0 ± 0.5
	6	10	13.6 ± 0.8	15.8 ± 0.5 *[a]	9.3 ± 0.3 *[a]	Mortality	9.5 ± 0.6 *[a]

Data are mean or % mean ± S.E.M.. (*), significantly different from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). (a), significant difference from control within rows at day 10 (ANOVA, $P < 0.05$). (b), significant difference between 20 µg l^{-1} of dissolved Cu and Cu-NPs at day 10 (nano-effects, *t*-test, $P < 0.05$). (c), significant difference between 100 µg l^{-1} of dissolved Cu and Cu-NPs at day 4 (nano-effects, *t*-test, $P < 0.05$). (+), significantly different from previous Cu-NPs within row at day 10 (dose effect within time point, *t*-test, $P < 0.05$). (#), significant difference between day 4 and 10 within high Cu-NPs concentration (time effects, *t*-test, $P < 0.05$). Note, there was no 100 µg l^{-1} $CuSO_4$ treatment at day 10 due to the termination of this treatment at day 4 following high mortality.

3.3.4 Kidney histopathology

The kidneys of control fish showed normal histology (Fig. 3.4). The incidental histology for veterinary purposes in the 100 μg l^{-1} Cu as $CuSO_4$ or Cu-NPs treatment at day 4 showed occasional degeneration of renal tubules, a few necrotic cells in the haematopoietic tissue, minor elevation in the number of melanomacrophage deposits throughout the kidney, as well as some enlargement in Bowman's space and numerous cytoplasmic vacuoles in the remaining epithelial cells in all three fish examined per treatment (Fig. 3.4). After 10 days of exposure to 20 μg l^{-1} of Cu as $CuSO_4$ or Cu-NPs and 100 μg l^{-1} of Cu-NPs, fish showed the same pathologies as above in five fish out of six fish examined from each treatment (Fig. 3.4).

A quantitative analysis of the alteration observed in the kidney confirmed these observations (Table 3.4). For example, after 10 days there was a statistically significant increase (ANOVA, $P < 0.05$) in the diameter of Bowman's corpuscle in all treatments compared to the unexposed control (μm, mean ± S.E.M., $n = 6$; control, 73.8 ± 1.4; 20 μg l^{-1} Cu as $CuSO_4$, 81.3 ± 1.6; 20 μg l^{-1} Cu as Cu-NPs, 79.9 ± 1.7; 100 μg l^{-1} Cu as Cu-NPs, 86.1 ± 0.9), with a concentration-effect within the nano treatments, but no material-type effect compared to $CuSO_4$. Copper exposure, regardless of material-type or concentration doubled the size of the Bowman's space (μm, mean ± S.E.M., $n = 6$): control, 5.3 ± 0.7; 20 μg l^{-1} Cu as $CuSO_4$, 13.7 ± 1.3; 20 μg l^{-1} Cu as Cu-NPs, 13.3 ± 2.2; 100 μg l^{-1} Cu as Cu-NPs, 14.6 ± 0.9, ANOVA, $P < 0.05$). The number of melanomacrophage deposits in the kidney after 10 days showed a statistically significant increase in all treatments compared to controls, and also with a material-type effect (ANOVA, $P < 0.05$; mean ± S.E.M., $n = 6$; counts/image at x 200 magnification,

total area image 212,400 μm^2): control, 277 ± 4; 20 $\mu g\ l^{-1}$ of Cu as $CuSO_4$, 341± 3; 20 $\mu g\ l^{-1}$ of Cu as Cu-NPs, 366 ± 4; 100 $\mu g\ l^{-1}$ of Cu as Cu-NPs, 352 ± 2.

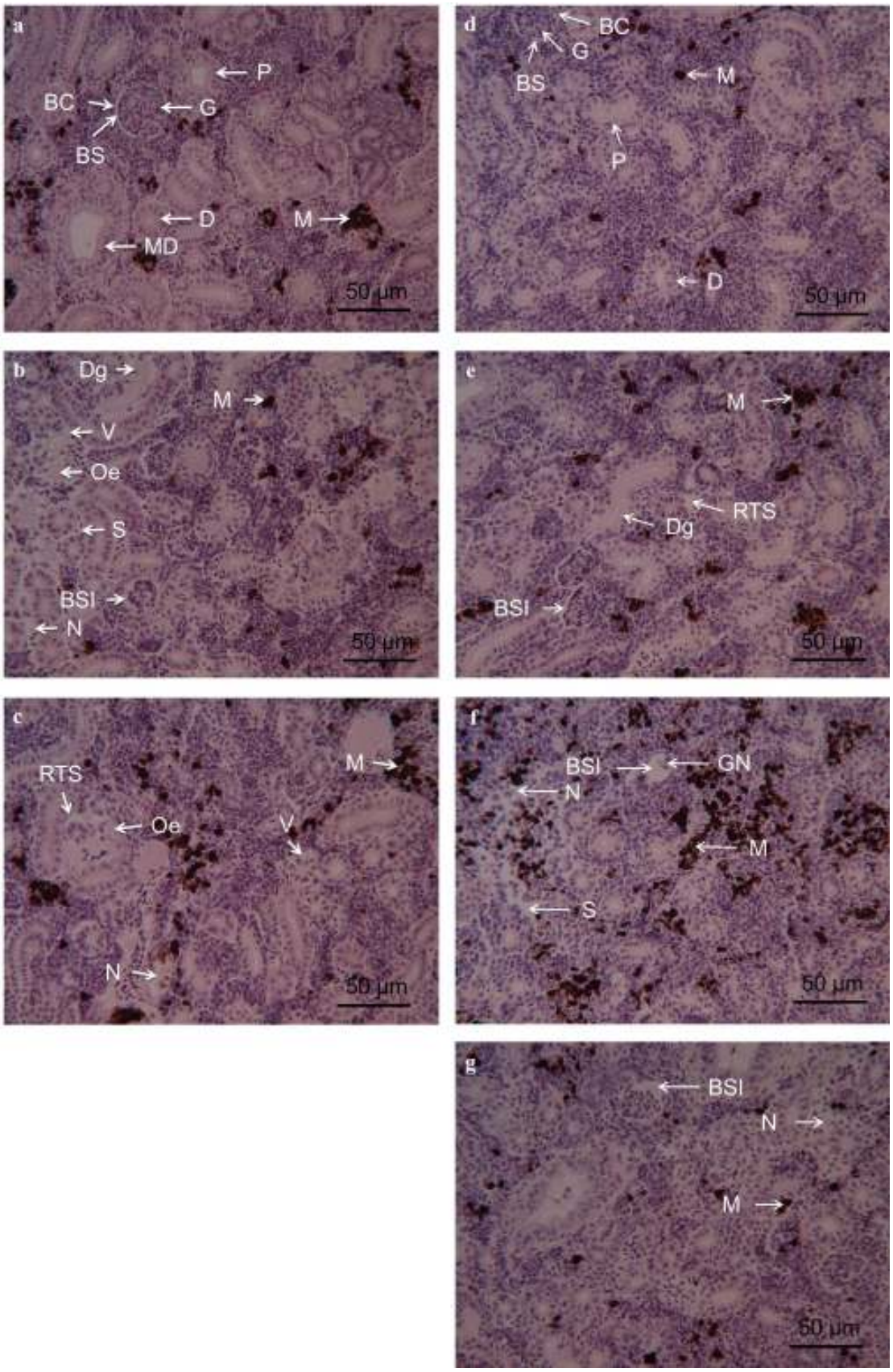
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Figure 3.4 Kidney morphology in rainbow trout following waterborne exposure to $CuSO_4$ or Cu-NPs for 4 days (left column) and 10 days (right column). For day 4 the panels include (a) control, (b) 100 μg l^{-1} $CuSO_4$, (c) 100 μg l^{-1} Cu-NPs. For day 10 the panels include (d) control, (e) 20 μg l^{-1} $CuSO_4$ (f) 20 μg l^{-1} Cu-NPs and (g) 100 μg l^{-1} Cu-NPs. Kidney of control fish showed normal histology with parietal epithelium of Bowman's capsule (BC), glomerulus (G), Bowman's space (BS), proximal tubules (P), distal tubules (D), mesonephric duct (MD) and melanomacrophages (M). The types of pathologies were similar for $CuSO_4$ and Cu-NPs, but with more melanomacrophage centres deposits in the latter. These injuries include degeneration of renal tubules (Dg), increased Bowman's space (BS), melanomacrophage aggregate (M), sinusoids were enlarged (S), renal tubular separation (RTS), oedema (Oe), cytoplasmic vacuolation (V) necrosis of hematopoietic tissue (N), and glomerular necrosis (GN). Scale bar indicates magnification, sections were 7 μm thick and stained with haematoxylin and eosin. Note the termination of the 100 μg l^{-1} $CuSO_4$ treatment after 4 days for ethical reasons.

Table 3.4 Alteration observed in the kidney content of rainbow trout after 0, 4, and 10 days of exposure to copper sulphate or copper nanoparticles.

Kidney parameters	No. of fish	Time/ days	Treatments				
			Control	20 µg l^{-1} $CuSO_4$	20 µg l^{-1} Cu-NPs	100 µg l^{-1} $CuSO_4$	100 µg l^{-1} Cu-NPs
% of renal corpuscle	5	0	4.0 ± 0.4				
	3	4				5.1 ± 0.4	3.2 ± 0.2 [c]
	6	10	4.1 ± 0.5	4.3 ± 0.4	4.0 ± 0.3	Mortality	4.7 ± 0.4 [‡]
% of proximal tubule	5	0	16.9 ± 2.7				
	3	4				23.6 ± 1.5 *	18.0 ± 1.8
	6	10	19.0 ± 2.0	22.1 ± 0.5	19.6 ± 1.6	Mortality	18.6 ± 1.6
% of distal tubule	5	0	11.5 ± 0.7				
	3	4				8.3 ± 0.4 *	9.9 ± 0.9
	6	10	7.2 ± 0.9 *	9.3 ± 0.8	4.3 ± 1.1 *[ab]	Mortality	5.4 ± 0.8 *[‡]
% of haematopoietic tissue	5	0	67.6 ± 2.6				
	3	4				63.0 ± 1.5	68.9 ± 1.1 [c]
	6	10	69.7 ± 2.9	64.3 ± 2.2	72.1 ± 1.9 *[b]	Mortality	71.3 ± 2.4
Number of melanomacrophage deposits	5	0	214 ± 1				
	3	4				322.3± 1.9 *	330.7 ± 4.6 *
	6	10	277 ± 4 *	341 ± 3 *[a]	366 ± 4 *[ab]	Mortality	352 ± 2 *[a‡]

Continued

Table 3.4

Diameter of Bowman's corpuscle (µm)	5 3 6	0 4 10	71.6 ± 1.9 73.8 ± 1.4	 81.3 ± 1.6 *[a]	 79.9 ± 1.7 *[a]	 81.3 ± 0.9 * Mortality	 69.8 ± 0.9 c 86.1 ± 0.9 *[a‡+]
Diameter of Glomerulus (µm)	5 3 6	0 4 10	66.3 ± 1.2 68.5 ± 1.6	 67.6 ± 2.0	 66.7 ± 1.9	 69.8 ± 0.9 Mortality	 62.7 ± 1.8 [c] 71.6 ± 1.4 *[‡+]
Diameter of Bowman's space (µm)	5 3 6	0 4 10	5.3 ± 0.8 5.3 ± 0.7	 13.7 ± 1.3 *[a]	 13.3 ± 2.2 *[a]	 11.5 ± 0.9 * Mortality	 7.1 ± 0.9 14.6 ± 0.9 *[a‡]
Total diameter of proximal tubule (µm)	5 3 6	0 4 10	45.2 ± 0.9 47.9 ± 0.7 *	 50.4 ± 1.0 *[a]	 51.0 ± 0.7 *[a]	 51.4 ± 1.0 * Mortality	 49.4 ± 0.2 * 51.0 ± 0.6 *[a]
Proximal lumen diameter (µm)	5 3 6	0 4 10	7.7 ± 0.4 9.7 ± 0.1 *	 12.4 ± 0.6 *[a]	 11.2 ± 0.4 *[ab]	 11.5 ± 0.8 * Mortality	 12.6 ± 0.2 * 12.0 ± 0.4 *[a]

Continued

Table 3.4

Proximal epithelial cell diameter (μm)	5	0	37.5 ± 0.8				
	3	4				39.9 ± 1.6	36.8 ± 0.3
	6	10	38.2 ± 0.7	38.0 ± 1.4	39.8 ± 0.6	Mortality	39.0 ± 0.8
Total diameter of distal tubule (μm)	5	0	54.3 ± 0.4				
	3	4				52.8 ± 0.7	53.8 ± 0.4
	6	10	53.0 ± 0.5	54.9 ± 0.7 [a]	53.8 ± 0.9	Mortality	58.3 ± 0.8 *[a‡+]
Proximal lumen diameter (μm)	5	0	16.7 ± 0.4				
	3	4				18.3 ± 0.1 *	17.3 ± 0.7
	6	10	17.4 ± 0.1	17.4 ± 0.3	17.1 ± 0.3	Mortality	17.4 ± 0.2
Proximal epithelial cell diameter (μm)	5	0	37.6 ± 0.4				
	3	4				34.5 ± 0.8 *	36.5 ± 0.5
	6	10	35.6 ± 0.4 *	37.5 ± 0.5 [a]	36.7 ± 0.9	Mortality	40.9 ± 0.7 *[a‡+]

Data are means or % means ± S.E.M. (*), significant difference from initial fish (stock fish at time zero, ANOVA, P <0.05). (a), significant difference from control within row at day 10 (ANOVA, P <0.05). (b), significant difference between 20 μg l^{-1} of $CuSO_4$ and Cu-NPs at day 10 (nano-effects, t –tests or ANOVA, P <0.05). (c), significant difference between 100 μg l^{-1} of $CuSO_4$ and Cu-NPs at day 4 (nano-effects, t –tests, P <0.05). (+), significant difference between 20 and 100 μg l^{-1} of Cu-NPs (dose effect within time point, t –tests, P <0.05). (‡), significantly different between day 4 and 10 for high concentration of Cu-NPs (time effect, t –tests, P <0.05). Note, there was no 100 μg l^{-1} $CuSO_4$ treatment at day 10 due to the termination of this treatment at day 4 following high mortality.

3.3.5 Spleen histopathology

3.3.5.1 Spleen morphology

Spleen morphology of trout was normal in the unexposed control animals (Fig. 3.5). Exposure to waterborne Cu as $CuSO_4$ or Cu-NPs for 4 days showed similar changes in the spleen that included the occasional necrotic cell, vacuole formation and depletion of lymphoid tissue (appears as space containing fewer cells) as well as foci of melanomacrophage deposits in all three fish examined compared to the control (Fig. 3.5). No spleens were examined from the 100 μg l^{-1} $CuSO_4$-treatment at day 10 due to mortality. After 10 days of exposure to 20 μg l^{-1} of Cu as $CuSO_4$ or Cu-NPs and 100 μg l^{-1} of Cu-NPs, the spleen showed similar types of changes as observed above in five fish out of six fish examined per treatment (Fig. 3.5).

A quantitative analysis confirmed changes in the proportion of red and white pulp as well as sinusoid space at day 4 with 100 μg l^{-1} of Cu as $CuSO_4$ or Cu-NPs treatments (Fig. 3.6). Ten days of exposure to 20 μg l^{-1} of $CuSO_4$ and 20 or 100 μg l^{-1} of Cu-NPs caused a decrease in the proportion of red pulp (2 fold) and an increase in the proportion of white pulp (1 fold) compared to control groups (all statistically significant; ANOVA, $P < 0.05$, Fig. 3.6). The alteration observed in the proportion of white pulp is considered as an indicator to immunological responses. The proportion of sinusoid space showed a statistically significant decrease with 100 μg l^{-1} of Cu-NP treatment only compared to the control, with a concentration effect (more changes with 100 μg l^{-1} than 20 μg l^{-1} of Cu-NPs, ANOVA, $P < 0.05$; Fig. 3.6). A material-type effect was observed in the proportion of red pulp by causing a greater decrease with the $CuSO_4$ than Cu-NPs treatment (Fig. 3.6).

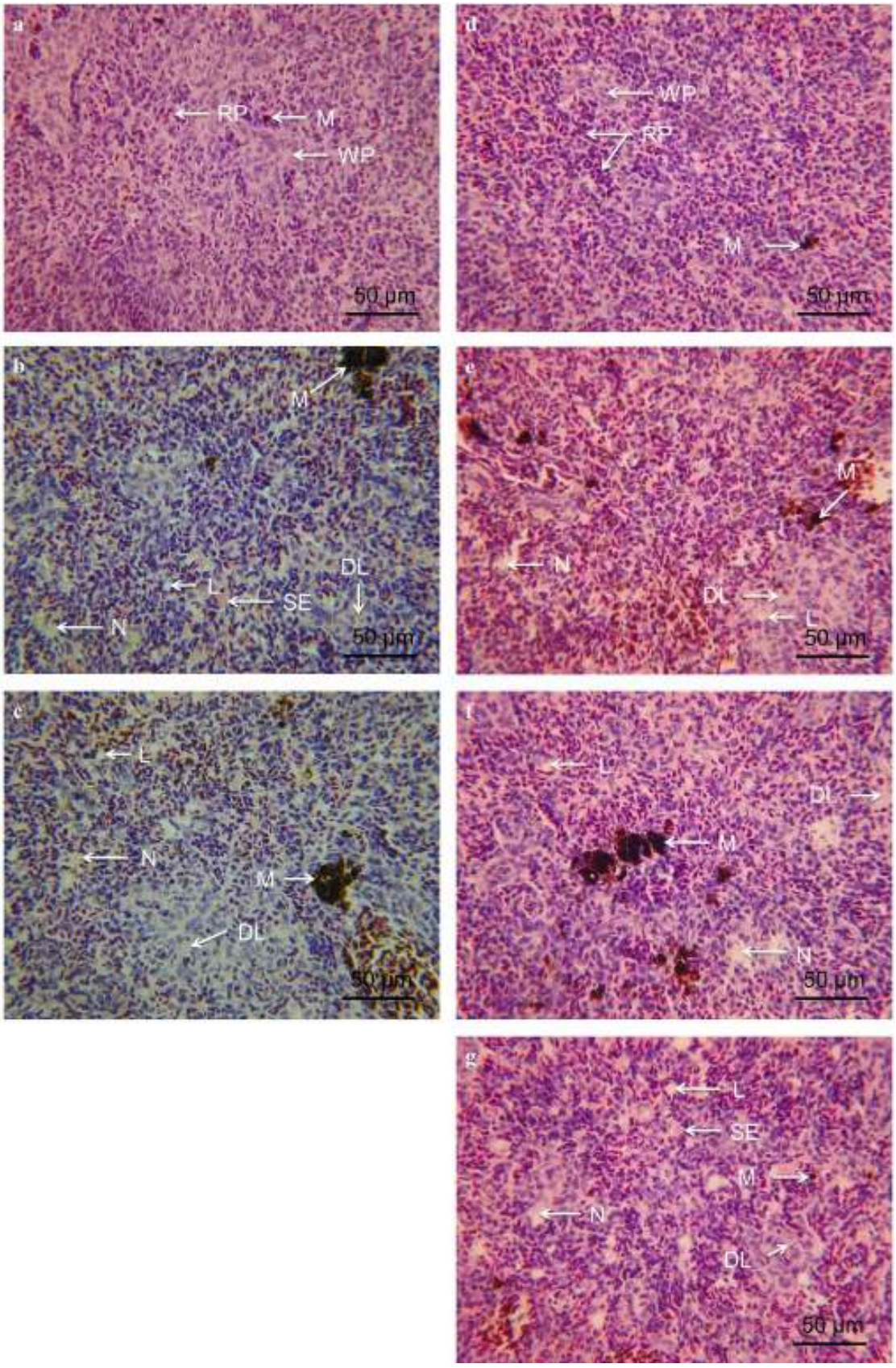
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Figure 3.5 Spleen morphology in rainbow trout following exposure to $CuSO_4$ or Cu-NPs for 4 days (left column) and 10 days (right column). For day 4 the panels include (a) control, (b) 100 µg l^{-1} $CuSO_4$, (c) 100 µg l^{-1} Cu-NPs. For day 10 the panels include (d) control, (e) 20 µg l^{-1} $CuSO_4$ (f) 20 µg l^{-1} Cu-NPs and (g) 100 µg l^{-1} Cu-NPs. Spleen from control fish showed normal histology, with defined red (RP) and white (WP) pulp. All treatments showed similar types of injuries. These injuries include necrosis (N), depletion of lymphoid tissues (DL), melanomacrophage deposits (M), vacuole formation (V), and swollen erythrocyte (SE). Scale bar indicates magnification; sections were 7 µm thick and stained with H&E. Note the termination of the 100 µg l^{-1} $CuSO_4$ treatment after 4 days for ethical reasons.

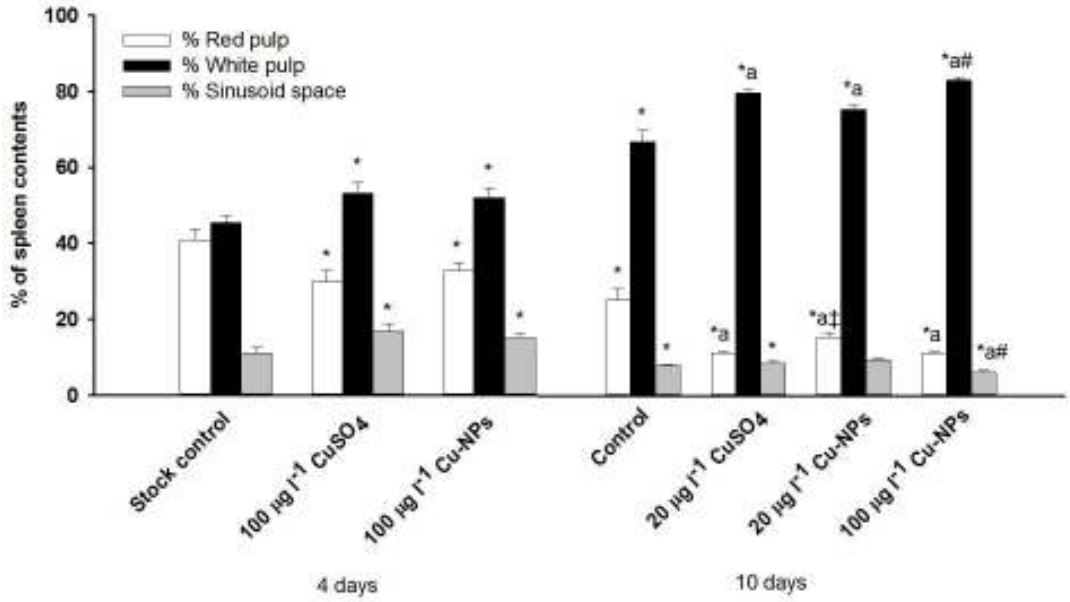

Figure 3.6 Changes in the proportions of red and white pulp as well as sinusoids space in the spleens of rainbow trout exposed to dissolved Cu as $CuSO_4$ (20 µg l^{-1}) or Cu-NPs (20 or 100 µg l^{-1}) for 0, 4, and 10 days. Data are means of proportional areas ± S.E.M. *n* = 6 fish/treatment. (*), significant difference from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). (a), significant difference from control within treatment (ANOVA, $P < 0.05$). (b), significant difference from the previous Cu-NPs concentration (dose effect, ANOVA, $P < 0.05$). (c), significant difference between low concentration of Cu and Cu-NPs (nano-effects, *t*-test, $P < 0.05$). Note the termination of the 100 µg l^{-1} $CuSO_4$ treatment after 4 days for ethical reasons.

3.3.5.2 Spleen prints

The proportion of mature and immature blood cells in the spleen were effected by exposure to $CuSO_4$ or Cu-NPs compared to the control images (Fig. 3.7; Table 3.5). An analysis of the different stages of the blood cells in the spleen of trout showed a small but statistically significant decrease in the proportion of immature cells (haemocytoblast, 0.6 fold and progranulocytes, 0.4 fold) and an increase in the proportion of mature immune cells (lymphocytes) after 4 days of exposure to 20 $\mu g\ l^{-1}$ of Cu as $CuSO_4$ compared to controls (ANOVA, $P < 0.05$; Table 3.5). In addition, the proportion of neutrophils showed a statistically significant decrease (0.5 fold) in the latter treatment compared to the control (ANOVA, $P < 0.05$). Exposure to 100 $\mu g\ l^{-1}$ of $CuSO_4$ treatment for 4 days caused an increase in the proportion of immature red blood cells (erythroblast, 2.6 fold) and a decrease in the proportion of mature red blood cells (erythrocyte, 0.8 fold) compared to controls (all statistically significant; ANOVA, $P < 0.05$; Table 3.5). After 10 days of exposure to 20 $\mu g\ l^{-1}$ of dissolved Cu as $CuSO_4$, a small but statistically significant decrease in the proportion of erythrocytes (0.9 fold) and an increase in the proportion of immune cells (lymphocytes, 3 fold) was observed (ANOVA, $P < 0.05$), although the proportion of immature red and white blood cells recovered and were the same as the controls (Table 3.5). No spleen samples were examined from 100 $\mu g\ l^{-1}$ $CuSO_4$ treatment at day 10 due to mortality.

Exposure to 20 $\mu g\ l^{-1}$ of Cu-NPs for 4 days showed minor changes that include a decrease in the proportion of mature immune cells (neutrophil, 0.5 fold) and an increase in the proportion of lymphocytes compared to controls (ANOVA, $P < 0.05$). However, at the higher concentration of the Cu-NPs, there were more effects including a statistically significant (ANOVA, $P < 0.05$) increase in the proportion of immature red blood cells (erythroblast, about 1.6 fold) and a decrease in the proportion of mature red

blood cells (erythrocytes, 1 fold) compared to the control (Table 3.5). In contrast, for the white blood cells, the proportion of progranulocytes (0.5 fold) and mature neutrophils (0.7 fold) decreased, while the proportion of lymphocytes increased in the spleen of 100 μg l^{-1} Cu-NP treatment compared to controls (all statistically significant; ANOVA, $P < 0.05$; Table 3.5). Ten days of exposure to 20 μg l^{-1} of Cu-NPs did not show any changes in the proportion of immature and mature red and white blood cells compared to controls. However, the 100 μg l^{-1} of Cu-NPs treatment showed a small but statistically significant increase in the proportion of neutrophils (1.8 fold) compared to the control (ANOVA, $P < 0.05$; Table 3.5). Overall, the changes in the proportion of red and white blood cells in the spleen of *O. mykiss* showed material-type effects (generally more severe with $CuSO_4$ than Cu-NPs), and a concentration-effect within each treatment with generally more changes at the higher metal concentration (all ststistically significant, ANOVA, $P < 0.05$; Table 3.5).

Exposure to $CuSO_4$ or Cu-NPs also caused changes in the morphology and levels of injury to the erythrocytes (Fig. 3.7; Table 3.6). At day 4, exposure to 20 or 100 μg l^{-1} of dissolved Cu as $CuSO_4$ caused an increase in the proportion of swollen erythrocytes and an increase in the incidence of membrane abnormalities at the highest $CuSO_4$ concentration compared to the controls (ANOVA, $P < 0.05$; Table 3.6). At day 10, surviving fish from the 20 μg l^{-1} of Cu as $CuSO_4$ treatment continued to show the erythrocyte abnormalities above, plus an increased incidence of cells with dividing nuclei compared to the control (ANOVA, $P < 0.05$; Table 3.6).

Exposure to 20 μg l^{-1} or 100 μg l^{-1} of Cu as Cu-NPs for 4 days also caused morphological abnormalities in the red blood cells, including a statistically significant increase (ANOVA, $P < 0.05$) in the proportion of swollen cells, and cells with

membrane abnormalities compared to controls (Table 3.6). By the end of the experiment (day 10), both Cu-NPs treatments continued to show a statistically significant (ANOVA, $P < 0.05$) increase in the proportion of cells with membrane abnormalities, but the proportion of swollen red cells was only increased with 20 μg l^{-1} of Cu-NPs treated compared to the control (Table 3.6). There were also some material-type effects. The proportion of red cells with dividing nuclei and membrane abnormalities were generally more severe with $CuSO_4$ than Cu-NPs (statistically significant, ANOVA, $P < 0.05$; Table 3.6).

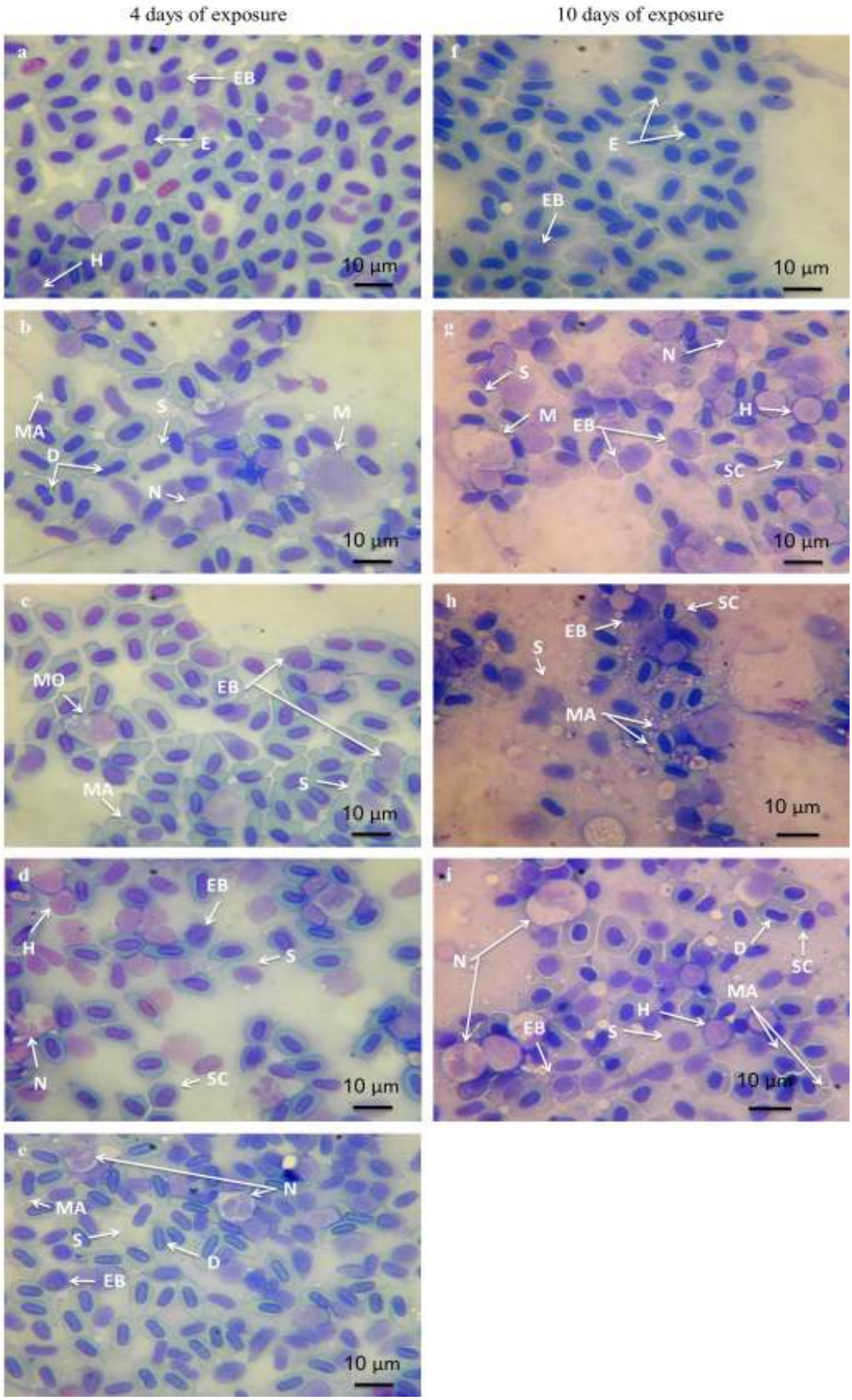
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Figure 3.7 Spleen prints of rainbow trout following exposure to $CuSO_4$ and Cu-NPs for 4 days (left column) and 10 days (right column). For day 4 the panels include (a) control, (b) 20 µg l^{-1} of $CuSO_4$, (c) 100 µg l^{-1} of $CuSO_4$, (d) 20 µg l^{-1} of Cu-NPs, (e) 100 µg l^{-1} of Cu-NPs. For day 10, the panels include (f) control, (g) 20 µg l^{-1} of $CuSO_4$, (h) 20 µg l^{-1} of Cu-NPs (i) 100 µg l^{-1} of Cu-NPs. Erythroblast (EB), erythrocyte (E), membrane abnormalities (MA), swollen cell (S), neutrophil (N), blood cells with a dividing nuclei (D), macrophage (M), monocyte (MO), small cell size (SC), haemocytoblast (H). Scale bar indicates magnification. Smear stained with May-Grünwald Giemsa. Note the termination of the 100 µg l^{-1} $CuSO_4$ treatment after 4 days for ethical reasons.

Table 3.5 Quantitative analysis of the proportion of haematopoietic cells in the spleen prints of rainbow trout that exposed to 20 and 100 µg l^{-1} of either dissolved Cu as $CuSO_4$ and Cu-NPs for 4 and 10 days.

Percentage of haematopoietic cells	Time (days)	Treatments				
		Control	20 µg l^{-1} $CuSO_4$	20 µg l^{-1} Cu-NPs	100 µg l^{-1} $CuSO_4$	100 µg l^{-1} Cu-NPs
Haemocytoblast	4	4.4 ± 0.4 (6)	2.8 ± 0.4 (6) *	4.3 ± 0.2 (6) +	5.0 ± 1.2 (3) #	4.3 ± 0.4 (6)
	10	5.5 ± 0.3 (6)	5.6 ± 0.4 (6) x	6.3 ± 0.4 (6) x	-	5.0 ± 0.2 (6) #
Erythroblast	4	7.8 ± 0.4 (6)	6.8 ± 0.4 (6)	7.9 ± 0.5 (6)	20.2 ± 3.4 (3) *#	12.8 ± 0.7 (6) *+#
	10	13.5 ± 1.0 (6) x	11.3 ± 0.4 (6) x	12.9 ± 0.5 (6) x	-	12.9 ± 0.2 (6)
Progranulocytes	4	2.4 ± 0.1 (6)	0.9 ± 0.1 (6) *	1.3 ± 0.4 (6) *	0.5 ± 0.3 (3) *	1.3 ± 0.2 (6) *+
	10	1.3 ± 0.2 (6) x	1.4 ± 0.2 (6)	1.3 ± 0.1 (6)	-	1.6 ± 0.2 (6)
Erythrocytes	4	79.8 ± 0.7 (6)	82.3 ± 0.8 (6)	82.0 ± 0.4 (6)	70.7 ± 3.4 (3) *#	76.1 ± 0.9 (6) *+#
	10	77.5 ± 1.3 (6)	73.8 ± 0.9 (6) *x	74.8 ± 0.6 (6) x	-	75.2 ± 0.6 (6)
Lymphocytes	4	Not observed (6)	5.0 ± 0.7 (6) *	1.9 ± 0.5 (6) *+	1.8 ± 0.4 (3) #	1.8 ± 0.4 (6) *
	10	Not observed (6)	3.3 ± 0.6 (6) *x	0.8 ± 0.4 (6) +	-	0.7 ± 0.2 (6)
Neutrophils	4	5.1 ± 0.3 (6)	1.8 ± 0.5 (6) *	2.7 ± 0.3 (6) *	1.5 ± 0.5 (3) *	3.4 ± 0.2 (6) *+
	10	2.2 ± 0.3 (6) x	3.5 ± 0.2 (6) *x	3.2 ± 0.7 (6)	-	3.9 ± 0.2 (6) *

Continued

Table 3.5

Percentage of haematopoietic cells	Time (days)	Treatment				
		Control	20 µg l^{-1} $CuSO_4$	20 µg l^{-1}Cu-NPs	100 µg l^{-1} $CuSO_4$	100 µg l^{-1} Cu-NPs
Macrophages	4	0.3 ± 0.2 (6)	0.4 ± 0.2 (6)	0.5 ± 0.2 (6)	Not observed (3)	Not observed (6)
	10	Not observed (6)	0.4 ± 0.2 (6)	0.3 ± 0.1 (6)	-	0.4 ± 0.2 (6)
Monocytes	4	Not observed (6)	Not observed (6)	0.2 ± 0.1 (6)	0.2 ± 0.1 (3)	0.2 ± 0.1 (6)
	10	Not observed (6)	Not observed (6)	Not observed (6)	-	Not observed (6)
Thrombocytes	4	Not observed (6)	Not observed (6)	Not observed (6)	Not observed (3)	Not observed (6)
	10	Not observed (6)	0.7 ± 0.3 (6) *^{x}	0.5 ± 0.3 (6) *^{x}	-	0.3 ± 0.2 (6)

Data are means of proportional cells ± S.E.M. (*), significantly different from control within row at each time point (ANOVA, $P < 0.05$). (#), significantly difference between 20 and 100 µg l^{-1} of $CuSO_4$ and Cu-NPs at the same time point (Dose effects, ANOVA, $P < 0.05$). (+), significantly different between $CuSO_4$ and Cu-NPs at the same time within concentration (material-type effects, ANOVA, $P < 0.05$). (x), significantly difference between day 4 and 10 within treatment (Time effects, ANOVA, $P < 0.05$). Note that there are no data for 100 µg l^{-1} $CuSO_4$ treatment at day 10 due to the stop of this treatment at day 4 following high mortality.

Table 3.6 Quantitative analysis of abnormal erythrocytes in the spleen prints of rainbow trout that exposed to 20 and 100 µg l^{-1} of either dissolved Cu as $CuSO_4$ or Cu-NPs for 4 and 10 days.

Percentage of abnormal erythrocytes	Time (days)	Treatments				
		Control	20 µg l^{-1} $CuSO_4$	20 µg l^{-1} Cu-NPs	100 µg l^{-1} $CuSO_4$	100 µg l^{-1} Cu-NPs
Swollen blood cells	4	1.1 ± 0.3 (6)	4.8 ± 0.3 (6) *	3.9 ± 0.4 (6) *	3.7 ± 0.4 (3) *#	2.8 ± 0.5 (6) *#
	10	1.0 ± 0.3 (6)	4.3 ± 0.2 (6) *	3.8 ± 0.6 (6) *	-	2.3 ± 0.4 (6) #
Small cell size	4	Not observed (6)	0.8 ± 0.5 (6)	1.7 ± 0.6 (6) *	1.5 ± 0.3 (3) *	1.4 ± 0.4 (6) *
	10	Not observed (6)	1.7 ± 0.2 (6) *	2.1 ± 0.2 (6) *	-	1.6 ± 0.2 (6) *
Cells with a dividing nucleus	4	9.8 ± 1.0 (6)	8.1 ± 0.5 (6)	8.5 ± 0.3 (6)	10.3 ± 2.2 (3)	7.3 ± 0.7 (6) *+
	10	7.2 ± 0.2 (6) x	10.8 ± 1.8 (6) *x	9.5 ± 0.7 (6)	-	7.8 ± 0.3 (6)
Membrane abnormalities	4	1.8 ± 0.2 (6)	2.5 ± 0.3 (6)	3.7 ± 0.3 (6) *+	6.7 ± 0.6 (3) *#	4.9 ± 0.3 (6) *#+
	10	2.8 ± 0.2 (6)	7.4 ± 0.4 (6) *x	7.0 ± 0.5 (6) *x	-	8.3 ± 0.5 (6) *#x

Data are means of proportional cells ± S.E.M. (*), significantly different from control within row at each time point (ANOVA, $P < 0.05$). (#), significant difference between low and high concentration of dissolved Cu as $CuSO_4$ and Cu-NPs at the same time point (ANOVA, $P < 0.05$). (+), significantly different between $CuSO_4$ and Cu-NPs at the same time within concentration (material-type effects, ANOVA, $P < 0.05$). (x), significant difference between day 4 and 10 within treatment (ANOVA, $P < 0.05$). Note that there are no data for 100 µg l^{-1} $CuSO_4$ treatment at day 10 due to the stop of this treatment at day 4 following high mortality.

3.3.6 Brain histopathology

The brains of fish were removed whole, and the histology is reported here for the forebrain (telencephalon), mid-brain (mesencephalon) and hind brain (metencephalon or cerebellum) regions. The telencephalon of control fishes showed normal structure, but exposure to $CuSO_4$ or Cu-NPs caused similar types of pathology, and this was evident in the 100 μg l^{-1} Cu as $CuSO_4$ or Cu-NPs treatment as early as day 4 with occasional necrotic nerve cell bodies, occasional cells with pyknotic nuclei and/or vacuole formation in the cell body as well as apparently enlarged nerve cells (hydropic change) in all three fish examined (Fig. 3.8). However, by day 10, the 20 μg l^{-1} Cu as $CuSO_4$ or Cu-NPs and 100 μg l^{-1} Cu-NPs treatment also showed the same pathologies as above in four to six fish out of six fish examined/treatment (Fig. 3.8).

A quantitative analysis of the telencephalon at day 10 confirmed a statistically significant increase in the proportion of enlarged (hydropic change) nerve cells (% mean ± S.E.M., $n = 6$): control, 3.0 ± 0.5; 20 μg l^{-1} of Cu as $CuSO_4$, 9.7 ± 1.3; 20 μg l^{-1} of Cu as Cu-NPs, 10.2 ± 1.4; 100 μg l^{-1} of Cu as Cu-NPs, 10.6 ± 0.7), and increased incidence of necrosis (% mean ± S.E.M., $n = 6$; control, 0.4 ± 0.3; 20 μg l^{-1} of Cu as $CuSO_4$, 7.0 ± 1.4; 20 μg l^{-1} of Cu as Cu-NPs, 10.5 ± 1.1; 100 μg l^{-1} of Cu as Cu-NPs, 9.9 ± 1.3) compared to the control (ANOVA or Kruskal-Wallis, $P < 0.05$), and with a small but statistically significant material-type effect for the incidence of necrosis (more in the nano treatments than $CuSO_4$).

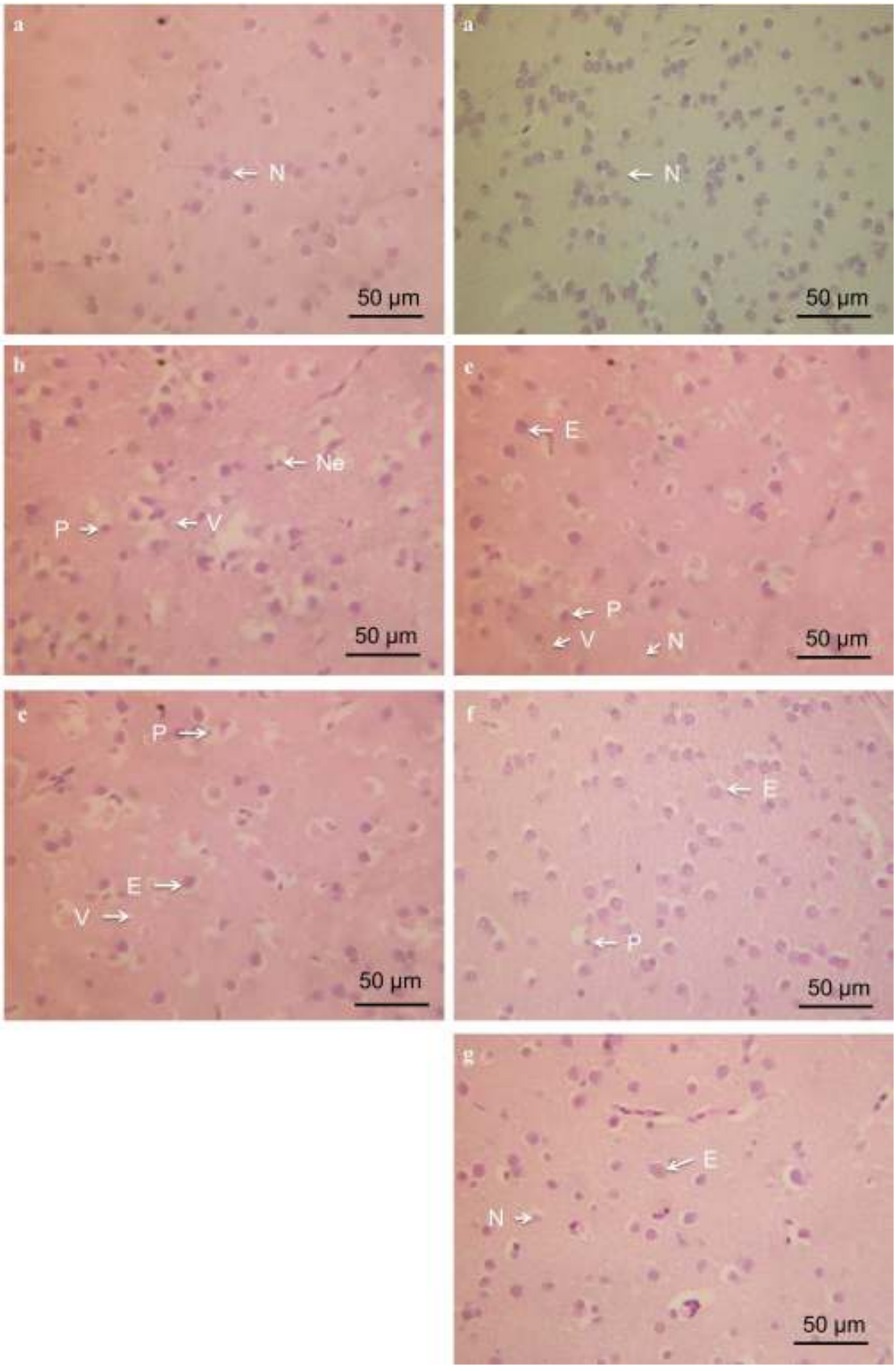
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Figure 3.8 Telencephalon morphology in the brain of rainbow trout following waterborne exposure to $CuSO_4$ or Cu-NPs for 4 days (left column) and 10 days (right column). For day 4 the panels include (a) control, (b) 100 μg l^{-1} $CuSO_4$, (c) 100 μg l^{-1} Cu-NPs. For day 10 the panels include (d) control, (e) 20 μg l^{-1} $CuSO_4$ (f) 20 μg l^{-1} Cu-NPs and (g) 100 μg l^{-1} Cu-NPs. The telenncephalon of control fish showed normal histology of nerve cell (N). All Cu-treatments showed similar types of injuries including enlarged nerve cells (E), occasional necrotic nerve cell (Ne), occasional pyknotic nuclei (P), and vacuole formation (V). Scale bar indicates magnification, sections were 7 μm thick and stained with haematoxylin and eosin. Note the termination of the 100 μg l^{-1} $CuSO_4$ treatment after 4 days for ethical reasons.

The mesencephalon region of the trout brain was normal in the control animals (Fig 3.9), but after 4 days of exposure to the highest $CuSO_4$ or Cu-NPs concentration showed similar injuries including apparent enlargement of blood vessel, minor alterations in the thickness of mesencephalon layers and occasional rupture of the periventriculare layer in all three fish examined. After 10 days, these alteration were also evident in five fish out of six fish from the 20 μg l^{-1} of Cu as $CuSO_4$ or Cu-NPs and 100 μg l^{-1} of Cu-NPs treatment. A quantitative analysis of the mesencephalon layers confirmed these observations (Fig. 3.9). Exposure to 100 μg l^{-1} Cu-NPs for 10 days caused a statistically significant increase in the thickness of stratum periventriculare and a decrease in the stratum fibrosum et griseum and stratum griseum centrale compared to the unexposed control (ANOVA or Kruskal-Wallis, $P < 0.05$) (Fig. 3.9). There was a concentration-effect within the nano treatments with the 100 μg l^{-1} Cu-NPs being worse than 20 μg l^{-1} Cu-NPs (Fig. 3.9).

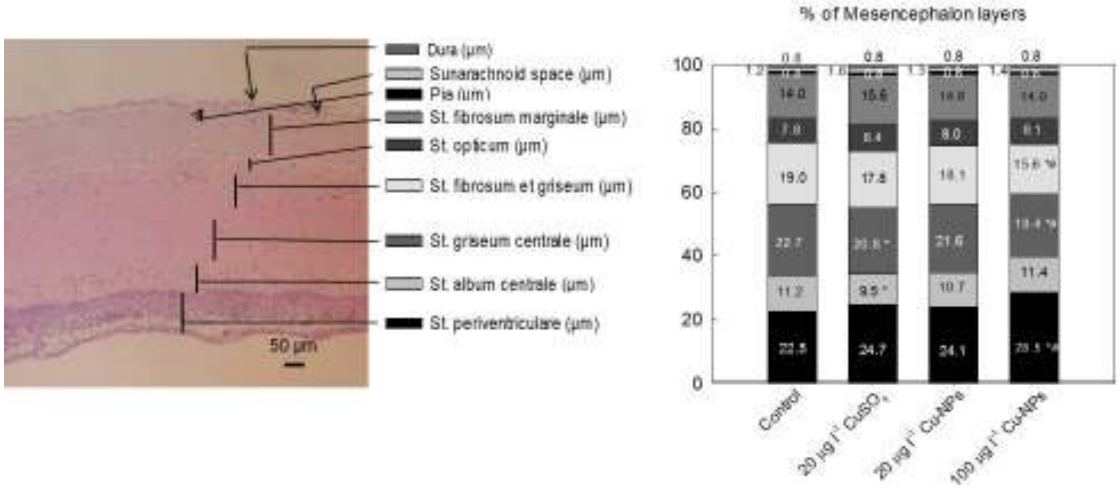

Figure 3.9 The proportion of alteration in the thickness of mesencephalon tissue layers of the brain in rainbow trout following waterborne exposure to control, 20 µg l^{-1} of Cu as $CuSO_4$, 20 or 100 µg l^{-1} of Cu as Cu-NPs for 10 days. Data are proportion means as a % ± S.E.M., n = 6 fish/treatment. (*), significant difference from control within treatment (ANOVA, $P < 0.05$). (#), significant difference between low and high concentration of Cu-NPs (t-test, $P < 0.05$). Note the termination of the 100 µg l^{-1} $CuSO_4$ treatment after 4 days for ethical reasons.

The metencephalon (cerebellum) regions of trout brain showed normal structure in the controls (Fig. 3.10). Exposure to 100 µg l^{-1} of Cu as $CuSO_4$ or Cu-NPs for 4 days showed similar alteration that included: increases in the blood vessel diameter on the ventral surface of the cerebellum and occasional necrotic cells between the molecular and granular layer of cerebellum cortex in all three fish examined (Fig. 3.10). The same alterations were also evident after 10 days of exposure to 20 µg l^{-1} of Cu as $CuSO_4$ (5/6 fish), 20 µg l^{-1} of Cu as Cu-NPs (4/6 fish), 100 µg l^{-1} of Cu as Cu-NPs (4/6 fish) (Fig. 3.10).

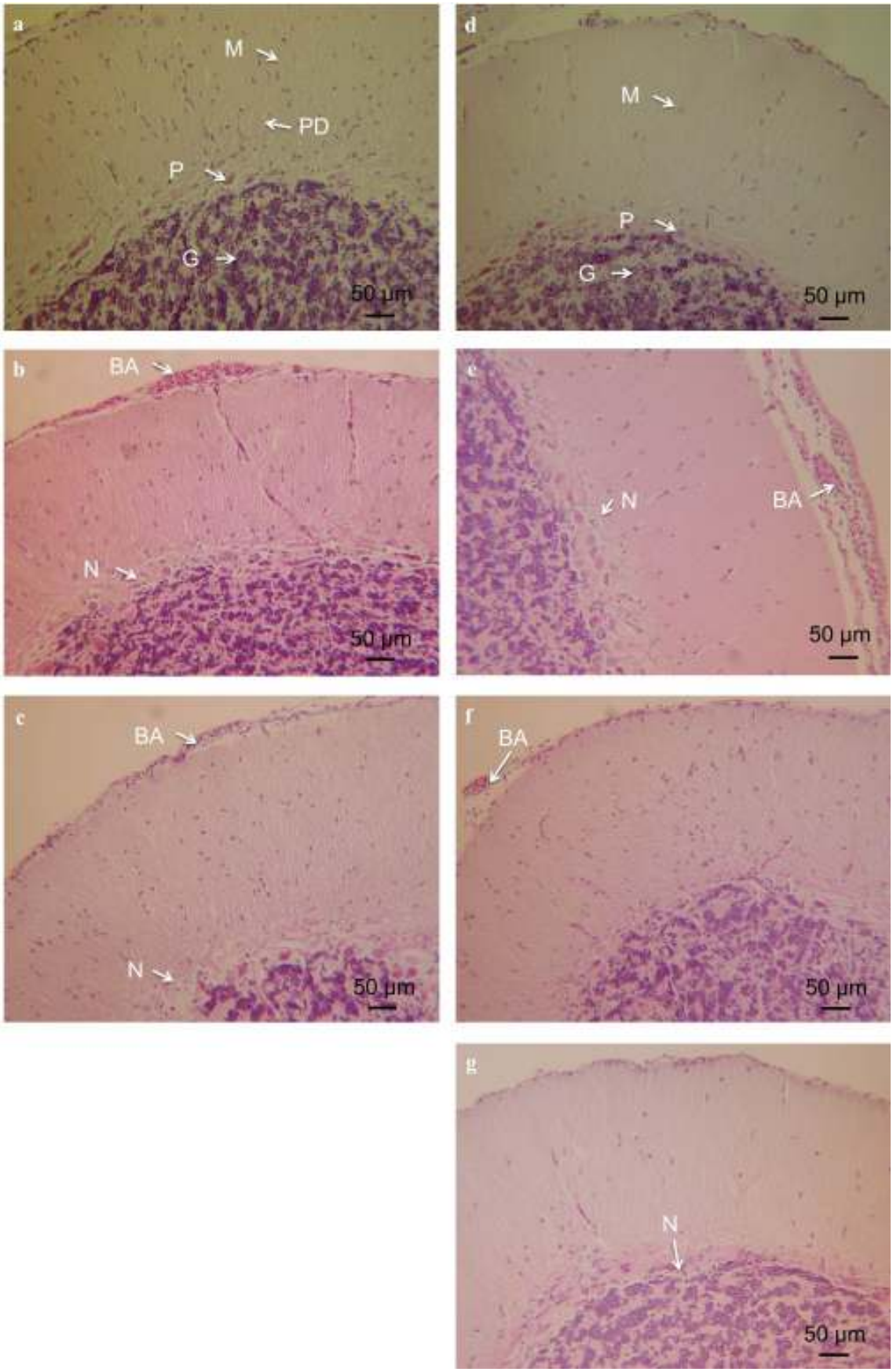
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Figure 3.10 Cerebellum morphology in the brain of rainbow trout following waterborne exposure to $CuSO_4$ or Cu-NPs for 4 days (left column) and 10 days (right column). For day 4 the panels include (a) control, (b) 100 μg l^{-1} $CuSO_4$, (c) 100 μg l^{-1} Cu-NPs. For day 10 the panels include (d) control, (e) 20 μg l^{-1} $CuSO_4$ (f) 20 μg l^{-1} Cu-NPs and (g) 100 μg l^{-1} Cu-NPs. The cerebellum of control fish showed normal histology with normal surface architecture and layers of the cerebellum. All Cu-treatments showed similar types of injuries, but the severity was worse for the equivalent Cu-NP exposure by day 10. These injuries include blood vessel abnormality on the ventral surface (BA), necrosis between the molecular and granular layers (N). Scale bar indicates magnification, sections were 7 μm thick and stained with haematoxylin and eosin. Note the termination of the 100 μg l^{-1} $CuSO_4$ treatment after 4 days for ethical reasons.

3.3.7 Muscle histopathology

Histology of the trout skeletal muscle shows normal anatomy in the controls (Fig. 3.11). Exposure to 100 μg l^{-1} of Cu as $CuSO_4$ or Cu-NPs for 4 days showed an increase in the extracellular space and a proportional decrease in the area of the sections muscle fibre in all three fish examined (Fig. 3.11). These changes were also evident by day 10 in the 20 μg l^{-1} of Cu as $CuSO_4$ or Cu-NPs and 100 μg l^{-1} of Cu-NPs treatment (between four to five out of six animals/treatment; Fig. 3.11). A quantitative analysis of the skeletal muscle confirmed these observations with the proportion of muscle bundle area decreasing in all the Cu-treatments compared to the unexposed control (ANOVA, $P < 0.05$), and with a small but statistically significant material-type effect at the 20 μg l^{-1} concentration (Table 3.7). Values for the proportion of muscle bundle area in the skeletal muscle were (%, mean ± S.E.M, $n = 6$): control, 68.6 ± 0.3; 20 μg l^{-1} Cu as $CuSO_4$, 64.8 ± 0.3; 20 μg l^{-1} Cu as Cu-NPs, 66.4 ± 0.3; 100 μg l^{-1} Cu as Cu-NPs, 65.4 ± 0.3, with the remainders being extracellular space and connective tissue.

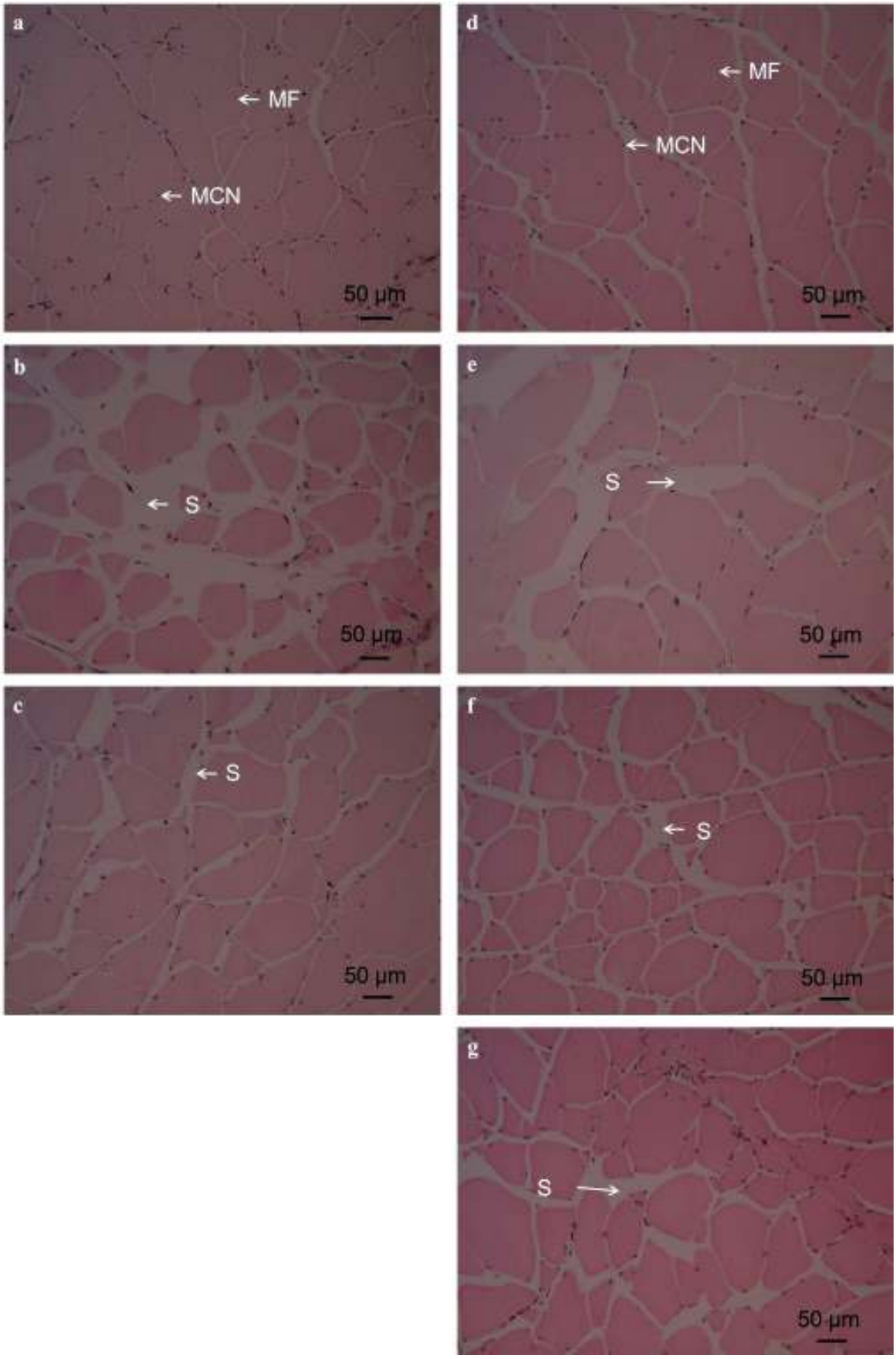
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Figure 3.11 Muscle morphology in rainbow trout following exposure to $CuSO_4$ or Cu-NPs for 4 days (left column) and 10 days (right column). For day 4 the panels include (a) control, (b) 100 μg l^{-1} $CuSO_4$, (c) 100 μg l^{-1} Cu-NPs. For day 10 the panels include (d) control, (e) 20 μg l^{-1} $CuSO_4$ (f) 20 μg l^{-1} Cu-NPs and (g) 100 μg l^{-1} Cu-NPs. The muscle of control fish showed normal architecture with the muscle cell nucleus (MCN), and muscle fibres (MF). All Cu treatments showed similar types of alteration, but with slightly more changes in the equivalent $CuSO_4$ treatment by day 10. These changes include an increased extracellular space between muscle bundles (S). Scale bar indicates magnification, sections were 7 μm thick and stained with haematoxylin and eosin. Note the termination of the 100 μg l^{-1} $CuSO_4$ treatment after 4 days for ethical reasons.

Table 3.7 Quantitative analysis of alteration in the muscle fibres of rainbow trout following exposure to control (no added Cu), 20 μg l^{-1} Cu as $CuSO_4$ or Cu-NPs and 100 μg l^{-1} Cu as Cu-NPs for 0 and 10 days.

Treatments	Time / Days	No. of fish	Proportion area of	
			Muscle fibres	Extracellular space
Control	0	5	69.5 ± 0.4	30.6 ± 0.4
Control	10	6	68.6 ± 0.3	31.4 ± 0.3
20 μg l^{-1} Cu as $CuSO_4$	10	6	64.8 ± 0.3 *[a]	35.3 ± 0.3 *[a]
20 μg l^{-1} Cu as Cu-NPs	10	6	66.4 ± 0.3 *[a‡]	33.6 ± 0.3 *[a‡]
100 μg l^{-1} Cu as Cu-NPs	10	6	65.4 ± 0.3 *[a]	34.7 ± 0.3 *[a]

Data are means of proportional area ± S.E.M. (*), significant difference from initial fish (stock fish at time zero, ANOVA or Kruskal Wallis, $P < 0.05$). (a), significant difference from control within column (ANOVA or Kruskal Wallis, $P < 0.05$). (‡), significant difference between 20 μg l^{-1} of $CuSO_4$ and Cu-NPs (*t* -test or ANOVA, $P < 0.05$).

3.4 Discussion

The current study details the effects of dissolved Cu compared to Cu-NPs on the organ integrity of rainbow trout. Overall, the results showed that dissolved Cu and Cu-

NPs cause similar types of injuries in gill, gut, liver, kidney, spleen, brain and muscle. At day 4, these injuries were more severe with $CuSO_4$ than Cu-NPs, but by day 10 the pathology with Cu-NPs was comparable or worse than equivalent $CuSO_4$ treatment, suggesting a delay in the appearance of Cu-NP pathology compared to $CuSO_4$.

3.4.1 Pathological observation in the gill tissue

Copper is a well-known respiratory and ionoregulatory toxicant in fish (reviews, (Grosell *et al.*, 2007; Handy, 2003) and there is some evidence that waterborne exposure to metal NPs may result in particle accumulation in or on the epithelial cells (*e.g.*, TiO_2, Moger *et al.*, 2008). This study showed that $CuSO_4$ and Cu-NPs caused similar types of gill injuries, but these pathologies appeared earlier (by day 4) in the highest $CuSO_4$ concentration and with more severe injuries than the equivalent concentration of Cu-NPs. The gills of $CuSO_4$-treated fish also had more Cu accumulation than the equivalent nano treatment by the end of the experiment. Together, these data suggest that the Cu salt is more bioavailable and/or bioreactive than the nano form.

Mucus secretion is often the first line of defence to metal exposure in the gills and can temporarily protect the underlying epithelium from injury (Handy and Maunder, 2009). Mucus secretion and swollen mucocytes were observed in this study, and has been noted in rainbow trout with other nanomaterials (SWCNT, Smith *et al.*, 2007; TiO_2 NPs, Federici *et al.*, 2007). However, in the current study mucus secretion was not sufficient to protect the gills from the pathology arising from either $CuSO_4$ or Cu-NP exposure. The types of gill pathology reported here for Cu-NPs have also been reported for waterborne exposures with other nanomaterials in trout (SWCNT, Smith *et*

al., 2007; TiO_2 NPs, Federici *et al.*, 2007), and for TiO_2 NPs in carp (Hao *et al.*, 2009). Oedema appears to be a common feature of the gill pathology for nano metals, and for dissolved metals, this is usually explained by inhibition of the branchial Na^+/K^+-ATPase which leads to solute accumulation in the epithelial cell and the osmotic influx of water. This explanation might also apply to Cu-NPs since they also inhibit branchial Na^+/K^+-ATPase (Shaw *et al.,* 2012), although measurements of intracellular free Cu ion activity from Cu-NPs in gill cells would be needed to confirm this hypothesis.

The gill also functions in respiratory gas exchange. Waterborne exposure to $CuSO_4$ is well known to cause swelling of the secondary lamellae of the trout gill, resulting in an increase in the diffusion distance across the epithelium for gas exchange and a systemic hypoxia (*e.g.*, arterial oxygen partial pressures of < 5 kP within 24 h, Wilson and Taylor, 1993). The hyperplasia, fusion and lifting of the gill epithelium suggests that Cu-NPs, like $CuSO_4$, (Mc Geer *et al.*, 2000), could also compromise oxygen uptake and contribute to a systemic hypoxia and/or add extra metabolic costs to respiration. Recently Bilberg *et al.* (2010) also showed that exposure to silver NPs can reduce blood pO_2 in fish. The increased incidence of aneurism in the branchial vasculature with Cu-NPs reported here would suggest some interruptions of gill perfusion that might contribute to a systemic hypoxia. Further research on the exercise performance and swimming behaviour of fish exposed to Cu-NPs is required to determine the functional significance of the gill pathology. Nonetheless, animals with acute branchial oedema and epithelial lifting would be unlikely to survive in the wild.

3.4.2 Pathological observation in the gut epithelium

The gut is an important route of Cu uptake in teleost fishes (*e.g.*, Handy *et al.*, 1999) and the anatomy of fish gut lends itself to particle uptake as a relatively leaky epithelium (compared to the gills) which also forms absorptive vacuoles for taking up nutrients (see Clearwater *et al.*, 2005 for an anatomical review relating to metals). In the current study, the fish were unfed and the normal gut morphology in time-matched controls at the end of the experiment suggests this short period without food was not a cause of pathology. Likely stress-induced drinking with gut pathology has been observed in trout with other nanomaterials (SWCNT, Smith *et al.*, 2007; TiO_2 NPs, Federici *et al.*, 2007). Notably while the types of gut pathologies for $CuSO_4$ and Cu-NPs were similar, the injuries were more severe in the Cu-NP treatment by the end of the experiment; and the quantitative analysis confirmed this difference in severity. The mechanism and biological importance of the difference in magnitude of the material-type effect is less clear. The copper concentrations in the gut tissue at the end of the exposure for the 20 μg l^{-1} of Cu treatments were similar for both $CuSO_4$ and Cu-NPs, and there was no material-type effect on intestinal Na^+/K^+-ATPase or thiobarbituric acid reactive substances (TBARS) at the end of the experiment (Shaw *et al.*, 2012); although the reduction in sodium pump activity and elevated TBARS implicated osmoregulatory disturbances and oxidative stress as mechanisms for both $CuSO_4$ and Cu-NPs (Shaw *et al.*, 2012).

3.4.3 Pathological observation in the liver

The Liver is a central compartment for Cu metabolism in fish and a target organ for Cu accumulation (Grosell *et al.*, 1996; Handy *et al.*, 1999). The types of injuries observed here for $CuSO_4$ (some patches of cellular necrosis, lipidosis, vacuole

formation in the cytoplasm of the hepatocytes, changes in sinusoid space, *etc*.) are well-known (Baker, 1969; Figueiredo-Fernandes *et al.*, 2007; Handy *et al.*, 1999; Mc Geer *et al.*, 2000) and often explained by Cu-induced oxidative stress in the liver tissue (Hoyle *et al.*, 2007). Exposure to Cu-NPs produced the same types of pathologies, but the severity was different. For example in the 20 μg l^{-1} treatments, Cu-NPs cause greater alteration in the proportion of hepatic area and sinusoid space than $CuSO_4$ after 10 days, even though the livers from the Cu-NP treatment showed no statistical increase of new Cu accumulation in the tissue. This suggests, more likely, that a secondary systemic hypoxia is contributing to changes in the liver with the Cu-NP treatments. This notion is supported by the decrease in sinusoid space in the Cu-NP treatment implying some redirection of blood flow to other internal organs. Functionally, the aspects of energy metabolism affected in the liver are likely to be similar for both materials given that the *types* of pathology are similar. For example, loss of sinusoid space with apparent increased glycogen storage (and cell size) has been associated with the inability to mobilise energy stores from the liver during Cu exposure (Handy *et al.*, 1999). The decrease in hepatocyte diameter relative to nuclei diameter suggests (with both substances) that liver cells are more metabolically active, and the increased presence of melanomacrophage deposits supports this notion as they are known to be involved with recycling of endogenous materials from damaged cells (Haaparanta *et al.*, 1996). In the absence of methods to identify Cu-NPs in the liver cells (trout livers can synthesis endogenous Cu-containing particles, Lanno *et al.*, 1987), it remains uncertain if these melanomacrophage deposits are also involved in the removal of Cu-NPs from the tissue.

3.4.4 Pathological observation in the kidney

There are relatively few reports of renal pathology from waterborne Cu exposure in rainbow trout (Handy, 2003), but the pathologies reported here including some damage to the epithelial cells of the renal tubules, changes in the Bowman's space, and an increase in the foci of melanomacrophage deposits. These pathologies are broadly similar to previous reports on fishes. For example, in the Nile tilapia (*Oreochromis niloticus*) following 7 days of exposure to 46 mg l^{-1} Cu pathologies included tubular swelling, atrophy of the glomerulus, and necrosis of the renal epithelium (Kosai *et al.*, 2009). Nano copper showed similar types of renal pathology as $CuSO_4$. At least one study in mice has also shown damage to renal proximal tubules, swollen glomeruli accompanied by loss of extracellular space in the Bowman's capsule after 72 h of oral gavage of 108-1080 mg kg^{-1} Cu-NPs (Chen *et al.*, 2006); and while some details may be different, these pathologies are broadly consistent with the findings here in trout. Unfortunately, partly due to the small size of the fish in the present study, it was not possible to determine total Cu in the urine or kidney tissue. It is therefore uncertain if the pathologies from the $CuSO_4$ or Cu-NPs are caused by direct target organ toxicity from internalized metal in the renal tissue, or an indirect effect.

In freshwater-adapted teleost fishes, the osmoregulatory strategy is to produce a large volume of dilute urine to compensate for the osmotic influx of water across the body surfaces. Renal filtration rate is therefore critical to survival. The almost doubling in the size of the Bowman's space with either $CuSO_4$ or Cu-NPs implies that water is being drawn osmotically into the space, presumably from glomerular filtration, but is not likely to be moving along the obstructed tubule lumens (damaged epithelial cells) and it seems likely that urine flow would be compromised. This is consistent with a dilution of plasma electrolytes in the same fish (Shaw *et al.*, 2012). However, at least

one study has shown the maintenance of creatinine clearance in trout exposed to mg levels of TiO_2 NPs (Scown *et al.*, 2009), implying that glomerular filtration rate (GFR) can be maintained in some exposures with less toxic NPs. The only notable material-type effect of Cu-NPs on renal pathology was an increase in the number of melanomacrophage deposits compared to the $CuSO_4$ treatment and unexposed controls. This is largely a haematopoietic response and is well known for metals in fish kidney and for $CuSO_4$ (Handy, 2003), but the observed material-type effect implies there may be some additional inflammation stress on the haematopoietic system with the nano form. However with so little known about the effects of metal NPs on the renal physiology of fishes (review on fish physiology, Handy *et al.*, 2011), much more research is needed.

3.4.5 Pathological observation in the spleen

The observed normal spleen structure in the controls of this study is consistent with our previous reports which showed distinct red and white pulp with the presence of a few melanomacrophage deposits in the splenic tissue (Handy *et al*., 2002c; Boyle *et al.*, 2013). There were only minor pathologies (necrotic cells) and melanomacrophage deposits observed in the current study, regardless of the type of Cu. These changes are consistent with increased splenic activity within the normal physiological scope of the fish for dealing with damaged cells. The finds in the present study are similar to Boyle *et al.* (2013) which showed an increase in the number of melanomacrophage deposits in the spleen of rainbow trout after 14 days of exposure to either 1 mg l^{-1} TiO_2 NPs or the bulk form of TiO_2.

These effects occurred without measurable increases in the Cu concentrations in the spleen for any treatment. There was also no histological evidence of NP aggregates in the spleen observed in this study. This suggests that indirect toxicity of Cu as $CuSO_4$ or Cu-NPs is a more likely explanation. Copper sulphate is well-known to cause oxidative stress in fishes. Hoyle *et al.* (2007) found that dietary Cu exposure causes oxidative stress in the internal organs of *Clarias gariepinus*. In *O. mykiss* this could arise from reactive oxygen species (ROS) generation due to hypoxia associated with gill injury (Al-Bairuty *et al.*, 2013).

In fishes, the haematopoietic response to systemic hypoxia is thought to include the release of stored red blood cells from the spleen with a subsequent decrease in the proportion of splenic red pulp (Handy *et al*., 2011); the latter was observed here for both $CuSO_4$ and Cu-NPs. The magnitude of the decrease in the red pulp in the present study was around a 9-14 % reduction relative to the time-matched control, and although there was a material-type effect with the $CuSO_4$ causing a few more percent contraction of the red pulp than Cu-NPs, this is well within the physiological scope of normal spleen. For example, the red pulp in the controls decreased over time by about 15% and is associated with not feeding the fish during the experiment. These findings are similar to our previous studies with *O. mykiss*. For example, Boyle *et al.* (2013) found a decrease in the proportion of red pulp from around 50% in controls to 45 and 46% in *O. mykiss* exposed to 1 mg l^{-1} bulk and TiO_2 NPs respectively for two weeks, with a concomitant increase in the sinusoid space of a few percent in the TiO_2 treatments. Similarly, the changes in the sinusoid space in the present study were small. One interpretation of the decrease in red pulp is a physiological response to maintain normal haematology during Cu exposures. Blood samples from fish from the same experiment showed normal blood

cell counts, haematocrits and haemoglobin concentrations, albeit with some variation within the normal range (Shaw *et al.*, 2012). This suggests the spleen was successful in this task for both $CuSO_4$ and Cu-NPs. An alternative interpretation, is that the increase in the proportion of white pulp is caused by an immune response with resulting congestion of the white pulp with extra immune cells. This explanation seems less likely. Although there were some small changes in the proportions of the types of white cells in the spleen (see below), these were not sufficient to interpret as a wide spread inflammatory response, and mainly involved one cell type.

3.4.6 Effects of dissolved Cu and Cu-NPs on blood cells stored in the spleen

Exposure to $CuSO_4$ or Cu-NPs caused some changes in the erythroblasts, progranulocytes, and neutrophils, but these effects are interpreted as normal background variation, as these small % changes (between 2-12 %) are often within the scope of temporal variation in the control. The most important changes were decreases in the proportions of mature red cells, and the appearance of lymphocytes in the spleen. The red cell depletion is consistent with the idea of systemic hypoxia from damaged gills and the need to deplete red pulp to keep circulating cell numbers normal (discussed above).

Injuries observed in erythrocyte morphology were mainly a few % of swelling in the cell population and membrane abnormalities. This is consistent with the idea of some transient osmotic disturbances occurring; such as inhibition of Na^+/K^+-ATPase activity and alteration of electrolyte concentrations in the tissue (Shaw *et al.*, 2012). A few cells were observed with a dividing nucleus and some shrinkage of the cytoplasm. This is not regarded as an indication of genotoxicity and defects in the cell cycle, but

merely as background noise within the normal variation of the controls. A small, but statistically significance increased in the proportions of lymphocytes were observed here with both Cu treatments. This may suggest that the haematopoeitic system is regarding the Cu-NPs as a foreign body or antigen. It is not yet clear how metallic NPs are immunogenic, and particles made from different metals may or may not be recognized as "self", but they can sometimes change the distribution of immune cells in the body (Fadeel and Garcia-Bennett, 2010). However, this would not explain why there is a stronger response to $CuSO_4$. The latter may be non-specific, and incidental with inflammation in the gills and other internal organs (Al-Bairuty *et al*., 2013). It is also possible that dissolved Cu is simply more bioavailable than the nano form to stimulate the immune system to produce these cells.

3.4.6 Pathological observation in the brain

There are relatively few detailed reports of regional brain pathologies in fishes from toxic metals, and most of these have been for dietary exposures (Hg, Berntssen *et al.*, 2003; Cu, Handy, 2003). In the present study, histological examination of the main regions of the brain following exposure to $CuSO_4$ showed injuries that include occasional necrosis of some nerve cells and the occasional vacuole formation in the telencephalon; minor alteration in the thickness of mesencephalon with parts of the preventriculare layer being damaged; as well as enlargement of blood vessels on the ventral surface of the cerebellum. Vascular injury on the surface of the brain, necrotic cell bodies and small foci of vacuoles in the optic lobe and cerebrum of trout have also been observed with SWCNT (Smith *et al.*, 2007). The functional consequences of changes in the brain for animal behaviour and locomotion remain unclear in this

experiment. However, previous studies on dietary Cu where the appearance of vacuoles and necrotic cells in the brain also occurred (without a measurable increase in brain Cu content) resulted in changes in circulating serotonin/melatonin levels (Handy, 2003) and loss of circadian locomotor activity patterns (Campbell *et al.*, 2002). The latter has potentially severe ecological consequences for a predator that feeds at dawn and dusk, as well as a big impact on their social status and competitiveness within a school of fishes (Campbell *et al.*, 2005). It is therefore a substantial ecological concern that the same brain pathologies are evident for Cu-NPs. Moreover, the quantitative analysis confirmed that the effects of Cu-NPs were greater at day 10 than the equivalent concentration of $CuSO_4$.

The accumulation of new Cu in the brain of fishes was not observed in the present study, and like previous report of Cu in fishes, the effects on the brain are best explained by indirect toxicity on neuro-endocrine function and/or hypoxia (Handy, 2003). Some recent work on rodents has shown that the administration of Ag^-, Cu^-, or Al/Al_2O_3-NPs can cause disruption of the BBB function and produces brain oedema (Sharma *et al.*, 2010). While the possibility of direct Cu or Cu-NP entry into the brain cannot be completely excluded here, systemic hypoxia resulting from damaged gills seems a more likely explanation. The enlarged blood vessels on the surface of the brain are consistent with an attempt to increase blood flow to offset the effects of hypoxia. Cu-NPs and $CuSO_4$ caused some changes in the relative thickness of the layers in the mid-brain. This cannot be due to differential tissue growth over 10 days and is almost certainly related to oedema. The rupture of preventriculare layer points to an enlargement of the adjacent ventricle (with fluid) or inflammation within the cerebral-spinal fluid compartment. The aetiology of Cu and Cu-NP pathology in the brain needs

further investigation, but it is clear from these observations that several physiological dysfunctions (osmotic, metal homeostasis, oxidative stress, vascular) may be contributing to the pathology.

3.4.7 Pathological observation in the muscle

Copper is known to cause a decrease in the size of skeletal muscle fibres with a concomitant increase in extracellular space in the muscles of gold fish (Vogel, 1959), and these observations are also consistent with the findings here for $CuSO_4$ in trout. The functional consequences for trout exposed to $CuSO_4$ are well known and can include spending more time at lower swimming speeds (Campbell *et al.*, 2002; Handy *et al.*, 1999), as well as depletion of muscle glycogen stores (Campbell *et al.*, 2005). These effects usually occur without appreciable increases of new Cu accumulation in the muscle (Handy *et al.*, 1999), implying indirect toxic effects of $CuSO_4$ on muscle function. This was also the case in the present study with no measurable increase in the total Cu concentrations in the muscle from either $CuSO_4$ or Cu-NP exposures. However, the effects of Cu-NPs on muscle have not been previously reported. Exposure to Cu-NPs caused similar types of injuries to $CuSO_4$ including a decrease in the relative proportion of muscle fibre area compared to controls. The quantitative histological analysis of trout muscle fibre proportion confirmed a small but statistically significant material-type effect, with the $CuSO_4$ causing slightly more changes in the muscle structure than the Cu-NPs. This implies the functional consequences for muscle function during Cu-NP exposure may be similar to those known for $CuSO_4$, but probably less severe. However, swimming speed and its relationship with muscle biochemistry needs further investigation with NPs.

Conclusion

In conclusion, this study has demonstrated from the viewpoint of pathology, that the target organs for Cu-NPs are similar to those for $CuSO_4$. It has also demonstrated that the types of pathologies caused by Cu-NPs are similar to those known for Cu metal salts, but there may be a short time delay in the appearance of the Cu-NPs pathologies. There are also some organ-specific material-type effects with Cu-NPs causing more severe injury in the intestine, liver, and brain than the equivalent concentration of $CuSO_4$ by the end of the experiment. However the reverse was true in the gill, spleen and muscle where Cu-NPs are less effective at producing pathology, and little difference between the materials in the kidney. Additionally, the present study also demonstrates that both Cu treatments caused similar alterations in the proportion of blood cells in the spleen, although there were a few material-type effects with metal salt being slightly more hazardous. However, the changes observed in the spleen are mainly within the scope of the physiological capacity of the spleen, and are best explained by systemic hypoxia due to gill injury and/or transient osmoregulatory disturbances (Shaw *et al.*, 2012). Detecting white blood cells, lysozyme, total plasma protein, and total immunoglobulin are still required to reflect the immune response following exposure to these materials. For copper sulphate, it is well known that organ pathology is not necessarily associated with direct Cu accumulation in all the internal organs, because of secondary oxidative stress and hypoxia during the metal exposure. This aspect needs further investigation for Cu-NPs, but like $CuSO_4$, it would be prudent to consider the possibility that some injuries could occur without the presence of exogenous Cu particles or Cu metal in some of the internal organs. Overall, the data of the present

study suggest that the pathology and immunotoxicity hazard to trout from $CuSO_4$ and Cu-NPs are similar at the exposure times and concentrations used here.

Chapter 4

The effects of pH on the uptake and bioavailability of either dissolved copper or copper nanoparticles on rainbow trout (Oncorhynchus mykiss): Physiological effects and organ pathologies

Hypotheses: Low water pH alters the physico-chemical properties of Cu-NPs in a different way to the effects of speciation chemistry on $CuSO_4$; leading to different biological effects of each form of the Cu during response to pH in trout.

Abstract

A few studies on fishes have investigated the interaction between copper toxicity and the pH value of the water, but little is known about the effects of low pH on the toxicity of copper nanoparticles (Cu-NPs). Therefore, this study aimed to describe the sub-lethal toxic effects of Cu-NPs compared to $CuSO_4$ at neutral and acidic pH on the main body system of fishes. Juvenile rainbow trout (*n* =198) were exposed in triplicate (11 fish/tank, 3 tank/treatment) to either a control or 20 µg l^{-1} of either Cu as $CuSO_4$ or Cu-NPs at pH 7 and 5 (mean primary particle size at pH 7, 60 ± 2 nm and pH 5, 54 ± 1 nm) in a semi-static aqueous exposure regime for up to 7 days. Dialysis experiments showed increased release of dissolved Cu from both $CuSO_4$ and Cu-NPs at pH 5 compared to pH 7. Copper accumulation was observed in the gills of trout from the $CuSO_4$ or Cu-NPs at pH 7 and 5, with a greater accumulation from the $CuSO_4$ treatment than Cu-NPs at each pH. The liver showed Cu accumulation with both Cu treatments at pH 7 only, whereas, spleen and kidney did not show measurable accumulation of Cu at any of the water pH values. There were some changes in haematology and depletion of plasma Na^+ at pH 7 and 5 due to Cu exposure, but there was no material- type or pH effects. The branchial Na^+/K^+-ATPase activity was significantly decreased with $CuSO_4$ treatment compared to Cu-NPs and control groups at pH 7 (0.2 fold at day 4) and pH 5 (0.6 fold at day 7). Statistically significant decreases in the Na^+/K^+-ATPase activity was also seen in the liver (with Cu-NPs treatment at pH 5) and kidney (with $CuSO_4$

treatment at pH 7 and pH 5; about 0.6 fold), with a material-type effect. In the liver, thiobarbituric acid reactive substances (TBARS) showed a decrease with the Cu-NP treatment at pH 7 and an increase with $CuSO_4$ treatment at pH 5 compared to the other treatments. Exposure to $CuSO_4$ or Cu-NPs at pH 7 and 5 caused similar types of organ pathologies, but these pathologies were more severe at pH 5 than pH 7. Pathologies include hyperplasia, clubbed tips and aneurism in the gill epithelium; pyknotic nuclei, lipidosis and hepatitis-like injury in the liver; vacuole formation and increased numbers of melanomacrophage deposits in the spleen; degeneration of renal tubules, necrotic haematopoietic cells and enlargement of the Bowman's space in the kidney. Overall, the data from the present study indicates that the particulate form is more toxic at pH 5 than at pH7. There is also a material-type effect on the Cu accumulation in the gills at either pH, but not for the internal organs. Sodium-potassium ATPase activity (in the liver and kidney) and TBARs (in the liver) also showed a material-type effect at different pH values.

4. 1 Introduction

Copper as an essential metal plays an important role in cellular metabolism (Handy, 2003; Taylor *et al.*, 2000), but it can be toxic to freshwater fish, invertebrates and amphibians in low concentration. Copper concentration in freshwater environments ranges from < 1 to 9000 μg l^{-1} (Leland and Kuwabara, 1985), and these ranges are depended on the level of natural and anthropogenic input. The toxicity of Cu to fish is influenced by water chemistry such as alkalinity, hardness, dissolved organic matter and pH (Erickson *et al.*, 1996). The water pH is an important factor in copper (Cu) toxicity and accumulation (Cusimano *et al.*, 1986). Hydrogen ions may compete for uptake with

Cu ions (Laurén and McDonald, 1985), but pH also alters Cu speciation of dissolved metals. For example, at low pH there are more free Cu ions (Cu^{2+} dominates speciation) which are toxic to fishes (Bury and Handy, 2010), whereas at higher pH values, copper hydroxide and other complexes are released as a dominant species (Kamunde and Wood, 2004). Acidic water is known to be toxic (Wood and McDonald, 1982) as well as Cu (Shaw *et al.*, 2012) to fish. Copper and acidic water seem to be similar in the toxicity, but combined Cu and low pH led to a reduction in toxicity. Cusimano *et al.* (1986) confirmed this observation on the effects of Cu in rainbow trout were decreased in acidic pH. The lower toxicity of Cu in acidic water could occur due to the competition of H^+ and Cu^{2+} ions on the same sites in the gill epithelium (Laurén and McDonald, 1985). Acidic water is associated with excessive mucus secretion and a proliferation of cells between gill lamellae (Plonka and Neff, 1969). Waterborne exposure to Cu (Figueiredo-Fernandes *et al.*, 2007) and acidic water (Chevalier *et al.*, 1985) can cause damage to the gill of fish (separation of the epithelial layer from underlying tissue, and hyperplasia in the primary lamellae). Damaging gill tissues at low pH which led in disturbances in ion regulation and gas exchange (Lauren and McDonald, 1985; Wood and McDonald, 1982).

Copper is known to accumulate in fishes. The accumulation of copper in tissues of aquatic animals increased with increasing the free copper ion concentration at acidic water pH values (Campbell and Stokes, 1985; Schubauer-Berigan *et al.*, 1993). Çogun and Kargın (2004) found that the highest levels of Cu were accumulated in the liver, followed by the gills and muscle of *Oreochromis niloticus* following exposure to Cu at pH 5.5, 7.8 and 9.5. These researchers also found the accumulation of Cu in all tissues was higher at pH 5.5 than pH 7.8 and 9.5.

Exposure to Cu is known to produce oxidative stress in fish (Hoyle *et al.*, 2007; Eyckmans *et al.*, 2011; Shaw *et al.*, 2012) that could be associated with ROS production from the metal accumulation (Cu-NPs, Shaw *et al.*, 2012). Systemic hypoxia originating from gill injury could produce secondary ROS leading to oxidative stress. Hoyle *et al.* (2007) found that dietary Cu exposure shows only a small transient osmoregulatory disturbance, but the increased TBARS level indicates some Cu-induced oxidative stress. The disturbance of electrolytes regulatory processes in the gill is the main toxic mechanism of metal and acid stress (Campbell *et al.*, 1999; Wood and McDonald, 1982). The uptake of hydrogen ions from the water inhibits the active uptake of sodium and chloride ions at the gill and stimulates the loss of electrolytes through both passive efflux and changes in the transepithelial potential at the gill membrane (Wood *et al.*, 1988). Nussey *et al.* (2002) showed that exposure of *Oreochromis mossambicus* to copper at neutral and acidic pHs caused physiological stress including haematological and osmotic changes. For example, these researchers found the acidic water caused the opposite result in plasma electrolyte (increased Na^+ concentration) to that induced by the neutral pH (decreased Na^+ concentration). The latter authors also found blood alkalosis and decreases in WBC (due to the bioconcentration of Cu that blocks or inhibits the leucopoietic tissue), RBC counts, haemoglobin, and hematocrit (due to hypoxia, gill injuries, and impaired osmoregulation) at acidic pH Cu concentration (Nussey *et al.*, 2002).

Organ pathologies are known in fish following exposure to Cu. Al-Bairuty *et al.* (2013) found that waterborne exposure to dissolved Cu as $CuSO_4$ for 10 days caused hyperplasia and necrosis in the secondary lamellae of the gills; hepatitis-like injury and a pyknotic nuclei in the liver; degeneration of renal tubules; and renal tubular separation

in the kidney. Necrotic cell and elevated the number of melanomacrophage deposits as well as a decrease in the proportion of red pulp and an increase in the proportion of white pulp were observed in the spleen of trout after 10 days of exposure to dissolved Cu (Al-Bairuty and Handy, unpublished).

Only a few recent studies have assessed the effects of copper nanoparticles (Cu-NPs) on fishes, and details of the mechanisms of toxicity at neutral and acidic pH are currently unclear. It remains to be seen if the pH-dependence of dissolved Cu toxicity will also apply to Cu-NPs. Copper ions (Cu^{2+}) may be released from the nano form of copper at different water pH values, but the toxicity of free Cu ions compared to Cu-NPs has only been reported in a few studies. Shaw *et al.* (2012) found that exposure to 20 and 100 $\mu g\ l^{-1}$ of Cu-NPs for 10 days caused 10 and 19 % mortality in trout respectively. Exposure to 0.25 $mg\ l^{-1}$ of dissolved Cu and either 0.25 or 1.5 $mg\ l^{-1}$ of Cu-NPs for 48 hours showed similar effects included inhibition of the branchial Na^+/K^+-ATPase activity and gill injury (Griffitt *et al.*, 2007). In rainbow trout, waterborne exposure to $CuSO_4$ or Cu-NPs caused similar effects, both showed Cu metal accumulation in the gills, transient changes in haematology and depletion of plasma Na^+, disturbances to tissue ion levels and decreased branchial Na^+/K^+-ATPase activity (Shaw *et al.*, 2012) as well as organ pathologies (Al-Bairuty *et al.*, 2013; Al-Bairuty and Handy, unpublished).

The overall aim of the present study was to determine whether or not the toxicity of Cu-NPs was affected by lowering the water pH, and to compare this with the effect of $CuSO_4$. Much research on the toxicity or accumulation of metals with pH change focused on pH values ranging from 4 to 7 in water, because of concern over the effects on acid rain on metal bioavilability (Campbell and Stockes, 1985). Whereas, another

investigated has determined the combined effect of Cu and pH 5 on antioxidant defences and biochemical parameters in fish (Garcia Sampaio *et al.*, 2008). Therefore, two values of pH (pH 7 and 5) were selected during this experiment in order to investigate the effect of these pH values on the toxicity of Cu-NPs compared to $CuSO_4$ and to investigate if acid pH values caused greater effects than neutral pH values on the dissolution of Cu from Cu-NPs form. The 20 μg l^{-1} of either $CuSO_4$ or Cu-NPs is selected as a sub-lethal concentration that allows both NP and dissolved Cu to be studied (Shaw *et al.*, 2012). Related to our previous work on the effects of $CuSO_4$ or Cu-NPs on fish (*e.g.*, Shaw *et al.*, 2012; Al-Bairuty *et al.*, 2013; Al-Bairuty and Handy, unpublished), a body systems physiological approach was used which estimated haematology and plasma ions, disturbances associated with osmoregulation (profiles for trace elements of the internal organs, and Na^+/K^+-ATPase activity) and oxidative stress (thiobarbituric acid reactive substances (TBARS), tissue glutathione content) as well as organs pathologies.

4.2 Materials and methods

4.2.1 Experimental design

Juvenile rainbow trout (*n* = 198) were obtained from Torre Fisheries Ltd, Watchet, Somerset, UK, and held for 4 weeks in a stock aquaria containing, aerated, dechlorinated Plymouth tap water (see below). Stock animals were fed to satiation on a commercial trout diet (EWOS, Westfield, UK; 2-3 mm pellets). This feed contained raw material (100 – 40 % inclusion) which was composed of fish oil, wheat, soya bean meal, beans, wheat gluten, sunflower meal, vitamins, minerals, copper (cupric sulphate 8 mg kg^{-1}) and ethoxyquin (a permitted antioxidant).

Fish weighing 27.4 g ± 0.9 and of total length (13.8 cm ± 0.1, mean ± S.E.M., *n* = 198) were used for the experiment. After morphometrics, fish were then transferred into nine aerated experimental glass aquaria containing 20 l of declorinated Plymouth tap water (11 fish/tank), in a triplicate design (3 tank/treatment), and acclimated for 24 h prior to the commencement of each pH experiment. Dechlorinated Plymouth tap water is used to do this experiment as described in the methodology chapter (see section 2.1). The stock water was adjusted to either pH 5 or pH 7. The lower pH was adjusted every 4 hours by adding several drops of 0.5 molar H_2SO_4 during the 12 hours exposure. At each pH value, fish were exposed in triplicate to one of the following treatments for up to 7 days using a semi-static exposure regime (80 % water change every 12 h with re-dosing after each change, twice/day): control (no added Cu), 20 μg l^{-1} of Cu as $CuSO_4$ or copper nanoparticles (Cu-NPs). The Cu concentration was selected after considering the concentration used to produce physiological effects (Shaw *et al.*, 2012) and organ injuries (Al-Bairuty *et al.*, 2013) as explained in chapter 3.

Fish were not fed 24 h prior to, or during the experiment in order to minimise the risk of the Cu-NPs absorbing to the food or faecal material, and to help maintain water quality. Water samples were collected before and after each water change for measurement of pH, hardness (HANNA, HI991301, UK), temperature, saturated oxygen (HACH HQ40d multi reader), total ammonia (HI95715, Hanna Instruments). There were no treatment differences in water quality between tanks (ANOVA, $P > 0.05$). Data were (mean ± S.E.M., *n* = 576 samples), temperature, 15.7 ± 0.2; total hardness (ppt), 0.12 ± 0.00; oxygen saturation %, 96 ± 0.5; total ammonia (mg l^{-1}), 0.97 ± 0.05. Photoperiod was 12 h light: 12 h dark. The electrolyte composition of the dechlorinated Plymouth tap water used in this experiment was measured by ICP-OES as

described in Chapter 2. Values were 17.86, 1.67, 9.90 mg l^{-1} for Ca^{+2}, K^{+} and Na^{+} respectively (0.45, 0.04, and 0.43 mmol l^{-1}). Background total Cu concentrations in the tank water were 2.94 ± 0.28 μg l^{-1} (0.046 ± 0.004 μmol l^{-1}). Fish were randomly sampled on day 0 (initial fish from the stock), day 4 and 7 for haematology and plasma ions, tissue electrolytes, as well as biochemistry. Samples for histopathology were collected at the end of the experiment (day 7) from all treatments.

4.2.2 Copper nanoparticles stock solution and dosing

The copper nanoparticles used here are from the same batch that has been previously reported in Shaw *et al.* (2012). Briefly, the powdered form of Cu-NPs was obtained from Sigma-Aldrich and had (manufacturer's information) an average particle size < 50 nm and 99.9 % purity. A fresh 1 litre stock solution of 1 g l^{-1} Cu-NPs was made twice times daily at 9 am and 9 pm by dispersing the NPs in ultrapure (Milli-Q) water without solvents and stirring (magnetic stirrer IKA Werke RET basic C, at 300 rpm) for 12 h in a low-density polyethylene (LDPE) plastic bottle. The LDPE plastic bottle was used rather than glass bottle when preparing the stock of Cu-NP in order to prevent the adhesion of the NPs to the glass and to make NP more stable in solution. This stock was then used to dose the fish tank following the water change. Samples were taken from the stock for analysis of total Cu concentration by ICP-OES (10 ml), mean aggregates by NanoSight LM 10 (10 ml), and primary particle size by transmission electron microscopy (TEM, JEOL 1200EXII, 1 ml). The remaining stock was discarded. TEM analysis of 1 g l^{-1} of stock suspensions showed a crystalline structure of particles (between rod and hexagonal shape) with a primary particle diameter of 61 ± 4 nm, 60 ± 2 nm and 54 ± 1 in stock with Milli-Q water dispersion,

buffered the stock at pH 7 and 5, respectively (mean ± S.E.M, *n* = 68 to 123, Fig 4.1). Nanoparticles tracking analysis (NTA, NanoSight LM10, Nanosight, Salisbury, UK, laser output set at 30 mW at 640 nm) was a second approach used to detect the mean aggregate size of Cu-NPs in 1 g l^{-1} of stock solution from the stock dispersion, buffered the stock at pH 7 and 5. This approach uses laser light scattering to visualise individual particles and tracks their Brownian motion. This technique has been used previously by Shaw *et al.* (2012) to characterise particle dispersions (*e.g.*, for Cu-NPs). A mean aggregate size of the NP stock dispersion in Milli-Q water was 138 ± 16 nm with a mode of 114 nm; stock buffered at pH 7, 143 ± 3 nm with a mode of 136 nm, and stock buffered at pH 5, 114 ± 11 nm with a mode of 97 nm.

A 1 g l^{-1} Cu metal stock solution was prepared by dissolving 3.929 g from $CuSO_4.5H_2O$ (Sigma-Aldrich) in 1 l of ultrapure (Milli-Q) water with stirring for 30 mins (magnetic stirrer IKA Werke RET basic C, at 300 rpm). The stock solution was used throughout the 7 days of exposure to dose the tanks. Dosing of all treatments was carried out at 9 pm following the water change and again at 9 am the following morning after a subsequent water change.

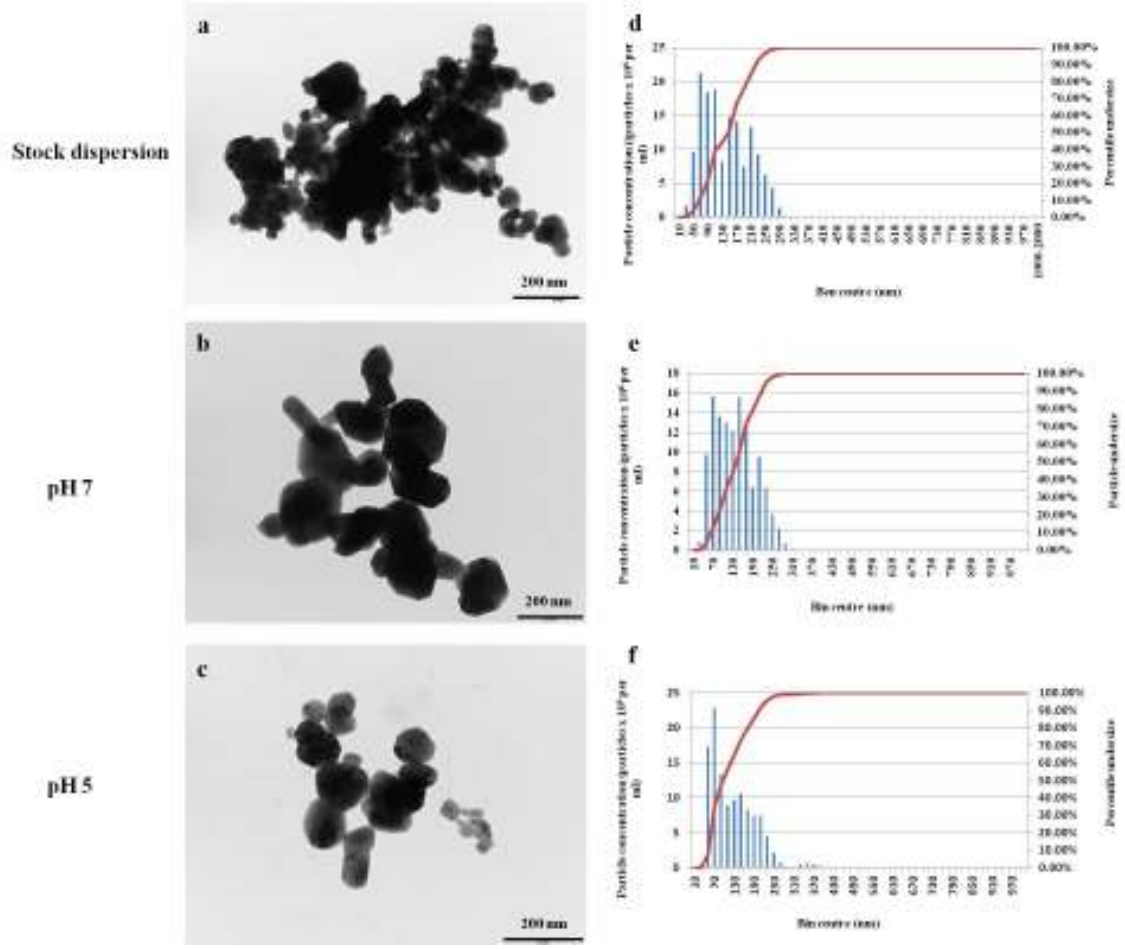

Figure 4.1 Primary particle size and particle size distribution as well as aggregation of 1 g l^{-1} Cu-NPs stock. Panels a, b and c are electron micrograph showing Cu-NPs with a mean primary particle diameter of 61 ± 4 from stock dispersion in MilliQ-water; 60 ± 2 in stock buffered at pH 7 and 54 ± 1 nm in stock buffered atpH 5 (mean ± S.E.M, n = 50 to 123). Panels d, e and f showed particle size distributions (NanoSight LM_{10}) with the concentration of particles at each size (vertical bars) and the percent of the total particles at each size (line). A mean aggregate size of NPs at the pH of MilliQ water was 138 ± 16 nm with a mode of 114 nm; pH 7, 143 ± 3 nm with a mode of 136 nm; pH 5, 114 ± 11 nm with a mode of 97 nm.

4.2.3 Dialysis experiment

The dialysis experiment (Fig. 4.2) was done to measure the dissolution rate of the Cu from the Cu-NPs or $CuSO_4$ in dechlorinated Plymouth tap water (left to stand in aeration for 24 h before use) and buffered at two values of pH (7 & 5). Briefly, 100 mg l^{-1} of metal as $CuSO_4$ or Cu-NPs solutions were prepared in Milli-Q water. All

glassware and equipment were acid washed (5 % nitric acid) and triple rinsed in deionised (Milli-Q) water. The dialysis method is based on Handy *et al.* (1989) with modifications for NMs. Briefly, 8 ml of 100 mg l^{-1} of Cu as $CuSO_4$ or Cu-NPs stock solutions or control (same external solution) were filled into dialysis bags which were made from 7 cm long x 25 mm wide dialysis tubing (product code: D9777, cellulose membrane with molecular weight cut off at 12,000 Da, Sigma-Aldrich, St. Louis, USA) and both ends of the dialysis bag secured with Medi-clips to prevent any leakage. The bags were then immediately placed in a beaker containing 492 ml of the appropriate solution (bringing the total volume to 500 ml). Care was taken to use beakers of identical shape/size for the experiments, and each experiment was performed in triplicate (3 separate beakers) for each material and external solution (Plymouth tap water pH 7 and pH 5). The solutions in the beakers were gently agitated with a multipoint magnetic stirrer (RO 15P power, Ika-Werke GmbH & Co. KG, Staufen, Germany) for 24 h at room temperature. Samples of the external solution (4.5 ml) were taken from each beaker at 0, 0.5, 1, 2, 3, 4, 6, 8 and 24 h, the pH value of the samples was recorded for each time point. At the end of the experiment, the remaining contents of the dialysis bag were also collected. Samples were analysed by ICP-OES for total Cu concentrations.

4.2.4 Haematology and blood plasma analysis

Blood is well-known as a useful diagnostic tool in veterinary and animal health sciences (Houston, 1990) and is the most accessible component of the vertebrate body fluid system (Houston, 1997). Two fish were randomly collected from each tank (six fish/treatment and initial fish) at day 4 and 7 at each pH experiment and carefully

anaesthetised with buffered MS222. Whole blood was firstly collected via the caudal blood vessel into heparinised (lithium heparin) syringes, and then the fish weighed and the total length was recorded before dissection. Haematocrit (packed cell volume, Hct) and haemoglobin (Hb) were determined immediately. Haematocrit of whole blood was determined as described in Handy and Depledge (1999). Briefly, Hct was calculated in duplicate by centrifuging whole blood in microhaematocrit tubes (13,000 rpm, 2 min, Heraeus pico 17 microcentrifuge) and calculating the ratio of packed red cells to supernatant by using Hawksley reader (Hawksley, Sussex, UK).

Haemoglobin content was determined by using the cyanmethaemoglobin method (Dacie and Lewis, 1995). Briefly, Drabkin's reagent were prepared (1.0 g sodium bicarbonate, 50 mg potassium cyanide, 200 mg potassium ferricynide and then add 1 litre of distilled water) and stored in borosilicate glass bottle for later use. Haemoglobin assay was performed in test tubes where 20 µl of freshly collected blood was mixed with 5 ml of Drabkin's reagent (diluents). The solution was inverted several times before using. Absorbance was measured using a spectrophotometer (Jenway 6300 Spectrophotometer) at 540 nm. The value of haemoglobin content was calculated against a standard calibration curve.

For counting the red and white blood cell, 20 µl from whole blood was fixed in 0.98 ml of Dacie's fluid (10 ml of 40% formaldehyde, 31.3 g trisodium citrate, 1.0 g brilliant crystal blue, diluted to 1 litre with distilled water). This provides a 1:50 dilution of the blood sample. Cell counts were performed on a Neubauer haemocytometer. The remaining blood was centrifuged (13,000 rpm for 2 min, Micro Centaur MSE), and serum collected and stored at -80 °C until subsequent analysis of plasma ions and osmometry. Briefly, serum Na^+ and K^+ were analysed by flame photometry (Corning

420 Flame photometer) while serum chloride analysed by automated titration (Jenway PCLM 3 Chloride Meter). For serum Na^+ and K^+, the aliquots homogenate sample was diluted as 1:200 (20 µl sample + 3.98 ml Milli-Q water) and then prepared standards for flame photometry (in mmol l^{-1}, 140 Na^+ and 5 K^+). Osmotic pressure was determined by the freezing-point depression method using 50 µl of plasma (Precision System micro osmometer, Natick, Massachusetts, USA).

4.2.5 Tissue ion analysis

Following blood sampling, fish were terminally anaesthetised with MS222 and dissected for tissue metal analysis. Gill, liver, spleen and kidney were harvested and washed with Milli-Q water and processed for ion analysis as described in chapter 2. Spike recovery tests were performed with both $CuSO_4$ and Cu-NPs using rainbow trout gill and liver as mentioned in Chapter 2. Recovery values were good in all tissues measured. Values were (% mean ± S.E.M, n = 6) 94 ± 1 (Cu-NPs) and 102 ± 1 ($CuSO_4$) in the gill tissues and 92 ± 2 (Cu-NPs) and 102 ± 1 ($CuSO_4$) in the liver.

4.2.6 Biochemistry

Samples (gill, liver, kidney and spleen) were collected and stored at -80 ^oC in order to assay for biochemical indicators of osmoregulatory disturbances or oxidative stress.

4.2.6.1 Sample collection and homogenisation

Fish tissues (gill, liver, kidney, and spleen) were harvested and immediately snap frozen in liquid nitrogen and stored at -80 °C until required. Tissues about 0.5 g were weighed and homogenised (3 x 10 s with 2 min rest at 17,500 rpm, Cat X520D

with a T6 shaft, medium speed, Bennett & Co, Weston-super-Mare) in five volumes (2.5 ml) of ice-cold isotonic buffer solution (in mmol l^{-1}; 300 sucrose, 0.1 ethylenediamine tetra acetic acid (EDTA), 20 HEPES [4-(2-hydroxyethyl) piperazine-1-ethane sulfonic acid], adjusted to pH 7.8 with a few drops of Tris (2-amino-2-hydroxyl-1,3-propanediol)). Crude homogenates were centrifuged for 2 min (13.000 rpm at 4 ºC) after which the supernatant was stored in 0.5 ml aliquots at -80 °C until required.

4.2.6.2 Determination of total protein

A Biorad Bradford assay was used to determine the total protein content of the tissue homogenates by a modification of the method of Bradford (1976). However, this assay was used in order to normalise enzyme activity for further biochemical analyses. Briefly, serial dilution of bovine serum albumin (BSA) standard solution was prepared (1.6-0.05 mg l^{-1}). The supernatant of homogenate tissues was diluted as 1: 10 (*e.g.*, 10 µl sample + 90 µl Milli-Q water) in Eppendorf tubes, and vortexed to ensure rapid mixing. After that 10 µl of blank, standard, and samples were dispensed to a 96 well microplate and then 200 µl of the Bradford reagent was added to each well followed by shaking. The plate was allowed to incubate for 5 minutes before reading absorbances at 595 nm using a plate reader (VERSA MAX plate reader, Molecular Devices (UK) Ltd, Wokingham, UK).

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

The Na^+/K^+-ATPase assay was performed according to Bouskill *et al.* (2006) with minor modification. Briefly, 15 µl, in triplicate from each sample (gill, liver, and kidney) were dispensed into 1.5 ml Eppendorf tubes containing either 400 µl of a K^+-containing medium (in mmol l^{-1}, 100 NaCl, 10 KCl, 5 $MgCl_2$, 1.25 Na_2ATP, 30 HEPES,

adjusted to pH 7.4 with 2 M Trisma Base) or a K^+-free medium (as above, but replacing KCl with 1.0 mmol l^{-1} ouabain, a sodium pump inhibitor), then incubated at 37 °C for 10 minutes. The reaction was stopped by adding 1 ml of ice-cold 8.6 % trichloroacetic acid (TCA). Samples Eppendorf were spin at 13,000 rpm for 2 mins to remove protein debris. After that, the supernatants (140 µl) were drawn from each labelled Eppendorf tubes (Blank, K^+- containing buffer or K^+- free buffer; standard, 0-2.0 mmol l^{-1} potassium phosphate, and samples) and added into a 96 well microplate. Then, 100 µl of freshly prepared colour reagent (9.6%, w/v $FeSO_4.6H_2O$; 1.15%, w/v ammonium heptamolybdate dissolved in 0.66 M H_2SO_4) was added to each well for measuring the free phosphate. The colour was allowed to develop for 20-30 minutes at room temperature. Absorbance was measured at 660 nm (VERSA MAX plate reader, Molecular Devices) against 0 to 2.0 mmol l^{-1} phosphate standards. The Na^+/K^+-ATPase activity is determined as the difference in inorganic phosphate released from Na_2ATP hydrolysis by the enzyme in the presence and absence of potassium (K^+-free with Ouabain as a sodium pump inhibitor), corrected for spontaneous non-enzymatic breakdown of ATP (in a sample blank).

4.2.6.4 Thiobarbituric acid reactive substances (TBARS)

The TBARS assay was performed according to Camejo *et al.* (1998), and used to determine the total lipid peroxidation products. Briefly, 40 µl aliquots of homogenate, standard or blank was added in triplicate to a labelled Eppendorf tube containing 10 µl of 1 mmol l^{-1} butylated hydroxytoluene (2,6-Di-O-tert-butyl-4-methylphenol or BHT) to stop any undesired further oxidation of samples. The final volume was made up to 190 µl by adding 140 µl of 0.1 M phosphate buffered saline (adjusted to pH 7.4). Following

this, 50 µl of 50 % (w/v) trichloroacetic acid (TCA) and 75 µl of 1.3 % (w/v) thiobarbituric acid (TBA, dissolved in 0.3 % (w/v) NaOH) were added to each tube. The eppendrof tubes were incubated at 60 °C for 60 minutes and then spin for 2 minutes at full speed (13.000 rpm). A 300 µl from each sample and standard were pipetted out and dispensed into 96-well microplate. Absorbances were read at 530 nm using a microplate reader (VERSA MAX plate reader, Molecular Devices) against 1,1,3,3-tetraethoxypropane standards (0.5 – 25 µmol l^{-1} and expressed per mg of protein in the initial volume of homogenate added).

4.2.6.5 Total glutathione

Glutathione (GSH) is a good indicator of oxidative stress, potentially leading to apoptosis or cell death. The total glutathione (*i.e.* reduced: GSH, and oxidised: GSSG) content in the aliquot homogenate samples (gill, liver, kidney, and spleen) were performed as describe in Al-Subiai *et al.* (2009). Briefly, samples were treated with dithionitrobenzoic acid (DTNB) by mixing them at a 1:1 ratio with buffered DTNB (10 mM DTNB in 100 mM potassium phosphate, pH 7.5, containing 5 mM EDTA) in triplicate. One hundred millimolar potassium phosphates, pH 7.5, containing 5 mM EDTA and glutathione reductase (0.6 U, Sigma G-3664 from *Saccharomyces cerevisiae*) and 40 µl of DTNB-treated samples were mixed in a 96-well microplate. After 1 min of equilibration the reaction was started by the addition of 60 µl of 1 mM NADPH. The rate of absorbance change at 412 nm was recorded over 5 minutes using a microplate reader (VERSA MAX plate reader, Molecular Devices). The total glutathione content (GSH/GSSG) (µmol g^{-1} wet weight tissue) was determined using a standard calibration curve (serial dilution of 40 µmol l^{-1} reduced glutathione solution).

4.2.7 Determination of histopathological changes

Gill, liver, spleen and kidney were dissected out at day 7 for histopathological examination as described in detail in Chapter 2 (see 2.5 - 2.9).

4.2.8 Statistical analysis

The resulting data were analysed by StatGraphics Plus version 5.1 and graphs were drawn using an Excel spreadsheet as described in Chapter 2 (see 2.11). Throughout the experiment, no tank effects were seen, so data were pooled by treatment for statistical analysis. Briefly, parametric data was analysed for treatment, pH, time, treatment x time x pH, treatment x time, treatment x pH, and pH x time interactions by General linear model. When a statistical significant effect was observed by this model a one way ANOVA (or equivalent non-parametric test) was used to assess for simple effects. The Student's *t*-test was sometimes used to investigate the differences between pairs of data, where appropriate. Results were presented as mean ± S.E.M. All statistical analysis used the default 5 % rejection level.

4.3. Results

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

Water samples were taken within 10 minutes following the dosing of the experimental tanks and total Cu metal concentrations were confirmed by ICP-OES. The total Cu concentrations were (mean ± S.E.M., n = 12 water samples per treatment) 2.94 ± 0.05, 20.36 ± 0.27, 19.42 ± 0.12 μg l^{-1} at pH 7 and 3.23 ± 0.12, 20.53 ± 0.08, 19.62 ± 0.21 μg l^{-1} at pH 5 (for the control, 20 μg l^{-1} of either Cu as $CuSO_4$ or Cu-NPs treatments respectively), representing recoveries of 101.8 and 97.1 % at pH 7 and 102.6

and 98.1 % at pH 5 of the nominal concentration of $CuSO_4$ or Cu-NPs respectively. Mean recoveries taken over the 12 hour exposure period between water changes were (data; mean %, *n* = 12 samples per treatment) 85.7 and 77.7 % at pH 7 and 88.6 and 83.8 % at pH 5 for $CuSO_4$ and Cu-NPs treatments respectively. Analysis of Cu-NPs stocks used to dose the fish showed that values never deviated by more than 3 % of the nominal concentration in the stock (mean values 97.5 % of nominal concentration).

Dialysis experiments were performed to determine the release of dissolved Cu from $CuSO_4$ or Cu-NPs in the dechlorinated Plymouth tap water at pH 7 and 5. The result of this experiment showed the maximum release of Cu from $CuSO_4$ treatment into the external media was 73 $\mu g\ l^{-1}$ at pH 7 and 61 $\mu g\ l^{-1}$ at pH 5 (Fig. 4.2). Whereas, the Cu-NPs treatment showed a maximum total dissolved Cu in the beakers of 57 $\mu g\ l^{-1}$ at pH 7 and 165 $\mu g\ l^{-1}$ at pH 5 (Fig. 4.2), indicating that the particles showed more dissolution of Cu from Cu-NP at pH 5 than at pH 7. Calculated cumulative Cu releases over 24 h at pH 7 were < 0.02 (value below the detection in controls), 0.615 and 0.468 mg of Cu metal (mean of triplicate measurements, absolute amount release) in the control, $CuSO_4$ and Cu-NPs, respectively. At pH 5, the cumulative values of Cu metal were < 0.04 (below the detection), 0.483 and 0.438 mg in control, $CuSO_4$ and Cu-NPs, respectively. The maximum dissolution rate of Cu (maximum slope, when the dialysis was not at equilibrium, time = 1 h) at pH 7, was 1.22 and 0.93 $\mu g\ min^{-1}$ for $CuSO_4$ and Cu-NPs respectively. Whereas the maximum slope values at pH 5 were 0.95 and 0.37 $\mu g\ min^{-1}$ for $CuSO_4$ and Cu-NPs respectively.

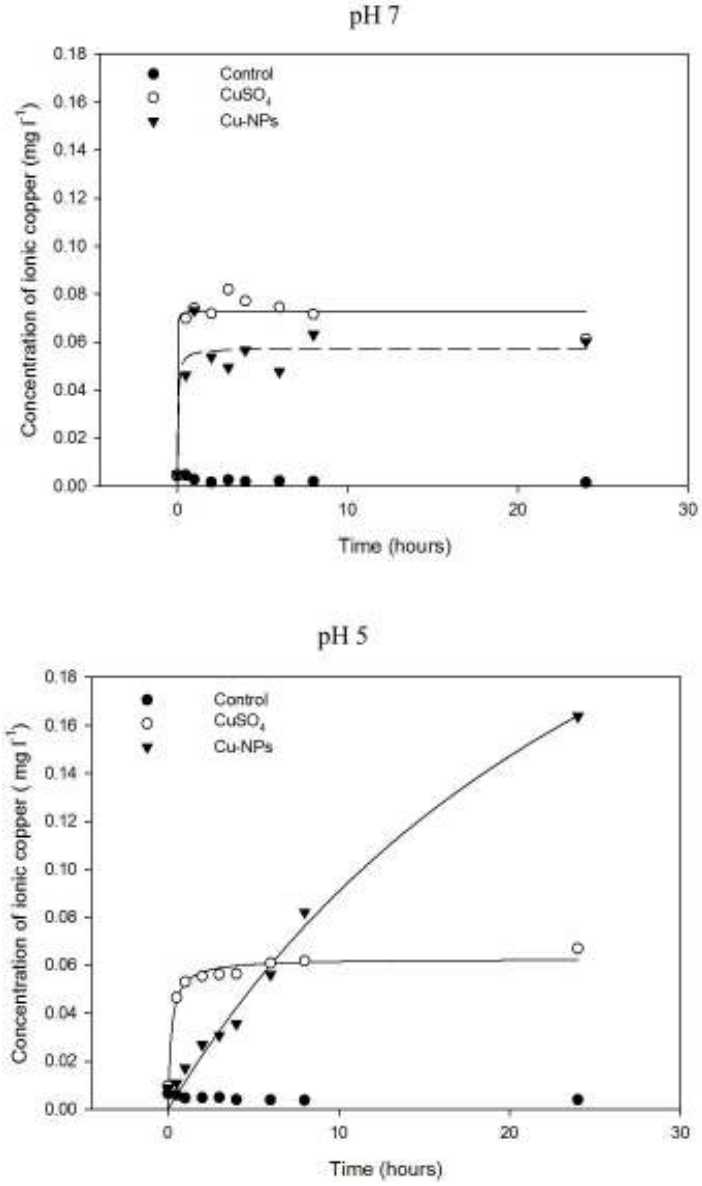

Figure 4.2 Results from the dialysis experiment showed the dissolution of total Cu concentration of control (Plymouth tap water) or either $CuSO_4$ or Cu-NPs in the dechlorinated Plymouth tap water at pH 7 and 5 (same water used in the experimental tanks) over 24 h. Data are means ± S.E.M, n = 3 replicates. Curves for dissolved Cu concentration that were fitted by using a rectangular hyperbole function (one site, ligand to saturation) in SigmaPlot version 12.2. At pH 7, the equation for $CuSO_4$: y = 0.077*x/(0.005+x), r^2 = 0.77, and for Cu-NPs: 0.057*x/(0.039+x), r^2 = 0.61. At pH 5, the equation for $CuSO_4$: y = 0.063*x/(0.195+x), r^2 = 0.91 and for Cu-NPs: y = 0.387*x/(32.583+x), r^2 = 0.99. The controls were at or below the detection limits, and therefore the curve is not fitted, but data points showed for convenience. The Cu-NP treatment showed more dissolution of Cu from Cu-NP form at pH 5 than pH 7.

Figure 4.3 shows copper accumulation in the gills of trout with either dissolved Cu as $CuSO_4$ or the Cu-NPs treatments at day 4 and 7; and at both pH 7 and 5. A pH effect was observed for branchial Cu accumulation from the $CuSO_4$ treatment which showed less accumulation of Cu at pH 5 compared to pH 7 (statistically significant, ANOVA, $P < 0.05$). Nano copper treated did not show pH effects. Both Cu treatments showed a statistically significant increase in Cu accumulation in the gill compared to the control, with a material-type effect (greater accumulation with $CuSO_4$ treatment than Cu-NPs, ANOVA, $P < 0.05$, Fig. 4.3). The liver also showed elevation of Cu concentrations with both Cu treatments at day 4, and only $CuSO_4$ treated at day 7 showed Cu accumulation when compared to controls of pH 7 (all statistically significant, ANOVA, $P < 0.05$, Fig. 4.3). At pH 5, the liver of trout exposed to $CuSO_4$ for 7 days showed a decrease in the Cu levels compared to the pH 7 (statistically significant, ANOVA, $P < 0.05$, Fig. 4.3). The material-type effect at pH 7 showed a decrease in the Cu accumulation with NP treatment at day 7 compared to the $CuSO_4$ (Student's *t*-test, $P < 0.05$, Fig. 4.3). In the spleen, no statistically significant Cu accumulates was observed from any Cu treatments when compared to the control (ANOVA, $P > 0.05$, Fig. 4.3). A decrease in the Cu concentration was observed in the spleen at pH 5 with both Cu treatments when compared to the pH 7 (pH effects, ANOVA, $P < 0.05$, Fig. 4.3). There was also a time effect on the spleen of NP treatment at pH 7 which showed more decline in the Cu levels at day 7 when compared to day 4 (Fig. 4.3). No treatment effect was observed in the kidney (Fig. 4.3). The kidney showed a pH effect with NP treatment at day 4 which caused a decrease in the Cu accumulation at pH 5 compared to the pH 7 (Fig. 4.3). The kidney also showed a time effect of NP treatment, with Cu increases over time (Fig. 4.3).

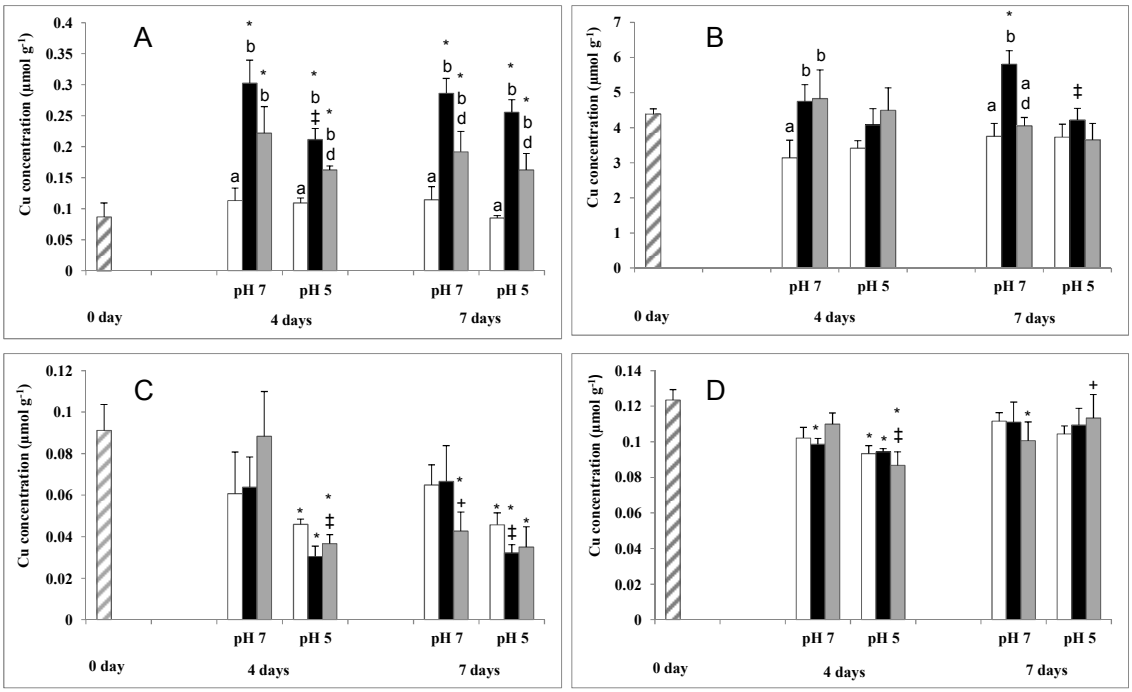

Figure 4.3 Cu concentrations in (A) gills, (B) liver, (C) spleen and (D) kidney of trout after exposure to control (white bars), 20 µg l^{-1} of either $CuSO_4$ (black bars) or Cu-NPs (gray bars) for 4 and 7 days at pH7 and 5. Diagonal hatched bars are initial (day 0) fish. Data are means ± S.E.M., dry weight tissue, n = 6 fish/treatment. (*) indicates a statistically significant different from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). Different letter (a & b) denotes a statistically significant difference between treatment within pH and time point (ANOVA, $P < 0.05$). (‡), indicates a statistically significant difference between pH 7 and 5 within a treatment at each time point (pH effects, ANOVA, $P < 0.05$). (+), indicates a statistically significant difference between day 4 and 7 within treatment and pH (time effects, ANOVA, $P < 0.05$). (d), indicates a statistically significant different between $CuSO_4$ and Cu-NPs within pH and time point (ANOVA, $P < 0.05$). Note: the gill and liver showed Cu accumulation whereas, the spleen and kidney did not show Cu accumulation.

4.3.2 Haematology and blood plasma analysis

Exposure of trout to Cu as $CuSO_4$ or Cu-NPs at neutral and acidic pH values caused a statistically significant change in some blood parameters (Table 4.1). The exposure to $CuSO_4$ showed a pH effect that exhibited an elevation in the haemoglobin levels, haematocrit values, red blood cell and white blood cell count at pH 5 compared

to the pH 7 (all statistically significant, ANOVA, $P < 0.05$; Table 4.1). Nano Cu treatment also showed pH effects which include a statistically significant increase in the haematocrit and white blood cells counts at pH 5 compared to the pH 7 (ANOVA, $P < 0.05$; Table 4.1). There was a material- type effect with a statistically significant increase in the haemoglobin, haematocrit and white blood cells of Cu-NP treatment compared to $CuSO_4$ (Table 4.1). A time effect showed a statistically significant increase in the haemoglobin, haematocrit and white blood cells at day 7 compared to day 4 of $CuSO_4$ treated (Table 4.1). Whereas, the nano Cu treatment showed a decrease in the haemoglobin levels and an increase in the white blood cells at day 7 when compared to the day 4.

Blood plasma electrolytes showed pH, treatment and time effects (Table 4.1). Exposure to $CuSO_4$ at pH 7, fish showed a statistically significant decrease in plasma Na^+ throughout the experiment compared to controls (ANOVA, $P < 0.05$). At pH 5, exposure to $CuSO_4$ caused a transient decrease in plasma Na^+ only at day 4 (statistically significant, ANOVA, $P < 0.05$). The pH- effect of Cu-NPs on plasma Na^+ was similar to that of $CuSO_4$, with more depletion at pH 5 compared to the pH 7 (Table 4.1). There was a material-type effect with a statistically significant decreased of plasma Na^+ in $CuSO_4$ treatment when compared to the Cu-NPs (Table 4.1). Time effects showed more depletion in the value of plasma Na^+ at day 7 compared to the day 4 with Cu treatment. Whereas, nano Cu showed transient changes in the plasma Na^+ at day 7 compared to the day 4. Plasma K^+ showed a small but statistically significant decrease in $CuSO_4$ treatment at pH 7 compared to the control (ANOVA, $P < 0.05$, Table 4.1). Exposure to Cu-NPs at pH 7 showed similar to that observed with $CuSO_4$ treatment which includes a decrease in the K^+ levels compared to controls (ANOVA, $P < 0.05$). At pH 5, exposure

to Cu-NPs showed a small increase in the plasma K^+ compared to the control (ANOVA, $P < 0.05$). The plasma K^+ levels showed a pH-effect with more elevation at acidic pH of both $CuSO_4$ and Cu-NPs treatments than neutral pH. Time- effect showed a small but statistically significant increase in the plasma K^+ at day 7 of both Cu treatments when compared to the day 4 (ANOVA, $P < 0.05$, Table 4.1). At day 4, the plasma Cl^- showed a decrease with $CuSO_4$ treatment at pH 5 when compared to the control. Exposure to Cu-NPs for 7 days at pH 7 showed similar to that of $CuSO_4$ which showed a decrease in plasma Cl^- compared to controls (Table 4.1). The pH-effect showed more depletion in the plasma Cl^- with both Cu treatments at pH 5 compared to the pH 7. The time-effect showed a statistically significant increase in the plasma Cl^- at day 7 of $CuSO_4$ compared to the day 4 (ANOVA, $P < 0.05$, Table 4.1). There were no treatment or pH effects on plasma osmolarity (Table 4.1).

Table 4.1 Haematology and plasma ions in rainbow trout exposed to control (no added Cu or Cu-NPs), and 20 µg l^{-1} of either $CuSO_4$ or Cu-NPs at two values of pH (7 and 5).

Parameters	Stock fish at day zero	Time /day	Control		20 µg l^{-1} $CuSO_4$		20 µg l^{-1} Cu-NPs	
			pH 7	pH5	pH 7	pH5	pH 7	pH5
Haemoglobin (mg dl^{-1})	17.4 ± 0.5	4	19.8 ± 0.8 ^{a}	16.6 ± 0.9 ^{a}	13.9 ± 0.5 ^{*b}	17.7 ± 0.9 ^{a‡}	18.8 ± 0.6 ^{ad}	20.5 ± 1.5 ^{*bd}
		7	19.3 ± 1.0	14.9 ± 1.2 ^{a‡}	16.9 ± 0.9 $^{+}$	19.0 ± 0.9 ^{b}	16.9 ± 1.2	18.5 ± 0.9 ^{b}
Haematocrit (%)	20.3 ± 0.8	4	23.3 ± 1.0 ^{a}	26.3 ± 1.0 *	19.0 ± 0.4 ^{b}	27.7 ± 1.2 *‡	23.7 ± 0.7 ^{*ad}	25.5 ± 0.3 *
		7	23.2 ± 1.3 ^{a}	24.7 ± 1.7 *	25.7 ± 0.5 $^{*b+}$	25.7 ± 1.3 *	21.2 ± 0.9 $^{a+d}$	25.2 ± 1.8 *‡
RBC cell count (cell * 10^6)	0.6 ± 0.0	4	0.7 ± 0.0 ^{*a}	0.7 ± 0.0 *	0.5 ± 0.0 ^{b}	0.6 ± 0.0 ‡	0.6 ± 0.0 ^{b}	0.7 ± 0.0 *
		7	0.7 ± 0.0 ^{*a}	0.6 ±0.0 ^{a‡}	0.6 ± 0.0 ^{b‡}	0.7 ± 0.0 ^{*b}	0.6 ± 0.0 ^{b}	0.7 ± 0.0 ^{*b}
White blood cell count (cells * 10^3)	15.6 ± 0.8	4	18.3 ± 1.9	25.3 ± 3.4 ^{*a‡}	17.3 ± 0.7	17.2 ± 0.7 ^{b}	15.8 ± 0.9	22.2 ± 2.4 ^{*a‡d}
		7	13.0 ± 0.2 ^{a‡}	28.5 ± 2.1 ^{*a‡}	17.2 ± 0.8 ^{b}	35.0 ± 2.7 $^{*b+‡}$	15.5 ± 0.5 ^{*a}	34.7 ± 0.8 $^{*b+‡}$
Plasma Na^+ (mmol l^{-1})	155.0 ± 0.5	4	148.8 ± 1.7 ^{a}	139.9 ± 2.9 ^{*a‡}	137.9 ± 1.6 ^{*b}	133.6 ± 0.6 ^{*b}	135.4 ± 1.7 ^{*b}	133.1 ± 1.9 ^{*b}
		7	151.6 ± 2.6 ^{a}	126.7 ± 1.6 $^{*+‡}$	130.0 ± 2.4 $^{*b+}$	126.6 ± 1.1 $^{*+}$	142.5 ± 4.5 $^{*bd+}$	126.9 ± 2.9 $^{*+‡}$
Plasma K^+ (mmol l^{-1})	3.0 ± 0.0	4	2.9 ± 0.1 ^{a}	2.66 ± 0.2 ^{*a}	2.5 ± 0.1 ^{b}	2.9 ± 0.1 ^{a}	2.5 ± 0.0 ^{*b}	3.4 ± 0.2 ^{bd‡}
		7	3.0 ± 0.0 ^{a}	3.66 ± 0.1 $^{*+‡}$	2.8 ± 0.1 $^{a+}$	3.7 ± 0.3 $^{+‡}$	2.7 ± 0.1 $^{b+}$	3.3 ± 0.1 ‡
Plasma Cl^- (mmol l^{-1})	160.3 ± 1.9	4	154.3 ± 3.2	146.8 ± 4.4 ^{*a}	151.4 ± 0.5 *	138.5 ± 5.4 ^{*b‡}	157.3 ± 2.0	145.8 ± 1.3 ^{*a‡}
		7	158.1 ± 2.7 ^{a}	142.9 ± 3.0 *‡	157.4 ± 2.9 ^{a}	147.7 ± 2.6 $^{*+‡}$	150.7 ± 2.6 ^{b}	145.9 ± 2.2
Osmol kg^{-1}	0.4 ± 0.0	4	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
		7	0.4 ± 0.0	0.3 ± 0.0 ‡	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0

Data are mean ± S.E.M. (n = 6 fish per treatment and per time point).(*), indicates a statistically significant different from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). Different letters (a & b) denote a statistically significant difference between treatment within time point and pH (ANOVA, $P < 0.05$). (‡), indicates a statistically significant difference between pH 7 and 5 within the type of Cu-treatment and time point (pH effects, ANOVA, $P < 0.05$). (+), indicates a statistically significant difference between day 4 and 7 within the type of Cu-treatment and pH (time effects, ANOVA, $P < 0.05$). (d), indicates a statistically significant different between $CuSO_4$ and Cu-NPs within pH and time point (ANOVA, $P < 0.05$).

4.3.3 Tissue electrolytes and water content

During the exposure to $CuSO_4$ or Cu-NPs at each pH value, there were some statistically significant differences of electrolyte levels in some tissues of trout especially the Na^+, K^+, and Ca^{2+} levels. In gills tissue, branchial Na^+ levels only increased in $CuSO_4$ at pH 7 compared to controls (statistically significant, ANOVA, $P < 0.05$; Table 4.2), and this elevation appeared early (only at day 4). The pH-effect showed a greater decrease in the Na^+ levels of $CuSO_4$ treatment at pH 5 than at pH 7 (Table 4.2). Nano Cu treated did not show any changes in the branchial Na^+ when compared to controls, but showed pH effects (more depletion at pH 5 than pH 7). Greater elevation of Na^+ was observed in the $CuSO_4$ at pH 7 than the nano Cu treated (Table 4.2). Branchial K^+ levels showed a statistically significant increase in $CuSO_4$ treatment at pH 5 compared to the control (ANOVA, $P < 0.05$). The nano Cu treatment showed elevation in the K^+ levels at both pH 7 and 5, with no pH effect (table 4.2). A time effect showed more increased in the branchial K^+ level of both Cu treatments at day 7 when compared to the day 4 (Table 4.2). Branchial Zn at pH 7 of both Cu treatments showed a statistically significant decrease compared to the control (ANOVA, $P < 0.05$, Table 4.2). The pH effects showed more elevation in the Zn level of both $CuSO_4$ and Cu-NP treatment at pH 5 than at pH 7.

Hepatic Na^+ showed a pH effect which includes a statistically significant decrease in the Na^+ levels of both $CuSO_4$ and Cu-NP treatment at pH 5 when compared to the pH 7, although this electrolyte did not show treatment or time effects (ANOVA, $P < 0.05$, Table 4.3). There was a material-type effect with a statistically significant decrease in Na^+ levels of Cu-NPs at pH 5 compared to the $CuSO_4$ treatment of the same

pH value (ANOVA, $P < 0.05$, Table 4.3). However, a decrease (day 4 only) in the hepatic K^+ was observed in the fish exposed to $CuSO_4$ treatment at pH 7 only compared to controls (Table 4.3). At pH 7 of exposure to Cu-NP treatment, the hepatic K^+ showed similar effects to that of $CuSO_4$ which include a statistically significant decrease in the K^+ levels compared to the control (ANOVA, $P < 0.05$). The pH-effects showed more depletion in the hepatic K^+ levels at the acidic pH of both controls (day 4 and 7), $CuSO_4$ (day 4 only) and Cu-NP treatment (day 7 only) than neutral pH (Table 4.3). The hepatic Ca^{2+} showed a small but statistically significant decrease at pH 7 of Cu-NP treatment only compared to controls (ANOVA, $P < 0.05$), with a material- type effect (worse with Cu-NPs than $CuSO_4$ treatment). No significant changes in the hepatic Mn levels were observed in any treatment at different pH values (ANOVA, $P > 0.05$, Table 4.3). A small but statistically significant increase in the hepatic Zn was observed at pH 5 with both $CuSO_4$ and Cu-NP treatments compared to the control (ANOVA, $P < 0.05$), with a pH-effect (more increase at pH 5 than at pH 7).

In the kidney, no significant changes were observed in the Na^+ levels of any Cu treatment at different pH values (ANOVA, $P > 0.05$, Table 4.4). Exposure to $CuSO_4$ treatment at pH 7 showed a decrease in K^+ levels in the kidney when compared to controls (ANOVA, $P < 0.05$), with a pH effect (more decrease at pH 7 than pH 5). The nano Cu treatment showed only a pH- effect which include more depletion in the kidney K^+ level at pH 5 than pH 7 (only at day 7). There was a material- type effects with a statistically significant decrease in the kidney K^+ level of $CuSO_4$ treatment compared to the Cu-NP (ANOVA, $P < 0.05$, Table 4.4). The kidney Ca^{2+} level showed a statistically significant increase at pH 5 of Cu-NPs compared to controls, with a pH-effect (more increased at pH 5 than pH 7) (ANOVA, $P < 0.05$). Exposure to $CuSO_4$ did not show

changes in the kidney Ca^{2+} level at each pH value. A material-type effect showed more elevation in the kidney Ca^{2+} level of nano Cu treatment at pH 5 than $CuSO_4$ treatment (Table 4.4). No significance difference was observed in the levels of Mn and Zn in the kidney of any Cu treatments at each pH value or time point (ANOVA, $P > 0.05$).

In the spleen, exposure to $CuSO_4$ at pH 5 showed an increase in the Na^+ level compared to controls (only at day 4; ANOVA, $P < 0.05$, Table 4.5). The Cu-NP treatment did not show changes in the Na^+ level in the spleen at any pH values or time point. No significant changes were observed in K^+ levels of the spleen in any Cu treatments at different pH values (ANOVA, $P > 0.05$). Exposure to $CuSO_4$ treatment at pH 7 (day 7 only) caused a statistically significant decrease in the Ca^{2+} level of the spleen compared to the control (ANOVA, $P < 0.05$). However, the Zn level of the spleen showed an increase with both $CuSO_4$ and Cu-NP treatments at pH 5 (only day 4) compared to the control (ANOVA, $P < 0.05$), with a pH effect (more elevation at pH 5 than at pH 7). The tissue moisture content did not differ significantly between treatments or over time with values remaining between 55-90 % depending upon the tissue.

Table 4.2 Electrolyte concentrations in the gill of rainbow trout following exposure to control (no added $CuSO_4$ or Cu-NPs) and 20 µg l^{-1} of either $CuSO_4$ or Cu-NPs at neutral and acidic pH.

Electrolyte concentrations	Stock fish at day zero	Time /day	Control		20 µg l^{-1} $CuSO_4$		20 µg l^{-1} Cu-NPs	
			pH 7	pH 5	pH 7	pH 5	pH 7	pH 5
Na (µmol g^{-1})	231.6 ± 9.1	4	196.3 ± 7.5 *[a]	183.1 ± 10.8 *	249.4 ± 5.9 [b]	200.3 ± 10.0 *‡	214.6 ± 7.8 [ad]	180.9 ± 14.3 *‡
		7	232.2 ± 10.2 [+]	196.2 ± 4.1 *‡	245.2 ± 3.1	209.0 ± 8.2 ‡	241.4 ± 6.7 [+]	211.5 ± 6.6 ‡+
K (µmol g^{-1})	385.4 ± 4.8	4	372.4 ± 2.9	376.5 ± 12.2	379.2 ± 8.9	390.8 ± 6.9	388.6 ± 9.0	371.5 ± 8.4
		7	391.9 ± 5.5 [a]	365.8 ± 3.5 [a‡]	409.8 ± 9.6 *[a+]	409.3 ± 3.4 *[b]	418.1 ± 7.5 *[b+]	408.6 ± 4.0 *[b+]
Ca (µmol g^{-1})	138.0 ± 12.8	4	109.4 ± 21.0	43.1 ± 5.9 *‡	127.3 ± 24.7	88.8 ± 22.1	95.2± 19.6	54.5 ± 10.4 *
		7	107.7 ± 23.1	77.7 ± 16.4 *	105.7 ± 25.7	52.6 ± 8.4 *	123.3 ± 23.0 [d]	104.8 ± 22.2
Mn (µmol g^{-1})	0.1 ± 0.0	4	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
		7	0.1 ± 0.0 [a]	0.1 ± 0.1	0.1 ± 0.0 [a]	0.1 ± 0.0	0.2 ± 0.0 [b d+]	0.1 ± 0.0
Zn (µmol g^{-1})	5.5 ± 0.9	4	6.7 ± 0.7	9.7 ± 2.0 *	6.3 ± 1.2	8.6 ± 2.1	6.1 ± 1.0	13.1 ± 2.2 *[d‡]
		7	9.5 ± 1.6 *[a]	9.1 ± 1.1	2.9 ± 0.1 [b]	10.6 ± 1.5 *‡	4.5 ± 1.2 [b]	10.3 ± 1.5 *‡

Data are mean ± S.E.M. (n = 6 fish per treatment and per time point).(*), indicates a statistically significant different from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). Different letters (a & b) denote a statistically significant difference between treatment within time point and pH (ANOVA, $P < 0.05$). (‡), indicates a statistically significant difference between pH 7 and 5 within the type of Cu-treatment and time point (pH effects, ANOVA, $P < 0.05$). (+), indicates a statistically significant difference between day 4 and 7 within the type of Cu-treatment and pH (time effects, ANOVA, $P < 0.05$). (d), indicates a statistically significant different between $CuSO_4$ and Cu-NPs within pH and time point (ANOVA, $P < 0.05$).

Table 4.3 Electrolyte concentrations in the liver of rainbow trout following exposure to control (no added $CuSO_4$ or Cu-NPs) and 20 μg l^{-1} of either $CuSO_4$ or Cu-NPs at neutral and acidic pH

Electrolyte concentrations	Stock fish at day zero	Time /day	Control		20 μg l^{-1} $CuSO_4$		20 μg l^{-1} Cu-NPs	
			pH 7	pH 5	pH 7	pH 5	pH 7	pH 5
Na (μmol g^{-1})	76.0 ± 3.2	4	72.6 ± 3.4	67.4 ± 1.5 *	75.1 ± 2.2	73.1 ± 1.8	74.1 ± 1.4	63.9 ± 1.9*[d‡]
		7	69.5 ± 0.3	64.8 ± 3.0 *	77.4 ± 0.8	67.9 ± 2.0 *[‡]	74.2 ± 5.5	64.8 ± 2.5 *[‡]
K (μmol g^{-1})	359.8 ± 14.5	4	414.7 ± 8.8 *[a]	363.6 ± 4.0 [‡]	393.5 ± 5.3 *[b]	362.9 ± 6.7 [‡]	384.2 ± 4.9 *[b]	362.1 ± 0.7
		7	406.4 ± 4.0 *	369.5 ± 3.0 [‡]	390.2 ± 5.6 *	371.0 ± 4.3	409.8 ± 7.6 *[+]	359.9 ± 4.3 [‡]
Ca (μmol g^{-1})	4.8 ± 0.2	4	5.2 ± 0.1 [a]	4.8 ± 0.1	5.9 ± 0.1 *[a]	4.1 ± 0.2 [‡]	4.3 ± 0.1 [bd]	4.3 ± 0.3
		7	5.5 ± 0.3 *	4.1 ± 0.2 [‡]	5.3 ± 0.5	4.2 ± 0.2 *[‡]	5.2 ± 0.2 [‡]	4.0 ± 0.2 *[‡]
Mn (μmol g^{-1})	0.1 ± 0.0	4	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
		7	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Zn (μmol g^{-1})	1.3 ± 0.1	4	1.4 ± 0.1	1.3 ± 0.0 [a]	1.5 ± 0.0 *	2.1 ± 0.1*[b‡]	1.5 ± 0.1 *	2.0 ± 0.1 *[b‡]
		7	1.5 ± 0.1	2.0 ± 0.0 *[+‡]	1.6 ± 0.1 *	2.2 ± 0.1 *[‡]	1.6 ± 0.1 *	2.0 ± 0.1 *[‡]

Data are mean ± S.E.M. (n = 6 fish per treatment and per time point).(*), indicates a statistically significant different from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). Different letters (a & b) denote a statistically significant difference between treatment within time point and pH (ANOVA, $P < 0.05$). (‡), indicates a statistically significant difference between pH 7 and 5 within the type of Cu-treatment and time point (pH effects, ANOVA, $P < 0.05$). (+), indicates a statistically significant difference between day 4 and 7 within the type of Cu-treatment and pH (time effects, ANOVA, $P < 0.05$). (d), indicates a statistically significant different between $CuSO_4$ and Cu-NPs within pH and time point (ANOVA, $P < 0.05$).

Table 4.4 Electrolyte concentrations in the kidney of rainbow trout following exposure to control (no added $CuSO_4$ or Cu-NPs) and 20 µg l^{-1} of either $CuSO_4$ or Cu-NPs at neutral and acidic pH.

Electrolyte concentrations	Stock fish at day zero	Time/ day	Control		20 µg l^{-1} $CuSO_4$		20 µg l^{-1} Cu-NPs	
			pH 7	pH 5	pH 7	pH 5	pH 7	pH 5
Na (µmol g^{-1})	147.9 ± 7.9	4	149.2 ± 13.9	125.7 ± 8.3	149.5 ± 5.3	130.3 ± 7.7	143.2 ± 5.7	123.3 ± 1.0
		7	157.2 ± 8.5	131.8 ± 7.1	158.3 ± 6.1	157.3 ± 10.3	154.1 ± 8.7	173.5 ± 11.5
K (µmol g^{-1})	405.5 ± 6.7	4	408.1 ± 10.1	380.5 ± 12.5	398.4 ± 8.2	389.6 ± 13.0	391.5 ± 11.3	380.2 ± 7.4
		7	418.3 ± 7.8 [a]	358.9 ± 13.9 *‡	245.3 ± 11.6 *[b+]	378.0 ± 7.9 ‡	420.8 ± 13.0 [ad]	356.3 ± 12.3 *‡
Ca (µmol g^{-1})	14.7 ± 1.2	4	18.0 ± 2.3	20.2 ± 4.0 [a]	18.5 ± 3.0	13.6 ± 2.3 [a]	22.0 ± 4.2	32.7 ± 10.8 *[bd‡]
		7	13.0 ± 1.4	17.0 ± 3.5	18.2 ± 2.3	12.6 ± 0.9	17.4 ± 2.5	12.0 ± 0.6‡
Mn (µmol g^{-1})	0.1 ± 0.0	4	0.1 ± 0.0	0.0 ± 0.0 *[a]	0.1 ± 0.0	0.1 ± 0.0 [b]	0.1 ± 0.0	0.1 ± 0.0 [b]
		7	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0 *‡	0.1 ± 0.0 *	0.0 ± 0.0 *
Zn (µmol g^{-1})	2.0 ± 0.1	4	2.0 ± 0.1	1.8 ± 0.1	2.2 ± 0.3	3.0 ± 0.4	2.1 ± 0.2	2.9 ± 0.1
		7	2.2 ± 0.2	3.3 ± 0.3	2.6 ± 0.3	3.0 ± 0.2	2.0 ± 0.1	3.7 ± 0.2

Data are mean ± S.E.M. (n = 6 fish per treatment and per time point). (*), indicates a statistically significant different from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). Different letters (a & b) denote a statistically significant difference between treatment within time point and pH (ANOVA, $P < 0.05$). (‡), indicates a statistically significant difference between pH 7 and 5 within the type of Cu-treatment and time point (pH effects, ANOVA, $P < 0.05$). (+), indicates a statistically significant difference between day 4 and 7 within the type of Cu-treatment and pH (time effects, ANOVA, $P < 0.05$). (d), indicates a statistically significant different between $CuSO_4$ and Cu-NPs within pH and time point (ANOVA, $P < 0.05$).

Table 4.5 Electrolyte concentrations in the spleen of rainbow trout following exposure to control (no added $CuSO_4$ or Cu-NPs) and 20 µg l^{-1} of either $CuSO_4$ or Cu-NPs at neutral and acidic pH.

Electrolyte concentrations	Stock fish at day zero	Time /day	Control		20 µg l^{-1} $CuSO_4$		20 µg l^{-1} Cu-NPs	
			pH 7	pH 5	pH 7	pH 5	pH 7	pH 5
Na (µmol g^{-1})	49.2 ± 2.1	4	54.6 ± 3.8	40.0 ± 0.6 [a‡]	54.7 ± 3.7	58.0 ± 10.3 [b]	48.8 ± 1.0	47.5 ± 4.4 [a]
		7	48.6 ± 2.5	39.2 ± 2.4	45.5 ± 2.6	40.8 ± 4.0 [+]	46.8 ± 2.9	38.0 ± 0.9
K (µmol g^{-1})	425.2 ± 11.7	4	373.3 ± 8.5 *	372.3 ± 14.1 *	375.1 ± 16.2 *	358.7 ± 22.1 *	375.5 ± 11.5 *	352.0 ± 13.8 *
		7	395.7 ± 8.4	358.2 ± 15.8 *	384.1 ± 20.6	350.2 ± 26.6 *	374.9 ± 2.6 *	344.9 ± 5.9 *
Ca (µmol g^{-1})	5.6 ± 0.6	4	4.3 ± 0.2	5.4 ± 1.7	3.9 ± 0.6	3.8 ± 1.0	2.9 ± 0.1 *	2.0 ± 0.3 *
		7	4.6 ± 0.6 [a]	2.5 ± 0.3 *[+‡]	2.6 ± 0.1 *[b]	2.5 ± 0.4 *	3.2 ± 0.4 *[a]	2.0 ± 0.2 *
Mn (µmol g^{-1})	0.0 ± 0.0	4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.00	0.0 ± 0.0	0.0 ± 0.0
		7	0.0 ± 0.0 [a]	0.0 ± 0.0	0.1 ± 0.0 *[b]	0.0 ± 0.0 [‡]	0.0 ± 0.0 [ad]	0.0 ± 0.0
Zn (µmol g^{-1})	2.2 ± 0.3	4	1.8 ± 0.1	1.3 ± 0.1 [a]	1.6 ± 0.3	3.8 ± 1.0 *[b‡]	1.4 ± 0.1	3.0 ± 0.6 [b‡]
		7	2.0 ± 0.3	2.7 ± 0.4 [+]	0.9 ± 0.0	2.7 ± 0.4 [‡]	1.2 ± 0.1	3.6 ± 0.6

Data are mean ± S.E.M. (n = 6 fish per treatment and per time point).(*), indicates a statistically significant different from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). Different letter (a & b) denotes a statistically significant difference between treatment within time point and pH (ANOVA, $P < 0.05$). (‡), indicates a statistically significant difference between pH 7 and 5 within treatment and time point (pH effects, ANOVA, $P < 0.05$). (+), indicates a statistically significant difference between day 4 and 7 within the type of Cu-treatment and pH (time effects, ANOVA, $P < 0.05$). (d), indicates a statistically significant different between $CuSO_4$ and Cu-NPs within pH and time point (ANOVA, $P < 0.05$).

4.3.4 Na^+/K^+-ATPase activity

The gills of trout exposed to Cu as $CuSO_4$ (only at day 4) at pH 7 showed a decrease in the Na^+/K^+-ATPase activity compared to controls (about 0.2 fold; ANOVA, $P < 0.05$), with a material-type effect (worse with $CuSO_4$ than Cu-NP treatment; Fig. 4.4). At pH 5, exposure to $CuSO_4$ for 7 days caused depletion (0.6-fold) in the Na^+/K^+-ATPase activity compared to the control. A pH-effect of $CuSO_4$ at day 4 only showed more elevation in the Na^+/K^+-ATPase activity at pH 5 than pH 7 (Fig. 4.4). Exposure to Cu-NPs did not show significant changes in the Na^+/K^+-ATPase activity at each pH value when compared to controls (ANOVA, $P > 0.05$). There was a pH effect in Cu-NPs treatment that continued to the end of the experiment which caused a greater elevation in the activity of Na^+/K^+-ATPase at pH 5 than pH 7 (Fig. 4.4). In the liver, exposure to $CuSO_4$ at pH 7 and 5 did not show changes in the Na^+/K^+-ATPase activity at each time point when compared to controls (ANOVA, $P > 0.05$), but there was a pH-effect (greater elevation at pH 5 than pH 7; only at day 4) (Fig. 4.4). Exposure to Cu-NPs at pH 5 for 7 days only caused a statistically significant decrease in the Na^+/K^+-ATPase activity of the liver (ANOVA, $P < 0.05$), with a material-type effect (more depletion with Cu-NP than $CuSO_4$). The kidney of trout showed a statistically significant decrease in the activity of Na^+/K^+-ATPase with $CuSO_4$ treatment at both pH values (ANOVA, $P < 0.05$), with a pH effect (greater elevation at pH 5 than at pH 7). A time-effect showed more depletion in the activity of Na^+/K^+-ATPase at day 7 of pH 7 with $CuSO_4$ treatment than at day 4 (Fig. 4.4). Exposure to Cu-NPs at pH 5 for 4 days only showed a statistically significant increase in the Na^+/K^+-ATPase activity compared to

the control (ANOVA, $P < 0.05$). There were no pH or time effects in the Na^+/K^+-ATPase activity of nano Cu treatment in the kidney (Fig. 4.4).

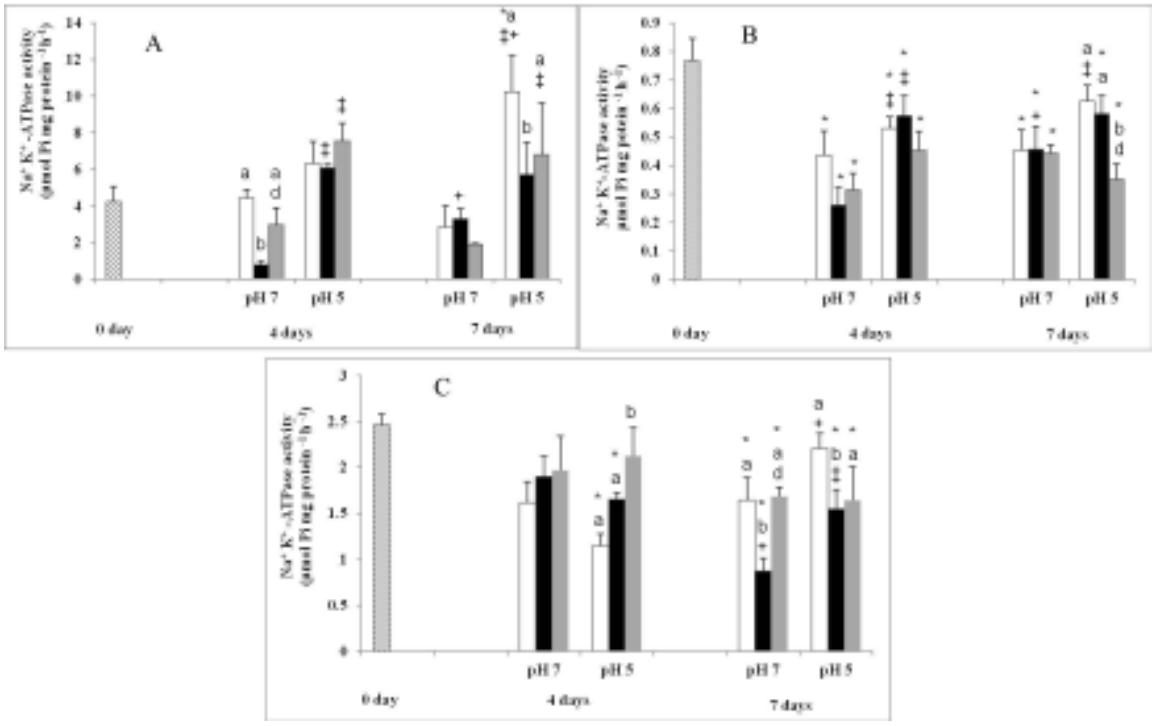

Figure 4.4 Na^+/K^+-ATPase activity in crude homogenates of (A) gills; (B) liver; and (C) kidney of trout after exposure to control (white bars), 20 μg l^{-1} of either $CuSO_4$ (black bars) or Cu-NPs (grey bars) for 4 and 7 days at pH7 and 5. Diagonal hatched bars are initial (day 0) fish. Data are mean ± S.E.M. (n = 6 fish per treatment). (*), indicates statistically significant different from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). Different letters (a & b) denote a statistically significant difference between treatment within time point and pH values (ANOVA, $P < 0.05$). (‡), indicates a statistically significant difference between pH 7 and 5 within the type of Cu-treatment and time point (pH effects, ANOVA, $P < 0.05$). (+), indicates a statistically significant difference between day 4 and 7 within the type of Cu-treatment and pH (time effects, ANOVA, $P < 0.05$). (d), indicates a statistically significant different between $CuSO_4$ and Cu-NPs within pH and time point (ANOVA, $P < 0.05$). Note the different y-axis scales on each graph.

4.3.5 TBARS and total glutathione content

Trout exposed to dissolve $CuSO_4$ or Cu-NPs at pH 7 and 5 for up 7 days did not show changes in the TBARS levels in the gill, kidney and spleen (Fig. 4.5). A time-effect was observed in the TBARS level of the gill at pH 7 (with both Cu treatments) and pH 5 (with $CuSO_4$ treatment only) which caused more depletion at day 7 than day 4 (Fig. 4.5). In the liver, exposure to $CuSO_4$ at pH 5 for 4 days showed a small but statistically significant increase in the concentration of TBARS compared to controls (ANOVA, $P < 0.05$). Exposure to the Cu-NP at pH 7 for 4 days showed a decrease in the TBARS level of the liver when compared to the control group (ANOVA, $P < 0.05$), and with a material-type effect (more depletion with Cu-NPs than $CuSO_4$). At pH 5, there was a material-type effect in the TBARS value of the Cu-NP treatment at day 7 compared to the $CuSO_4$ treatment (more decrease with NP treatment, Fig. 4.5). A time-effect was observed in the liver by causing more depletion in the TBARS level at day 7 of exposure to $CuSO_4$ (at pH 7 only) and Cu-NPs (at pH 5 only) compared to day 4 (all statistically significant, ANOVA, $P < 0.05$).

The total glutathione (GSH) content of the gill showed a statistically significant decrease with $CuSO_4$ at pH 7 (only at day 4) compared to controls (ANOVA, $P < 0.05$), with a material-type effect (more depletion with $CuSO_4$ than Cu-NPs) (Fig. 4.5). There was a time-effect on the branchial GSH content of the $CuSO_4$ which showed more increase at day 7 than day 4 (Fig. 4.5). Exposure to the Cu-NP treatment at day 7 showed only a pH-effect which includes more depletion in the branchial GSH content at pH 5 than pH 7. The hepatic GSH content did not show treatment or time effects, although there was a pH-effect

which includes a more depletion at pH 5 with both Cu treatments than pH 7. In the spleen, there was a pH-effect with $CuSO_4$ only (at day 4 only) that showed a statistically significant decrease in the total GSH content at pH 5 when compared to the pH 7 (ANOVA, $P < 0.05$, Fig. 4.5). In the kidney, exposure to $CuSO_4$ at pH 7 and 5 for up 7 days did not show changes in the total GSH (Fig. 4.5). Exposure to Cu-NPs at pH 5 (day 4 only) showed a statistically significant decrease in the GSH content of the kidney compared to the control (ANOVA, $P < 0.05$). There were no pH or time effects observed in the total GSH of the kidney with both Cu treatments (Fig. 4.5).

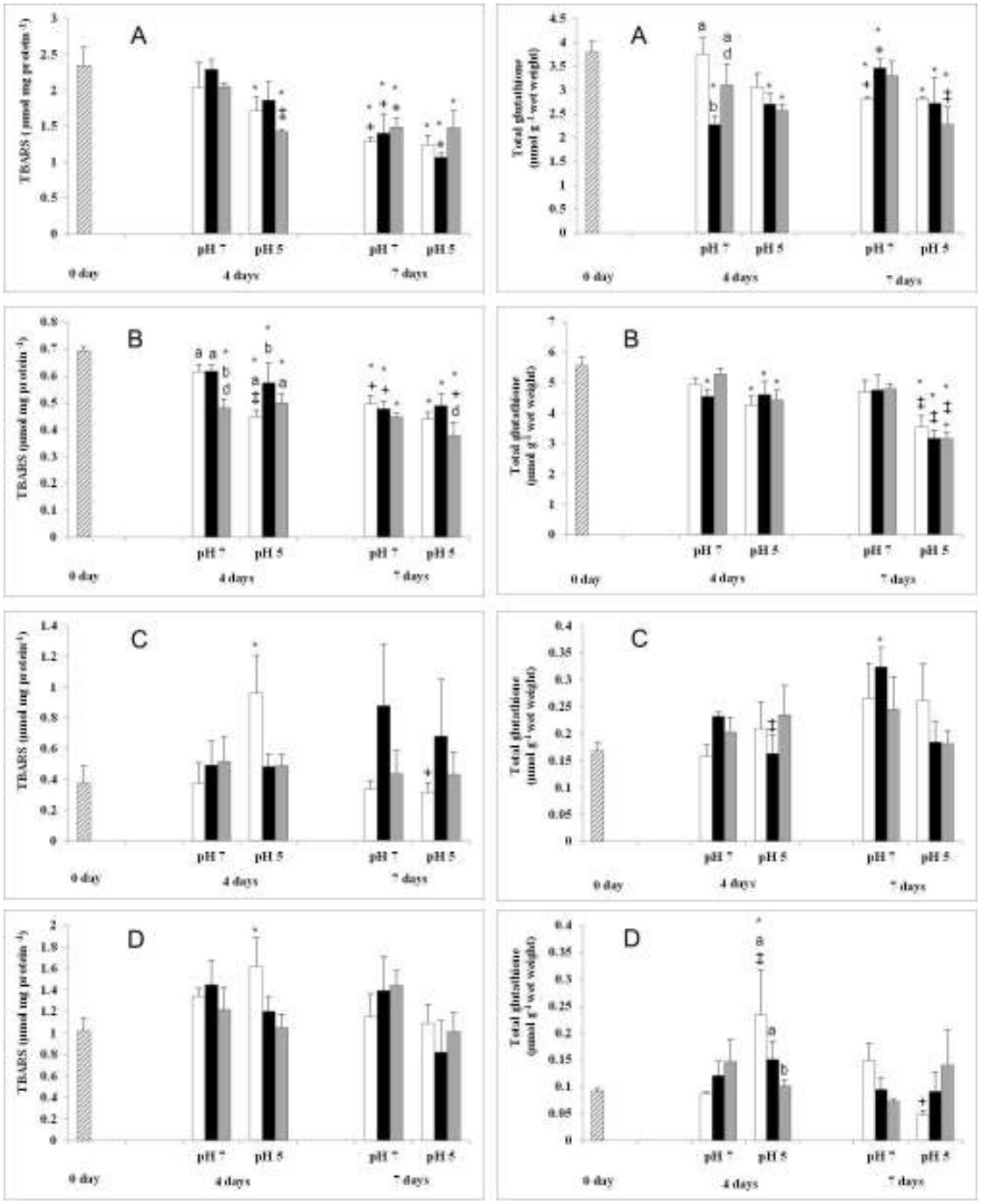

Figure 4.5 Thiobarbituric acid reactive substances (TBARS) (left panel) and total glutathione content (right panel) in crude homogenates of (A) gill; (B) liver; (C) spleen and (D) kidney after exposure to control (white bars), 20 μg l^{-1} of either $CuSO_4$ (black bars) or Cu-NPs (grey bars) for 4 and 7 days at pH 7 and 5. Diagonal hatched bars are initial (day 0) fish. Data are mean ± S.E.M. (n = 6 fish per treatment). (*), indicates a statistically

significant different from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). Different letters (a & b) denote a statistically significant difference between treatment within time point and pH (ANOVA, $P < 0.05$). (‡), indicates a statistically significant difference between pH 7 and 5 within the type of Cu-treatment and time point (pH effects, ANOVA, $P < 0.05$). (+), indicates a statistically significant difference between day 4 and 7 within the type of Cu-treatment and pH (time effects, ANOVA, $P < 0.05$). (d), indicates a statistically significant different between $CuSO_4$ and Cu-NPs within pH and time point (ANOVA, $P < 0.05$). Note the different y-axis scales on each graph.

4.3.6 Histopathological studies

Gill, liver, spleen and kidney were examined at the end of each treatment (control, $CuSO_4$, and Cu-NPs) at pH 7 and 5. The gill morphology of control groups showed normal structure at pH 7 and a minor lesion observed with the control of pH 5 (Fig. 4.6). Exposure to waterborne $CuSO_4$ treatment at pH 7 and 5 showed similar types of lesions included an occasional area of hyperplasia with evidence of necrotic cells in the primary filament, swollen mucocytes, clubbed tips and aneurism in the secondary lamellae as well as epithelium lifting compared to unexposed control groups (Fig 4.6). Generally, these lesions were worse at pH 5 than pH 7 of $CuSO_4$ treatments. Typically, each type of lesion was observed in at least four fish out of six fish examined per treatment at pH 7 and all 6 fishes per treatment at pH 5 (Fig. 4.6). The types of lesions observed at pH 7 and 5 of Cu-NP treatment was similar to that of $CuSO_4$ treatment (Fig. 4.6), with a severe injury at pH 5 than pH 7.

A quantitative analysis of the incidence of gill lesions confirmed these observations with a statistically significant increase in the incidence of clubbed tips with both dissolved Cu as $CuSO_4$ or Cu-NPs compared to controls, with a pH effect (worse with pH 5,

ANOVA, $P < 0.05$) (Fig. 4.7). Values for pH 7 were (% means ± S.E.M. $n = 6$) 10.5 ± 1.3 in control, 23.8 ± 0.5 in $CuSO_4$, and 20.0 ± 1.0 in Cu-NPs treatments. While, the values at pH 5 were (% means ± S.E.M. $n = 6$) 20.7 ± 2.0 in control, 36.5 ± 1.7 in $CuSO_4$, and 33.7 ± 1.7 in Cu-NPs treatments. Similarly, the incidence of oedema increased from (% means ± S.E.M. $n = 6$) zero (not observed) in the controls to 3.0 ± 0.4 and 3.7 ± 0.6 in the $CuSO_4$ and Cu-NPs treatments respectively at pH 7 and from 7.8 ± 1.0 in the control to 16.3 ± 1.2 and 14.3 ± 0.8 in the $CuSO_4$ and Cu-NPs treatments respectively at pH 5, with much worse with pH 5 (ANOVA, $P < 0.05$) (Fig. 4.7). The incidence of swollen mucocytes was a statistically significant increased only with pH 5 from (% means ± S.E.M. $n = 6$) 5.2 ± 0.8 in the control to 11.8 ± 1.8 and 9.3 ± 0.9 in the $CuSO_4$ and Cu-NPs treatments respectively (ANOVA, $P < 0.05$) (Fig. 4.7). There was also a statistically significant increase in the incidence of necrosis at pH 7 with either $CuSO_4$ or Cu-NPs treatments compared to controls, but the value remained below 2 % incidence rate (data not shown).

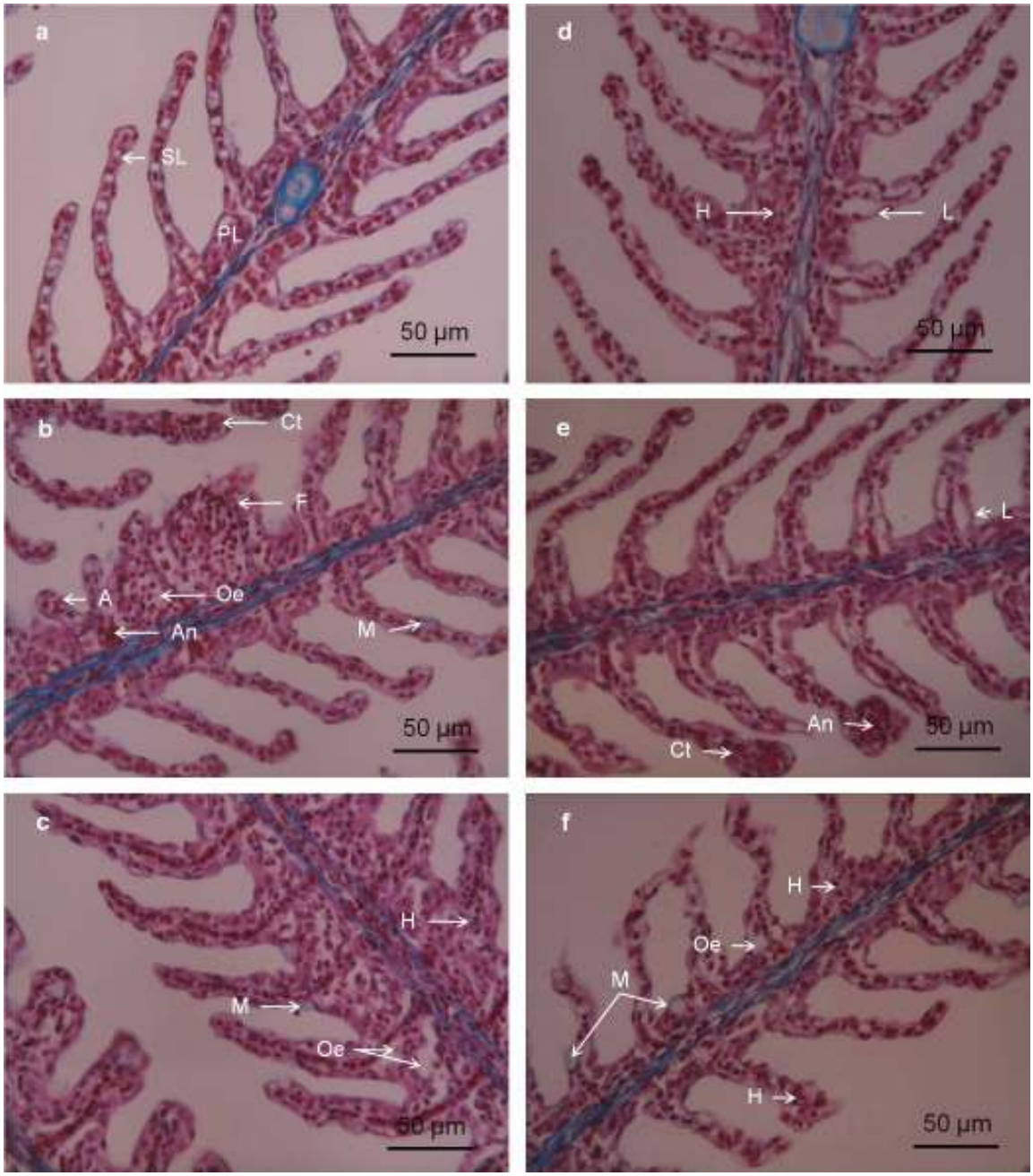

Figure 4.6 Gill morphology in rainbow trout after 7 days of waterborne exposure to (a) control (b) $CuSO_4$, (c) Cu-NPs at pH 7 and (e) control (f) $CuSO_4$ (g) Cu-NPs at pH 5. The gill of control groups showed the normal structure of primary (PL) and secondary lamellae (SL) at pH 7, with minor lesions at pH 5. Exposure to Cu as $CuSO_4$ or Cu-NPs at pH 7 and 5 caused similar types of injuries, with worse at pH 5 than 7. These injuries include clubbed tips (Ct), lamellar fusion (F), oedema (Oe), atrophy (A), aneurism (An), mucocytes (M),

and hyperplasia (H). Scale bar indicates magnification, sections were 7 µm thick and stained with Mallory`s trichrome.

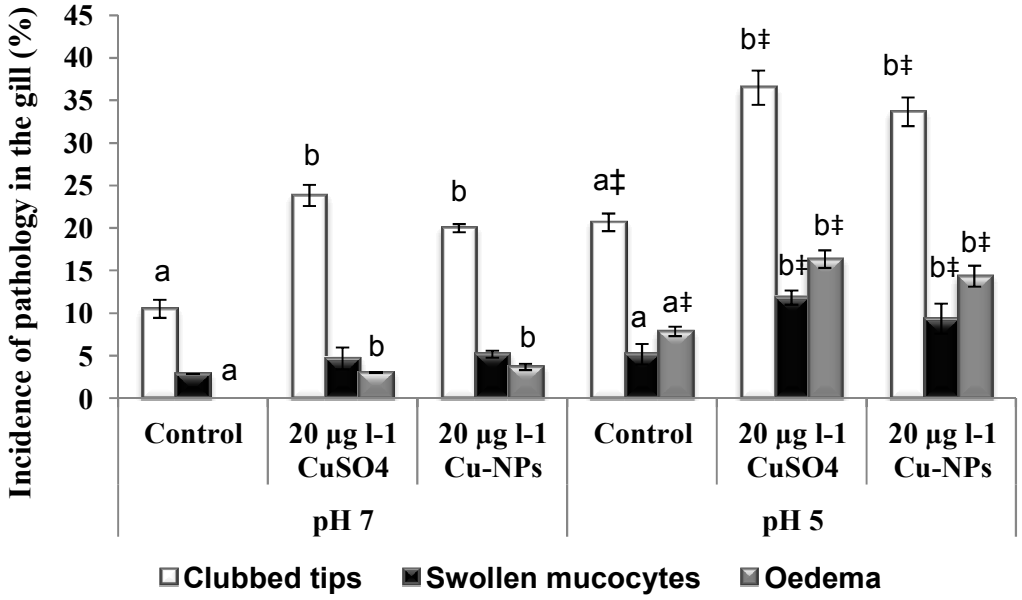

Figure 4.7 Proportion of secondary lamellae showing pathology in the gills of rainbow trout after 7 days of exposure to Cu as $CuSO_4$ or Cu-NPs at pH 7 and 5. Data are means of proportional injuries ± S.E.M.; n = 6 fish/treatments. Different letters denote a statistically significant difference between treatment within the pH value (ANOVA, $P < 0.05$). (‡), indicates a statistically significant difference between pH 7 and 5 within the type of Cu-treatment (pH effects, ANOVA, $P < 0.05$).

The livers of control groups showed normal histology at pH 7 and minor lesion was observed at pH 5 of the control (Fig. 4.8). Exposure fish to the $CuSO_4$ at pH 7 and 5 showed similar types of lesions, with more severity at pH 5 than pH 7. These lesions include incidence of pyknotic nuclei, occasional necrotic cells, lipidosis, hepatitis-like cell injury, and increase the presence of melanomacrophage deposits in five fish out of six fish examined per treatment (Fig. 4.8). The types of lesions in the Cu-NPs exposed fish at pH 7 and 5 were similar to that observed in $CuSO_4$ treatment (Fig. 4.8). The number of

melanomacrophage deposits in the liver showed a statistically significant increased with both $CuSO_4$ and Cu-NPs at different pH values compared to controls (ANOVA, $P < 0.05$), but there was no material-type or pH effects. At pH 7, the values of these deposits were (means ± S.E.M, counts/field of view of 53,200 μm^2, at x 400 magnification, $n = 6$) 15.5 ± 1.7 (control), 34.2 ± 4.7 ($CuSO_4$) and 35.7 ± 3.7 (Cu-NPs). At pH 5, the values were 22.3 ± 2.8 in the control and 33.2 ± 1.6 in Cu-NPs. The liver also showed an increase in the proportion of sinusoid space (5.9 ± 0.4 in control; 7.4 ± 0.2 in $CuSO_4$; 6.9 ± 0.3 in Cu-NPs) with a small decrease in the proportion of hepatic area (94.1 ± 0.4 in control; 92.6 ± 0.2 in $CuSO_4$; 93.1 ± 0.3 in Cu-NPs) with both Cu treatments ($CuSO_4$ or Cu-NPs) at pH 7 compared to the control (mean (μm) ± S.E.M; $n = 6$; all statistically significant; ANOVA, $P < 0.05$).

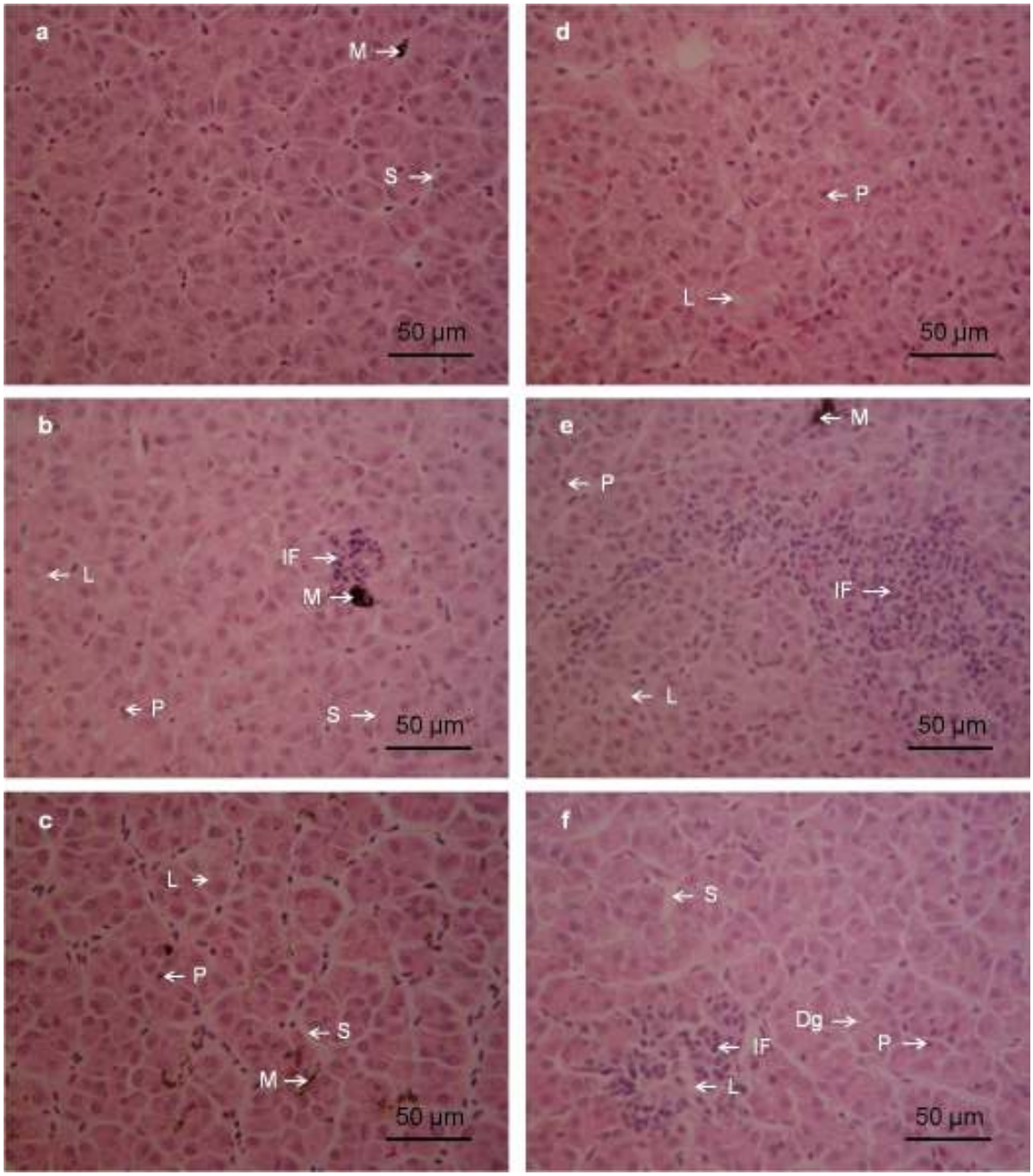

Figure 4.8 Liver morphology in rainbow trout after 7 days of waterborne exposure to (a) control (b) $CuSO_4$, (c) Cu-NPs at pH 7 and (e) control (f) $CuSO_4$ (g) Cu-NPs at pH 5. The liver of control groups showed normal structure with sinusoid space (S) and small deposit of melanomacrophage (M). Both Cu treatments ($CuSO_4$ or Cu-NPs) at pH 7 and 5 showed similar lesions, but pH 5 caused greater severity effects than pH 7. These lesions include, lipidosis (L), occasional inflammatory cells like hepatitis (IF), cell with pyknotic nuclei (P),

degeneration of hepatic cell (Dg). Scale bar indicates magnification, section were 7 μm thick and stained with haematoxylin and eosin.

Histology of the spleen was shown in Fig. 4.9. Spleens from control groups showed normal histology structure at pH 7 with minor lesions at pH 5, but some lesions were noted in fish exposed to both $CuSO_4$ and Cu-NPs at pH 7 and 5. At pH 7 and 5, the exposure to $CuSO_4$ treatment showed similar types of lesions included the occasional vacuole formation and increased the presence of melanomacrophage deposits in five out of six fish examined at each pH value (Fig. 4.9), but these lesions were worse at pH 5 than pH 7. Exposure to Cu-NPs at pH 7 and 5 showed similar lesions to that observed with the $CuSO_4$ treatment (Fig. 4.9). The fractional volume of red pulp was statistically significant increased in trout exposed to either $CuSO_4$ or Cu-NPs at pH 7 with a concomitant decreased in the white pulp and sinusoid space when compared to controls (ANOVA, $P < 0.05$, Fig. 4.10). At pH 5, the fractional volume of red pulp was significantly decreased in trout exposed to either $CuSO_4$ or Cu-NPs with an associated increase in the white pulp (only with $CuSO_4$ treatment) and sinusoid space when compared to controls (ANOVA, $P < 0.05$, Fig. 4.10). The pH-effects showed a greater change at pH 5 than pH 7 in the spleen content (proportion of red, white pulp and sinusoid space) (Fig. 4.10). Notably, there was also a statistically significant increased in the number of melanomacrophages (means ± S.E.M., counts/field of view of 53,200 μm^2, at x 400 magnification, $n = 6$) with either $CuSO_4$ or Cu-NPs treatments at pH 7 and 5 (Fig. 4.10), but there was no material-type or pH effects.

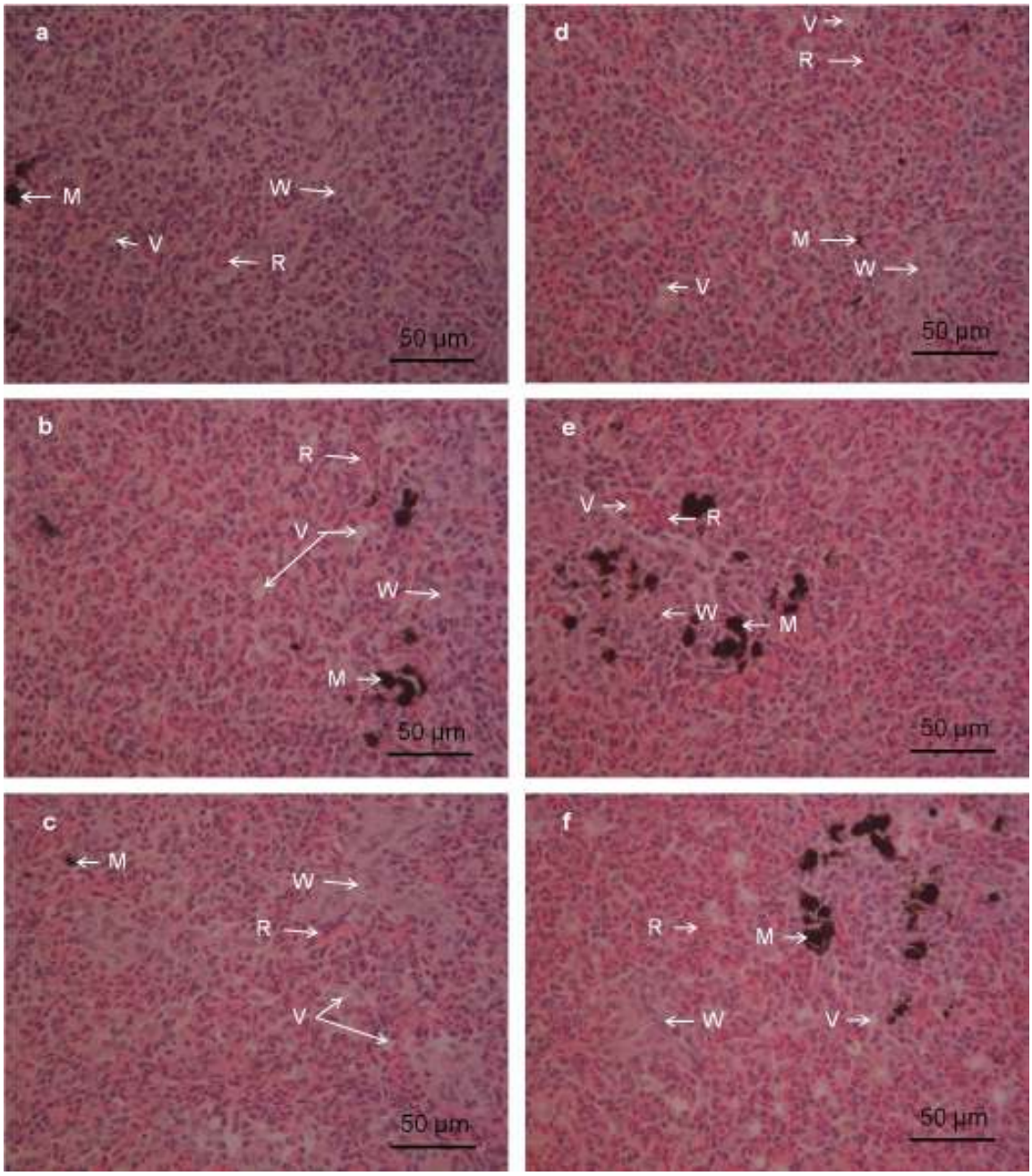

Figure 4.9 Spleen morphology in rainbow trout after 7 days of waterborne exposure to (a) control (b) $CuSO_4$, (c) Cu-NPs at pH 7 and (e) control (f) $CuSO_4$ (g) Cu-NPs at pH 5. The spleen of control groups showed normal structure of white (W) and red (R) pulp, and melanomacrophage deposits (M) with minor changes at pH 5. Both Cu treatments ($CuSO_4$ and Cu-NPs) at pH 7 and 5 showed lesions that include vacuole formation (V) and increased the number of melanomacrophage deposits. These lesions were worse at pH 5

than 7. Scale bar indicates magnification, section were 7 µm thick and stained with haematoxylin and eosin.

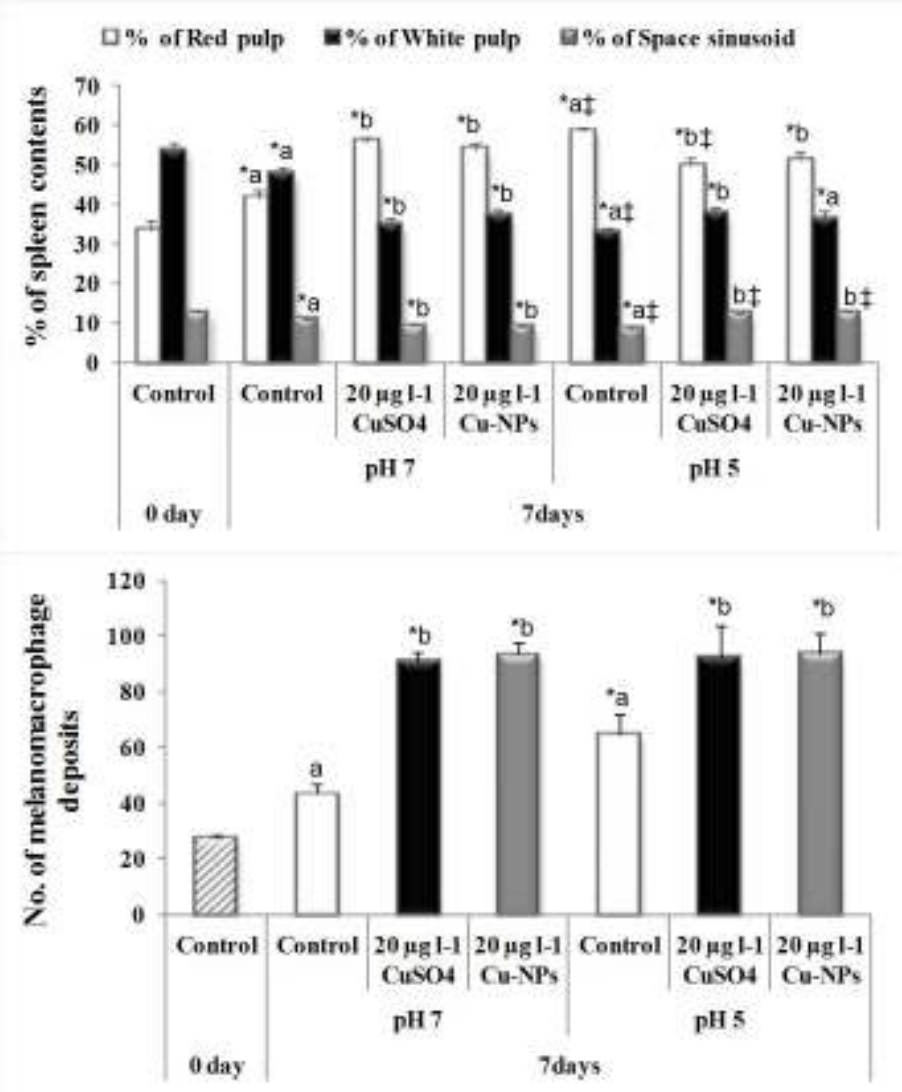

Figure 4.10 Alteration observed in the proportion of red and white pulp, sinusoid space and the number of melanomacrophage deposits in the spleen of trout after 7 days of exposure to either $CuSO_4$ or Cu-NPs at pH 7 and 5. Data are means of proportional areas, or means ± S.E.M., $n = 6$ fish per treatments. (*), indicates statistically significant different from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). Different letters (a & b) denote a statistically significant difference between treatments within pH values (ANOVA, $P < 0.05$). (‡), indicates a statistically significant difference between pH 7 and pH 5 within the type of Cu-treatment (pH effects, ANOVA, $P < 0.05$).

The posterior part of the kidney from the freshwater control showed normal histology at pH 7 with minor lesions at pH 5 (Fig. 4.11). Exposure to Cu as $CuSO_4$ at pH 7 and 5 showed similar types of lesion in five out of six fish examined at each pH values, with worse effects at pH 5 than 7 (Fig. 4.11). These lesions included occasional necrotic cells in the haematopoietic area, vacuole formation in the renal epithelial tubule and haematopoietic area, degeneration of renal tubule and enlargement of the Bowman's space. The kidney of trout exposed to Cu-NPs at both pH values showed similar lesions to that observed with $CuSO_4$ treatment. The number of melanomacrophage deposits in the kidney at pH 7 and 5 of exposure to $CuSO_4$ showed a statistically significant increase compared to controls, with a material-type and pH effects (ANOVA, $P < 0.05$). At pH 7, the values were (means ± S.E.M., counts/image at x 200 magnification, total area image 212,400 μm^2, n = 6) 303.5 ± 0.8, 458.3 ± 19.6 and 326.3 ± 27.5 in the control, $CuSO_4$ and Cu-NPs, respectively. At pH 5, the values were 310.2 ± 18.8 in controls; 364.5 ± 13.4 in $CuSO_4$; and 308.7 ± 24.2 in Cu-NPs treatments. The quantitative kidney analysis also showed minor changes in the proportion of proximal tubules and haematopoietic tissues (data not shown).

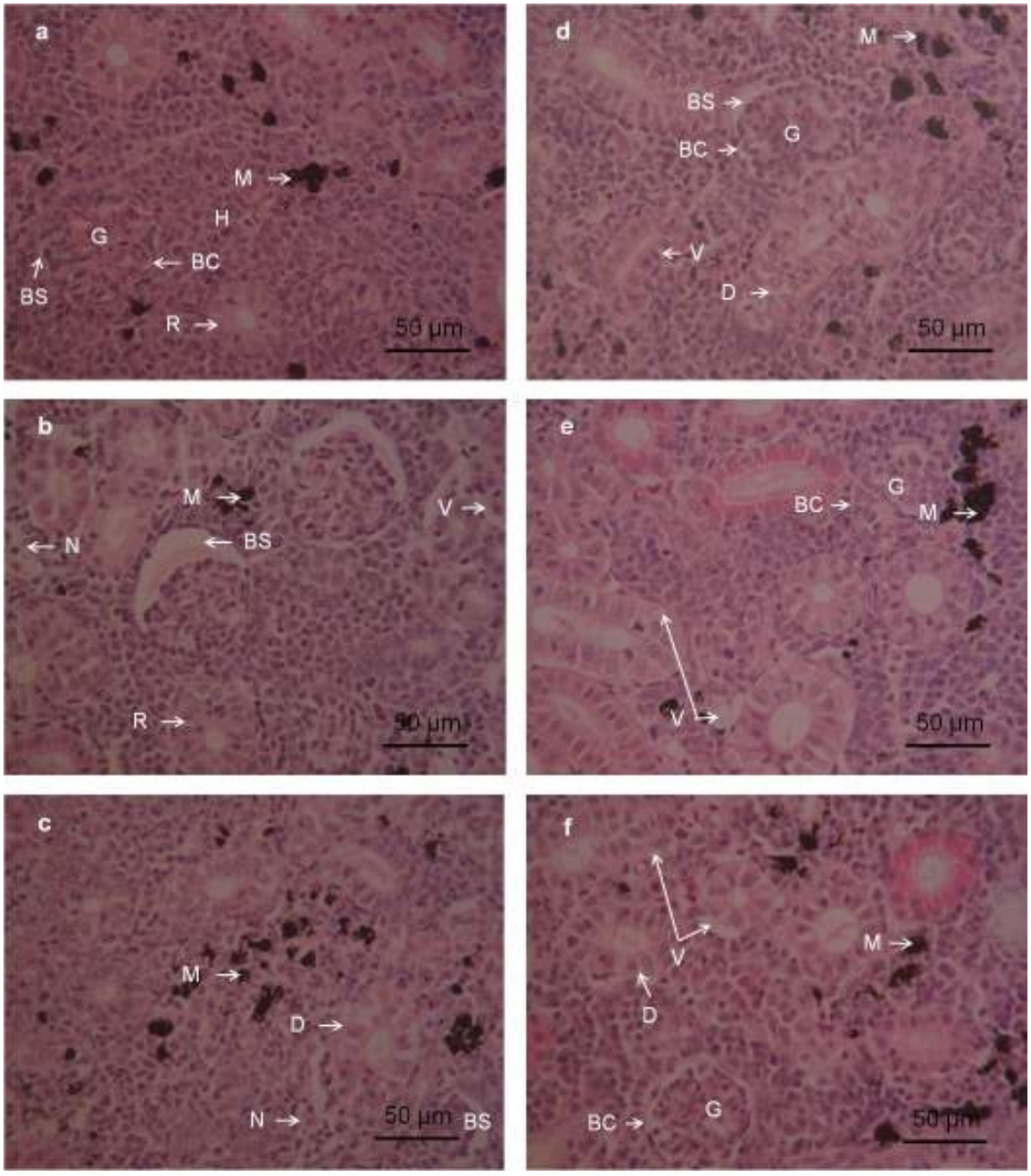

Figure 4.11 Kidney morphology in rainbow trout after 7 days of waterborne exposure to (a) control (b) $CuSO_4$, (c) Cu-NPs at pH 7 and (e) control (f) $CuSO_4$ (g) Cu-NPs at pH 5. Kidney of control fish showed normal histology with parietal epithelium of Bowman's capsule (BC), glomerulus (G), Bowman's space (BS), renal tubules (R), haematopoietic tissues (H) and melanomacrophages deposits (M). Similar pathologies were observed with $CuSO_4$ and Cu-NPs at pH 7 and 5, with worse effects at pH 5 than 7. These pathologies

include necrosis (N), vacuolisation (V), and degeneration of renal tubule (D). Scale bar indicates magnification, section were 7 µm thick and stained with haematoxylin and eosin.

4.4 Discussion

So far, there are very few studies investigating the effects of neutral and acidic water pH on the toxicity or uptake of waterborne Cu-NPs on rainbow trout. This study presents a comparison with a similar amounts of Cu as $CuSO_4$ or Cu-NPs on the sub-lethal effects of acid exposure on trout. Overall, acidic water caused a decrease in the mean of primary particle size of Cu-NPs that was associated with a reduction in the rate of particle aggregation sizes. Dialysis experiments confirmed these results showing more Cu dissolution from Cu-NPs at pH 5 than pH 7. Waterborne exposure to $CuSO_4$ or Cu-NPs showed the accumulation of Cu in the gills (at both pH values) and livers (at pH 7 only). This indicates that Cu-NP accumulate in the same organs as dissolved Cu. Exposure of trout to Cu-NPs also caused a transient change in the plasma and tissue electrolytes, alteration in TBARS, as well as pathologies in the gill, liver, spleen and kidney at pH 7 and 5 like dissolved Cu. Regardless of the form of the Cu, there were greater effects observed at pH 5 than pH 7. This could indicate that the particulate form (Cu-NPs) is more toxic at pH 5 than at pH 7.

4.4.1 Effect of pH on particle characterisation and metal ion dissolution

Acidic water pH does make the aggregate size of Cu-NPs smaller. This suggests that acidic pH could change the characterisation of Cu-NPs such as surface charges to alter the aggregation kinetics of the particles. Handy *et al.* (2008b) reported that surface charge

screening on particles can be changed by H^+ ions concentration in the solution, so measuring the zeta potential as a function of pH on particles can give a rational prediction of colloidal NP stability. Therefore, more studies need to measure and investigate the surface charge of Cu-NPs at different pH values.

Unfortunately, TEM and nanoparticle tracking analysis could not be used to verify the Cu-NP particle size distributions directly in the exposure tanks. Interestingly, the TEM images of the Cu-NPs stock buffered at pH 5 in the current study showed a reduction in the primary particle size of Cu-NPs; suggesting the acid conditions may slightly dissolve the particles. While electron microscopy is reliable for primary particle size measurements, one limitation of electromicroscopy is that the dehydration of the specimen prevents any information being obtained about particle size distribution of the aggregates; because these are simply artefacts of the specimen preparation. The nanoparticle tracking analysis (NanoSight) is a useful tool in estimating particle size distributions of NPs (distance and speed of the particles moving under Brownian motion), but there are some limitations. For measuring stock solutions of Cu-NPs in Milli-Q water it is a reasonably quick and reliable method to use. However, the Nanosight was not able to give reliable data for the particle size distributions of Cu-NPs in tank water because of intereference of natural colloids already in the tank water (dissolved organic matter, protein from fish mucus, *etc.*,). In the natural tank water samples, the range of particle sizes was simply too large and dynamic for the instrument to recognize them all. Against the background noise of natural materials there was not a sufficient number of continuously visible particles to gain a sufficient number of successful tracks. In tank water that contains fish, the aggregation of Cu-NPs

may well differ compared to the pure water because the tank water has higher ionic strength and contains organic matter from the mucous secretions of the fish. This could lead to change in the surface charge of the Cu-NPs in the tank water, and therefore their aggregation behaviour. Nonetheless, the results on the stock dispersions indicate the reasonable dispersion of the stock at acidic and neutral water pH.

Exposure to $CuSO_4$ or Cu-NPs was confirmed by ICP-OES analysis of water samples for total Cu concentrations. These analyses showed that 12 h water changes were sufficient to maintain an exposure consistent with regulatory test parameters. Waterborne exposure to both forms of Cu at pH 7 and 5 was also confirmed by increases of branchial Cu concentrations, with a material-type effect (more elevation with $CuSO_4$ than Cu-NPs). This could suggest that $CuSO_4$ has more bioavailability than Cu-NPs at both pH values. However, the dialysis results showed that acidic pH caused an increase in the dissolution of Cu from Cu-NPs. The present findings seem to be consistent with other research which found the metal-NPs in acidic water may dissolute to free metal ions in solution and result in the formation of smaller NPs sizes during dissolution (Borm *et al.*, 2006).

4.4.2 Accumulation of copper at pH 7 and 5 during waterborne exposure

Following waterborne exposure, Cu normally accumulated in the gills (as a route of exposure) and the liver (as a central compartment for Cu accumulation and homeostasis, Grosell *et al.*, 1998a; 1998b), with limited Cu concentration in the other internal organs (*e.g.*, Kamunde and Wood, 2004). The Cu accumulation pattern for waterborne exposure of $CuSO_4$ at pH 7 and 5 was therefore as expected. Acidic water of $CuSO_4$ treatment for 4

days only showed more depletion in Cu concentrations in the gills than pH 7, and this could suggest that H^+ ions compete with free metal ion at the cell surface for available binding sites. Waterborne exposure to Cu-NP at both pH values showed also accumulation in the gills with no pH effect. Nevertheless, there were some differences between $CuSO_4$ and Cu-NPs at both pH values which showed more accumulation of Cu with $CuSO_4$ treatment than Cu-NPs. This finding is agreement with Shaw *et al.* (2012) who also saw Cu concentrations were higher in the gill of $CuSO_4$ treatment compared to the equivalent Cu-NP treatment at neutral pH (pH = 6.9). The branchial Cu accumulation noted in this study was associated with the presence of gill injuries, which was more severe at pH 5 than pH 7. The severity gill injuries observed at pH 5 with both Cu treatments were accompanied by behavioural changes. These changes included the loss of position holding and swimming fatigue as well as increased mucus secretion which could indicate respiratory toxicity. The gill injury could lead to disturbances in gill function such as gaseous exchange and osmoregulation, explaining the observed swimming fatigue. Gill injury is a common response in fish acutely or chronically exposed to a variety of harmful agents, including $CuSO_4$ and Cu-NPs (Al-Bairuty *et al.*, 2013) and acidic pH (Chevalier *et al.*, 1985; Evans, 1987). The current study showed that $CuSO_4$ and Cu-NP at both pH values caused similar types of gill injuries (*e.g.* hyperplasia, necrosis, swollen mucocytes, *etc.*), with more severe pathologies at pH 5 than pH 7. These results suggest that the acidic water could cause respiratory or osmoregulatory failure. Recently, a study by Al-Bairuty *et al.* (2013) illustrated that exposure to 20 μg l^{-1} of either $CuSO_4$ or Cu-NPs at a neutral pH (6.9) caused similar types of gill injuries with a

material-type effect (more severity in the gill pathology with $CuSO_4$ than Cu-NP treatments).

Mucus secretion is mostly the first line of defence to metal and acidic exposure in the gills and can temporarily protect the underlying epithelium from injury (Plonka and Neff, 1969; Handy and Maunder, 2009). Mucus secretion and some swollen mucocytes were observed in this study, and had been seen in rainbow trout with $CuSO_4$ or Cu-NPs treatment at neutral pH condition (Al-Bairuty *et al.*, 2013) as well as with acidic pH (brook trout, Plonka and Neff, 1969). However, the mucus secretion observed in the current study was not sufficient to protect the gill epithelium from the injury arising from either $CuSO_4$ or Cu-NPs exposure at both pH values. Gill injury types that reported here for $CuSO_4$ or Cu-NPs at pH 7 and 5 have also been reported for waterborne exposure with other nanomaterials in trout (SWCNT, Smith *et al.*, 2007; TiO_2 NPs, Federici *et al.*, 2007) and low pH in carp (Chezhian *et al.*, 2011). A common type of the gill injury resulting from the exposure to nano metals and dissolved metals was oedema. Oedema is usually explained by inhibition of the branchial Na^+/K^+-ATPase that leads to solute accumulation in the epithelial cell and the osmotic influx of water. This explanation might apply to $CuSO_4$ which produced inhibition of branchial Na^+/K^+-ATPase activity at pH 7 and 5. The explanation for nano metals needs more investigation, but there may be a disturbance of normal values for hydrostatic pressure in the blood vessel and/or osmotic pressure due to damage the blood capillary system. The gill also showed hyperplasia, fusion, atrophy (shortening of lamellae), and lifting epithelium due to $CuSO_4$ or Cu-NPs exposures at pH 7 and 5, with a greater effect at pH 5 than 7. This extra effect of the Cu-NPs at pH 5 might be

due to the acidic pH causing the release of Cu ions from the surface of Cu-NP. Thus increasing the bioavailable Cu on the gill surface which could compromise oxygen uptake or add extra metabolic costs to respiration. Recently, exposure to silver nanoparticles has been shown to reduce blood pO_2 in fish (Bilberg *et al.*, 2010). The present incidence of aneurism in the branchial vasculature with $CuSO_4$ or Cu-NPs exposures at pH 7 and 5 that were noted here would suggest some interruption of gill perfusion that might contribute to a systemic hypoxia.

However, unlike the trout exposed to $CuSO_4$ for 7 days at pH 7, there was an internal accumulation of Cu in the livers of Cu-NP exposed trout only at day 4 from the same pH value. This suggests that Cu-NPs on/in the gill tissue were translocated to the blood in small quantities to detect subsequent Cu accumulate in the liver. The absence of measurable Cu from Cu-NPs in other internal organs (spleen and kidney) would also suggest any Cu-NPs were trapped in the liver. Grosell *et al.* (1998a) found that a newly gained Cu was accumulated quickly in the liver. There were no significant elevation of Cu accumulations in the spleen and kidney of trout from any Cu treatments at different pH values. The absence of measurable increases of Cu accumulation in the spleen is consistent with previous reports at normal pH condition (Shaw *et al.*, 2012).

4.4.3 Haematology and ionoregulatory disturbances during waterborne exposure

Haematology and blood chemistry in many cases may be used to assess the health of animals. Waterborne Cu exposure at neutral and acidic pH have been shown to have an effect on haematological parameters in fishes. The exposure to $CuSO_4$ at neutral pH

showed a decrease in the Hb, HCT, and RBC levels, which predicts induction of anaemia and haemodilution due to the gill injury, or impaired osmoregulation (see kidney injury). Exposure to Cu-NPs at pH 7 showed only a decrease in the number of RBC, which resulted due to inhibition of the production process of erythrocytes. At pH 5, the significant increase in the Hb after $CuSO_4$ exposure was accompanied by an increase in the number of RBC. Rainbow trout were likely subjected to reduced oxygen tension (due to the gill injury) and responded to that by an increased production of RBCs by the erythropoietic tissue to raise the oxygen carrying capacity of the blood. The same results for $CuSO_4$ exposure were observed with Cu-NP treatment at pH 5, with greater elevation in Hb levels compared to the $CuSO_4$ treatment. This could suggest that acidic pH caused increased risk of haemolysis of Cu-NP treatment. The trout could become acidotic, resulting in some changes in the haemoglobin. For example, the fish may try to produce more haemoglobin to replace the oxidised or denatured haemoglobin formed due to toxic metal and acid exposure. Nussey *et al.* (2002) showed transient changes in blood parameters of *Oreochromis mossambicus* following exposure to copper and zinc at neutral and acidic pH by causing opposite result (*e.g.*, acidic Cu exposure caused decreases in RBCs whereas at the neutral pH showed increased). The WBC or leukocytes are the immune reaction to counteract the chemicals. Increasing the WBC counts, especially after $CuSO_4$ exposure at pH 7 and pH 5, could be attributed to the stimulation of the immune system to produce immune cells. Although, these results differ from some published study by Nussey *et al.* (2002) that found an increase in WBC at pH 7 and a decrease at pH 5 of Cu exposure in *O. mossambicus*. Nano-Cu treatment at pH 7 showed a small transient change in WBCs count, but not a statistically

significant compared to the control. These could indicate that NPs may produce initial stimulation to the immune system, but this stimulation was not enough to increase the immune cells in the circulation. Acidic exposure to nano Cu treatment showed similar observation to that of $CuSO_4$ treatment which showed elevation in the WBC count. However, the nano Cu haematology results have not previously been described.

Copper is known to interfere with Na^+ balance, which is also affected by pH (Nussey *et al.*, 2002). In the current study, no pH effect was observed with $CuSO_4$ treatment in the plasma Na^+ because both pH values caused a decline in the Na^+ concentration. A similar observation was observed in Cu-NPs treatment, but more depletion of Na^+ was noted at pH 5 than 7. This could indicate that acidic water caused increased release of free Cu ions from Cu-NPs. Decreasing of Na^+ levels can be ascribed to the net loss of Na^+ through damaged gills and kidneys. Damage to the structure of gills following exposure to $CuSO_4$ or Cu-NPs at both pH values that was observed in the current study, with more severe lesions at pH 5 than pH 7 could cause the excretion of Na^+. Observation of a decline in the branchial Na^+/K^+-ATPase activity with exposure to $CuSO_4$ only at both pH values could indicate some pH-dependent inhibition of the Na^+ pump and a reduced ability to take up ions from the external medium. Chezhian *et al.* (2011) found that exposure *Cyprinus carpio* to acidic water (pH 4) caused a reduction in plasma Na^+ and Cl^- levels, and this may be due to partial inhibition of influx or direct acid stress on the gills. However, the Na^+ concentration in the gill tissues showed a pH effect which includes greater depletion at pH 5 than pH 7 with both $CuSO_4$ and Cu-NP treatments. This could be attributed to the severity gill lesions that were observed with both Cu treatments at pH 5

when compared to pH 7. One other study has shown that exposure of trout to $CuSO_4$ and Cu-NPs at neutral pH caused decline in the Na^+ concentration that are associated with a depletion in the branchial Na^+/K^+-ATPase activity (Shaw *et al.*, 2012). However the Na^+ levels in the kidney did not show any changes with both Cu treatments, but there is a reduction in the activity of Na^+/K^+-ATPase with the $CuSO_4$ treatment only at both pH values, with more depletion observed at pH 7 than pH 5. This could indicate that $CuSO_4$ is more bioavailable at neutral pH than acidic pH in the kidney, and this could induce depletion in the Na^+/K^+-ATPase activity which led to reduce the re-absorption of Na^+ via the renal tubules.

The plasma potassium concentration (K^+) in fish exposed to both Cu treatments at neutral pH showed a small decrease, with a transient change in K^+ levels of the gill, kidney and liver. This could be attributed to ion losses via the urine, with subsequent decrease influx due to inhibition of branchial Na^+/K^+-ATPase activity (Lauren and McDonald, 1985). At pH 5 of the Cu-NP treatment only showed a small elevation in the plasma K^+, with a greater effect at pH 5 than pH 7. A similar increase of K^+ levels was observed in the kidney. These elevations could induce to balance the osmotic differences in the intracellular fluid caused by the decline in Na^+ concentration.

A transient decrease in the plasma chloride with $CuSO_4$ (at pH 5 only) and Cu-NPs (at pH 7 only) that was observed in the current study and another study (Cu, Nussey *et al*., 1995) could be attributed to an excretion of Cl^- ions via the gill. Copper and Cu-NPs are known to produce gill injury at neutral pH (Al-Bairuty *et al.*, 2013). Similar types of gill injuries were observed with both Cu treatments at pH 7 and 5, with more severe effects at

acidic pH, and these injuries could induce impair in the function of chloride cells, which would lead to an impaired active ion uptake.

4.4.4 Oxidative stress during waterborne exposure

Oxidative stress is a well-known result of copper exposure (Bopp *et al.*, 2008; Hoyle *et al.*, 2007; Eyckmans *et al.*, 2011) and acidic toxicity (*e.g.*, caused DNA damage through excessive ROS production that have effects on blood cell development, Mai *et al.*, 2010) in fish. In the current study, the TBARS levels of the $CuSO_4$ treatment at both pH 7 and 5 did not show any changes in the gill. Changed in antioxidant status was observed here with fish exposed to $CuSO_4$ treatment at pH 7 which showed a decrease in the glutathione content in the gills compared to the control, with a material-type effect. The depletion of glutathione may be related to the ROS formation due to the accumulation of Cu at both pH values. Most notably, the Cu-NP treatment at both pH values did not show alterations in the TBARS, glutathione in the gills, unlike the $CuSO_4$ treatment which showed a decrease in the glutathione levels at pH 7. This may be related to the low bioavailability of the Cu-NPs via the water compared to the metal salt. In the liver, exposure fish to the $CuSO_4$ treatment at pH 5 showed an increase in TBARS levels compared to controls; although the glutathione content of the liver was conserved. At acidic pH of $CuSO_4$ treatment, the liver did not show detectable Cu accumulation, but a systemic hypoxia arising from gill injury due to the Cu exposure (Mustafa *et al.*, 2012) or acidic exposure could generate secondary ROS leading to oxidative stress in the liver. The nano-Cu treatment at pH 7 showed a decrease in the TBARS levels in the liver compared to the control, though the GSH content remained constant. However, the liver showed metal

accumulation from Cu-NPs treatment at pH 7 and therefore oxidative stress could be associated with ROS arising from the particles. In the kidney, exposure to Cu-NPs at acidic water showed a decline in the GSH contents when compared to the control, whereas the TBARS level did not show alteration. This could be attributed to a systemic hypoxia due to the gill injury.

However, there are no studies about the effect of acidic water on the mechanism of Cu-NPs toxicity, only one study by Shaw *et al.* (2012) illustrated the effect of $CuSO_4$ compared to Cu-NPs at neutral pH. Shaw *et al.* (2012) found that exposure of trout to $CuSO_4$ or Cu-NP at neutral pH showed material-dependent changes in the TBARS levels of the gill tissue (depletion with $CuSO_4$ than Cu-NP). Other metal NPs studies showed that exposure trout to TiO_2 NPs caused oxidative stress in the gill, gut and brain, but without noticeable metal accumulation in the affected organs (*e.g.*, increased TBARS levels; Federici *et al.*, 2007). Overall, the organs injury and alteration in the antioxidant enzyme activity observed here could have effects on the organ functions of fish and therefore on fitness, fish health and production characteristics of fish populations. Clearly, the health of aquatic organisms exposed to Cu-NPs may be affected by oxidative stress, as is the case for many other pollutants (Di Giulio *et al.*, 1989).

4.4.5 Internal organ pathology during waterborne exposure

4.4.5.1 On the liver

In fish, the liver is a main compartment for Cu metabolism and a target organ for Cu accumulation (Grosell *et al.*, 1996; Handy *et al.*, 1999). Similar types of injuries observed

here with either $CuSO_4$ or Cu-NPs at neutral and acidic pH (a pyknotic nuclei, lipidosis, hepatitis) is known for $CuSO_4$ or Cu-NPs treatments at the natural condition of pH (Al-Bairuty *et al.*, 2013). Pathological observation showed that exposure to $CuSO_4$ or Cu-NPs at acidic pH caused more severe injury in the liver than neutral pH, even though the livers from the acidic pH showed less Cu accumulation. This could suggest that acidic pH caused more systemic hypoxia than neutral pH. Minor changes observed here in the diameter of hepatocytes and hepatocytes nuclei that associated with increasing the number of melanomacrophage deposits with both Cu treatments at pH 7 and 5 could suggest the energy metabolism in the liver may be affected, and the function of these deposits involved with recycling of endogenous materials from damaged cells (Haaparanta *et al.*, 1996). Research by Suresh (2009) found that exposure *Tilapia mossambica* to cadmium chloride for 120 hours at pH 7.4 - 7.8 caused an increase in the average number and size of melanomacrophage, and Suresh (2009) suggest that these deposits could be considered as a biomarker of stress induced by the various toxicants which are present in the aquatic environment. Little detail about the pathological effect of metal or metal nanoparticles accompanied with acidic or neutral pH on the liver is known. Therefore, more investigation needs to explain the mechanism toxicity of Cu-NPs at acidic pH.

4.4.5.2 On the spleen

The spleen is one of the fish immune system involved in counteracting the effects of toxicants. Injuries reported here following exposure to $CuSO_4$ or Cu-NPs at pH 7 and 5 (foci of necrotic cell, vacuole formation) are known in fishes exposed to $CuSO_4$ (Osman *et al.*, 2009) or $CuSO_4$ and Cu-NP at neutral pH treatments (Al-Bairuty and Hundy,

unpublished). Less is known about the effect of acidic pH on the toxicity of $CuSO_4$ and Cu-NP on the spleen. Exposure to $CuSO_4$ or Cu-NPs at pH 7 and 5 showed similar injuries with more severe effects at pH 5 than pH 7. This severity could be ascribed to the systemic hypoxia at acidic pH. In the present study, both Cu treatments at pH 7 caused elevation in the proportion of red pulp with a concomitant depletion in white pulp and sinusoid space, whereas, reverse effects were observed at pH 5 in the proportion of red, white pulp and sinusoid space. Changes in the proportions of red and white pulp and the presence of lesions in the spleen are typical during waterborne exposure to $CuSO_4$ or Cu-NPs at neutral pH condition (Al-Bairuty and Handy, unpublished). Increasing the proportion of red pulp with both Cu treatments at neutral pH could suggest that the spleen was working to produce new blood cells to release into the circulation. However, a decrease in the proportion of red pulp that observed at pH 5 with both $CuSO_4$ and Cu-NPs treatments would suggest that the spleen is working to release blood cells to the circulation after removing damage blood cells from the circulation. An increase in the proportion of white pulp with both Cu treatments at pH 5 would suggest an immunological stress (*e.g.* Cu and Cu-NPs). Also, our study showed an increase in the presence of melanomacrophages deposits in the spleen with both $CuSO_4$ and Cu-NPs treatment at pH 7 and 5. This may suggest to the normal phagocytic functions. Increasing the number of melanomacrophage deposits may be due to involvement of melanomacrophage in detoxification processes or recycling of endogenous and exogenous materials (Herráez and Zapata, 1991).

4.4.5.3 On the kidney

The kidney of fish is involved in important functions relating to electrolyte and water balance and the maintenance of a stable internal environment. Following the exposure of fish to different toxic agents, histological changes have been found at the level of the tubular epithelium and glomeruli (Teh *et al.*, 1997). Similar types of pathologies observed here following exposure to $CuSO_4$ or Cu-NPs at both pH values are broadly similar to previous reports on fish. For example, in the rainbow trout (*Oncorhynchus mykiss*) following 10 days of exposure to 20 μg l^{-1} from either $CuSO_4$ or Cu-NPs at neutral pH condition, pathologies included degeneration of the renal tubule, necrotic cells in the haematopoietic tissue, elevated the number of melanomacrophage deposits throughout the kidney, as well as increased Bowman's space (Al-Bairuty *et al.*, 2013). However, the renal contents may become acidified in some renal segments, which may provide an interaction with toxic substances. Therefore, the pH accompanied with metal or metal nanoparticles could have an effect on kidney function, such as glomerular filtration and tubular re-absorption. Renal tubular cells contain a variety of transport enzyme systems, such as carbonic anhydrase, Na^+/K^+-ATPase, Na^+/H^+ exchange and Cl^-/HCO_3^- exchange (Seldin and Giebisch, 1985), and these enzymes could be affected by these materials at neutral and acidic pH. This argues is consistent with a depletion of Na^+/K^+-ATPase activity with $CuSO_4$ treatment at pH 7 and 5. The only prominent material-type effect of $CuSO_4$ on renal pathology was an increase in the number of melanomacrophage deposits compared to the Cu-NPs treatment and control group at pH 7 and 5. This is principally a haematopoietic response and is well known for $CuSO_4$ in fish kidney (Handy, 2003).

Conclusions

The current study has demonstrated that water with acidic pH caused more dissolution of Cu from Cu-NPs and reduced the size of aggregation than neutral pH. This could suggest that acidic water could change the surface charge of nanoparticles. Therefore, measuring the zeta potential of Cu-NP at acidic water is needed to further investigate these effects. Additionally, this study has provided the first detailed overview of organ pathologies and the physiological effects of Cu-NPs at acidic pH, and shows these are similar or less than that caused by $CuSO_4$ treatment in rainbow trout. Some physiological results showed the effect of $CuSO_4$ was greater than Cu-NPs treatments at both pH values. For example, Cu accumulation in the gill (increased), haematology (transient changes) and plasma electrolytes (decreased) showed greater changes with $CuSO_4$ than Cu-NPs, whilst, similar organ injuries were observed with both Cu materials. This could suggest that $CuSO_4$ is more bioavailabe than NP form at both pH values. It is well known that organ pathology of the $CuSO_4$ is not associated with direct Cu accumulation in the internal organs, because of secondary oxidative stress and hypoxia (gill injuries) during the metal exposure. The current study also showed pH-effects by causing more severe effects on organ structure with both Cu treatments at acidic pH than neutral pH, and this could be attributed to the acidic stress on the organs. Overall, the effects of acidic pH on metal toxicity may be due to several factors such as: stimulated mucous secretions due to H^+ ions which resulting free mucous able to chelate the metals, changes in toxic species of metal at different pH values, and/or competitive inhibition of metal ion (Cu^{2+}) uptake by H^+ ions at gill surface binding

sites. Therefore, further studies required to investigate the toxic mechanism of Cu-NPs at acidic and alkaline water pH.

Chapter 5

Histopathological and haematopoietic studies of waterborne exposure to bulk or titanium dioxide nanoparticles on gill and other major organ of rainbow trout (Oncorhynchus mykiss)

Hypotheses: Waterborne exposure to bulk TiO_2 and TiO_2 NPs induce different pathological effects in different target organs of trout as well as immunological effects due to differences in particle size or crystallinity.

Abstract

Engineered nanoparticles (NPs) are used in a wide range of consumer products and exposure modeling predicts releases of ng to low μg l^{-1} level of NPs into surface waters. Studies have demonstrated biochemical and ionoregulatory disturbance, as well as organ pathologies in fish during waterborne exposure to TiO_2 NPs, but less is known about the pathologies from ordinary bulk powder forms (micron scale) of the same materials. Therefore, this study aimed to describe the pathologies in gill, gut, liver, kidney, spleen, brain and muscle of juvenile rainbow trout exposed in a four replicate design (14 fish/tank) to either a control (no added Ti), or 1 mg l^{-1} of either bulk or TiO_2 NPs for 14 days using a semi-static exposure test. Fish were sampled at day zero and 14 for histology and spleen prints. Both treatments caused organ injuries, but bulk TiO_2 produced slightly more severe injuries than the TiO_2 NPs. Pathologies included hyperplasia and clubbed tips in the gills; necrosis and vacuole formation in the gut mucosa; pyknotic nuclei and hepatitis-like injuries in the liver; renal tubule separation and necrosis in the haematopoietic tissue in the kidney; evidence of necrosis and elevated numbers of melanomacrophage deposits in the spleen were observed. The spleen also showed a decrease in the proportion of red pulp and an increase in the proportion of white pulp and sinusoid space with both TiO_2 treatments compared to the control (all statistically significant, $P < 0.05$). Additionally, a quantitative analysis of spleen prints showed both TiO_2 treatments caused an increase in the proportion

of erythrocytes abnormalities (swollen erythrocytes, cells with a dividing nuclei and membrane abnormalities), immature cells (haemocytoblast) and a decrease in the proportion of mature cells (erythrocytes) (all statistically significant, ANOVA, $P < 0.05$). The proportion of neutrophils was increased with bulk TiO_2 only (ANOVA, $P < 0.05$). The brain showed regional differences in the injuries that included necrosis, and vacuole formation in the telencephalon of the brain, as well as enlarged blood vessel on the surface of cerebellum. The trout muscle showed also an increase in the extracellular space of the muscle. Overall, the injuries observed in most organs were similar, but slightly more severe with bulk treatment than TiO_2 NPs.

5.1 Introduction

So far, little is known about the fate and behaviour of NPs that enter into the bodies of aquatic organisms such as fishes or their subsequent biological effects (reviews, Handy *et al.*, 2011; Shaw and Handy, 2011). Nonetheless, this research indicates that nanomaterials, including metal-containing NPs can have adverse effects on the body systems of fishes (*e.g.*, Cu-NPs, Griffitt *et al.*, 2007; TiO_2 NPs, Federici *et al.*, 2007). However, the toxicity data have not considered all the possible sizes and forms of TiO_2. Titanium dioxide naturally exists in anatase, rutile and brookite crystal forms (Mo and Ching, 1995), and is used commercially as a bulk micron scale powder, and now also as a nano scale TiO_2.

The ordinary form of bulk TiO_2 powder that is mostly micron scale particles and not considered toxic, has been used as an inert digestibility indicator in fish nutrition studies

(Mamun *et al.*, 2007), and as a negative control in respiratory toxicity experiment (Warheit *et al.*, 1997). Titanium dioxide in nano-form is used in a variety of consumer products, such as toothpastes, sunscreens, cosmetics, food products (Gurr *et al.*, 2005), paints and specialist coatings (Kandavelu *et al.*, 2004; Guarino *et al.*, 2008), and in industrial photocatalytic processes (Guillard *et al.*, 2003a; Guillard *et al.*, 2003b; Zhang *et al.*, 2006). Therefore TiO_2 NPs are probably already present in the environment, but a current level in water is unclear. Modelling approaches have suggested that the concentrations of TiO_2 NPs in the aquatic environment could be as high as 0.7-16 $\mu g\ l^{-1}$ (Mueller and Nowack, 2008). The pathological effects of aqueous exposure to bulk TiO_2 or TiO_2 NPs and information on the target organs in fish are only partly known. Waterborne exposure to 1 mg l^{-1} of TiO_2 NPs for 14 days caused respiratory distress gill pathologies including oedema and a hyperplasia-like thickening of the primary lamellae in rainbow trout (*Oncorhynchus mykiss*). Ingestion of the TiO_2 NP water also caused fusion and erosion of the villi tips in the gut. Internal organs also showed pathology including fatty change and condensed nuclear bodies in the liver of rainbow trout (Federici *et al.*, 2007). Juvenile carp also showed gill pathology (oedema and thickening of the gill lamellae) and foci of necrosis in the liver after 20 days of exposure to 100 and 200 mg l^{-1} TiO_2 NPs (Hao *et al.*, 2009), suggesting that TiO_2 NP pathology is evident in more than one species of fishes. In mice, the intra-gastric administration of 250 mg kg^{-1} (BW) of anatase TiO_2 NPs for 30 days caused a decline in the body weight of the animals and liver pathology (hepatotoxicity with congestion of interstitial vessels) that lead to a loss of liver function (Duan *et al.*, 2010). Whilst, the intraperitoneal injection of TiO_2 NPs (5, 50, 150 mg kg^{-1} BW) for 45 days

caused spleen injuries and apoptosis that leading to the reduction of immunity in mice (Li *et al.,* 2010). Handy *et al.* (2011) reviewed the alteration of the proportion of red and white pulp in the spleen of fishes exposed to TiO_2, raising concerns for the function of the haematopoietic system. Injection studies with fish have also been done. Scown *et al.* (2009) found that trout intravenously injected with 100 µg of TiO_2 NPs accumulated Ti in the kidney, but this had a minimal effect on kidney function. In mice, a single oral gavage of 5 g kg^{-1} body weight of TiO_2 NPs for 2 weeks exhibited swelling in renal glomerulus and filling of renal tubule with proteinic liquids (Wang *et al.*, 2007).

Brain pathologies have been observed in fish and mice following exposure to NPs. In fish, the exposure to 0.1 mg l^{-1} TiO_2 NPs for 14 days exhibited a few necrotic cells in the cerebrum (Federici *et al.*, 2007); whilst the exposure to other NPs (0.25-0.5 mg l^{-1} of SWCNT for 10 days) also showed necrotic cell bodies and small vacuole formation in the optic lobe and cerebrum of trout (Smith *et al.*, 2007). Mice, exposure to 5 g kg^{-1} body weight of TiO_2 NPs by a single gut gavage exhibited brain injury such as vacuole formation in the neurons of the hippocampus two weeks post-ingestion. The number of these vacuoles was increased in the 80 nm TiO_2 NPs and 155 nm of fine TiO_2 particles compared with control, which indicated that fatty degeneration was induce in this region of brain by TiO_2 (Wang *et al.*, 2007). Pathological effects of waterborne exposure to bulk TiO_2 or TiO_2 NPs on muscle of fish are mostly unknown.

In order to investigate the intrinsic hazard potential of TiO_2 NPs that may have entered into humans and animals from the environment, the histopathological and haematopoietic effects of the bulk material or TiO_2 NPs on rainbow trout after 14 days of

waterborne exposure was investigated. The goal of the present study was to compare and contrast the effects of bulk TiO_2 particle with TiO_2 NPs on organ integrity to determine any nano-specific pathologies and to investigate the effects of these materials on the immune system by using the spleen prints method.

5.2 Materials and methods

5.2.1 Experimental design

The design of this experiment is based on the method used by Federici *et al.* (2007) for TiO_2 NPs with minor modifications. Juvenile rainbow trout (mean ± S.E.M., n = 182, 23.96 ± 0.70 g fish) were obtained from a local supplier (Torre fishery, Watchet, Somerset) and maintained in flowing, aerated, dechlorinated Plymouth tap water (see below for ionic composition) for three weeks prior to onset of experimentation. Background concentrations of Ti in Plymouth tap water was below the instrument detection limit (< 1.3 μg l^{-1} n = 6). Stock fishes were fed twice daily with a commercial trout diet (EWOS, Westfield, UK) until 48 h prior to experimentation. Fish were individually weighed and then transferred to twelve experimental glass aquaria containing 20 l of continuously aerated water (14 fish/tank, four tanks/treatment, equating to 56 fish/treatment), and left overnight (16 h) prior to the beginning of the experiment. Fish were exposed in a quadruplicate to one of the following treatments for 14 days using a semi-static exposure regime (80 % water change every 12 h with re-dosing after each change): control (freshwater only, no added Ti), 1 mg l^{-1} of either bulk TiO_2 or TiO_2 NPs (see below for stock solutions). Briefly, each tank was dosed with 2 ml of the appropriate stock dispersion (see below) with the aeration rapidly

spreading the material in the tank water. An 80 % water change was conducted every 12 h, and the relevant tanks re-dosed with either 1.6 ml bulk TiO_2 and TiO_2 NP as appropriate A semi-static exposure regime was used to maintain water quality and the exposure concentrations. The concentration of TiO_2 NPs was selected after considering the concentrations used to produce oxidative stress and organ pathologies in rainbow trout in a previous study (Federici *et al.*, 2007). The exposure time of 14 days was chosen to reflect this dosimetry and allow some physiological or biochemical responses to the exposure, but also considering the ethical constraint of using the minimum exposure period possible to achieve the scientific objectives. Fish were fed twice (1.5 % body weight) during the experiment (day 8 and 12), immediately after the water change and prior to re-dosing to minimise the risk of ingestion of TiO_2 during feeding. Samples of water were collected once daily immediately after the water change and pH (7.2 ± 0.2); temperature (15 ± 1°C), dissolved oxygen concentration (HACH HZ40d multi meter, 8.3 ± 0.5 mg l^{-1}) and total ammonia (HI95715, Hanna Instruments, 0.008 ± 0.003 mg l^{-1}) measured as mean ± S.D, *n* = 36-156 samples. There were no treatment differences in water quality between tanks (ANOVA, $P > 0.05$).

Photoperiod was 14 h light: 10 h dark. The electrolyte composition of the dechlorinated Plymouth tap water used was (Means ± S.D., *n* = 36-156 measurements, in mmol l^{-1}) Ca^{2+}, 0.52 ± 0.07; K^+ 0.06 ± 0.01; Mg^{2+}, 0.17 ± 0.01; Na^+, 0.75 ± 0.11. Fourteen fish were randomly sampled at day 0 (initial fish from the stock) for tissue ions, tissue biochemistry (presented in Boyle *et al.*, 2013) and histopathology as well as spleen prints

(presented here, see below). After 14 days, samples were collected from 8 fish (2 fish/tank) from each treatment to assess the same parameters that measured at day zero.

5.2.2 Titanium dioxide nanoparticles and bulk stock dispersion

Nano particle and bulk forms of TiO_2 were obtained from DeGussa AG ("Aeroxide" P25, supplied by Lawrence Industries, Tamworth, UK) and ACROS (New jersey, USA) respectively. The P25 material was the same batch used by Federici *et al.* (2007), and as stated by the manufacturer's revised information, was 25 % rutile and 75 % anatase TiO_2, with > 99 % purity (as TiO_2, maximum impurity stated was 1 % Si), with a mean particle size of 21 nm and a specific surface area of 50 ± 15 m^2 g^{-1}. According to the manufacturer's information, the purity of bulk TiO_2 was 98-100.5 %. Analysis of transmission electron micrographs (TEM, JEOL 12000EXII, Tokyo, Japan) showed a mineral composition was (means ± S.D., n = 10 micrographs) 25 ± 5 % rutile and 75 ± 5 % anatase. Stock dispersions (500 ml) of 10 g l^{-1} of either bulk TiO_2 or TiO_2 NPs (nominal concentrations) were prepared in ultrapure water (Milli-Q®, Millipore Corporation) and stored in acid washed Nalgene bottles. Stocks were stirred overnight (2400 rpm, Magnetic Stirrer SM1, Stuart Scientific, UK) and then subsequently the entire 500 ml was sonicated (500 ml, 4 °C, 1 h, 35 kHz frequency, Fisherbrand FB 11010, Germany). The sonication step was repeated each day immediately prior to dosing experimental tanks to ensure reasonably dispersed material was added to the tanks. TEM analysis of nominal 1 g l^{-1} aliquots of stock suspensions (Fig. 5.1) showed the crystalline structure of particles with a primary particle size of (means ± S.D., n = 100) 134 ± 42 nm and 24 ± 10 nm of bulk TiO_2 and TiO_2 NPs respectively. Dispersion of bulk TiO_2 and TiO_2 NPs in MilliQ water were

analysed in nominal 10 mg l^{-1} aliquots of the stock suspensions (prepared and dispersed as above prior to dilution in MilliQ water) using nanoparticle tracking analysis (Nanosight LM 10, Nanosight, Salisbury, UK). Mean aggregate sizes of TiO_2 were (means ± S.D., n = 3): 211 ± 3 nm and 96 ± 40 nm for bulk TiO_2 and TiO_2 NPs respectively. Particle size distributions of bulk TiO_2 and TiO_2 NPs were significantly different (P = 0.023, n = 3, two sample Kolmogorov-Smirnov test) (presented in Bolye *et al.*, 2013).

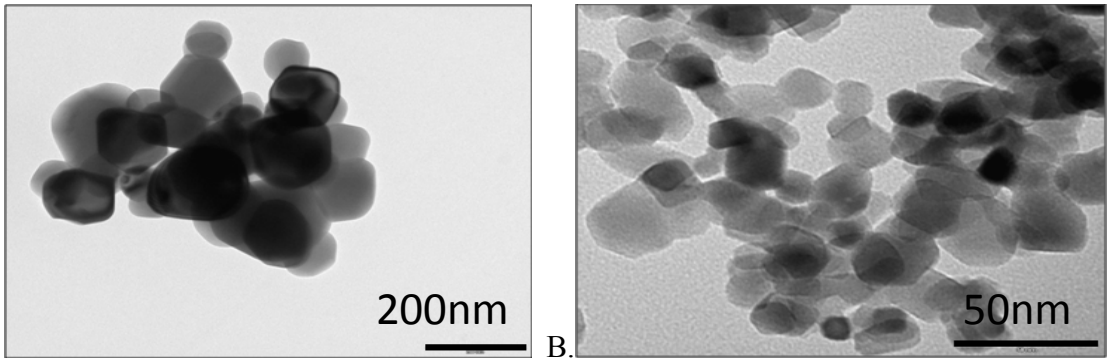

Figure 5.1 Transmission electron microscope (TEM) images showing (A) bulk TiO_2 and (B) TiO_2 NPs respectively, scale bar = 200 and 50 nm in 1 g l^{-1} stock dispersion made in Milli-Q water of bulk and TiO_2 NPs, respectively.

5.2.3 Histopathology

5.2.3.1 Structural analysis using light microscopy

Histological examinations were performed exactly as described in Chapter 2 (see 2.5 & 2.9). Briefly, at day 0 and 14, fish (eight initial fish at day 0, and eight fish/treatment at day 14, two fish/tanks) were terminally anaesthetised with MS222 and organs (gill, gut, liver, spleen, kidney, muscle and whole brain) carefully collected into buffered formal saline and then the specimens were processed in an ascending grades of alcohol. Tissue

samples were impregnated in paraffin and cut with a microtome at 7 μm thickness. Quantitative histological measurements were also made as described in Chapter 2 (see 2.9).

5.2.3.2 Ultrastructural analysis using transmission electron microscopy (TEM)

For utrastructural studies, gill samples were prepared as described by Mustafa *et al.* (2012). Samples from 6 rainbow trout fish were cut in small pieces and immediately fixed in glutaraldehyde (2.5 % glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2 made up with filtered freshwater) for overnight at 4 °C. After 2 washes in cacodylate buffer (2 minutes in each step), samples were post-fixed in 1 % aqueous osmium tetroxide (OsO_4) in the same buffer for 2 hours to provide contrast to the image. Samples were rinsed thoroughly with sodium cacodylate buffer (2 times at 15 minutes) followed by dehydration through a graded serial ethanol concentration (70 to 100 %) for 2 minute in each concentration. Then samples were placed in a mixture of absolute alcohol and Spur's resin as follows: 75 % alcohol + 25 % resin, 50 % for both alcohol and resin, 25 % alcohol and 75 % resin subsequently in each step for 24 h (for all 3 days). At day 4, samples were left in 100 % resin for overnight. Finally, the samples were embedded in resin to make the capsule for sectioning. Semi-thin sections were cut with an ultramicrotome on grids before embedding and then positively stained with methylene blue/azur II to enable orientation of the tissue for ultrathin sectioning. Then, the ultrathin sections for gill TEM were stained with 2 % uranyl acetate, followed by 1 % lead citrate for 15 minutes in each stain, to produce high contrast stain for cellular and tissue components. These sections were examined under a transmission electron microscope (JEOL, TEM-1200 EX-2) at 120 kV, and imaged taken by using a soft imaging system, Mega View 3.

5.2.4. Spleen print

Spleen prints were prepared exactly as described in Chapter 2 (see 2.10). Briefly, spleen prints were collected from the trout at day zero and 14, then stained with May-Grünwald Giemsa stain and scored the cellular pathologies as well as haematopoietic cells as described in Chapter 2 (see 2.10.1).

5.2.5 Statistical analysis

All resulting data from the experiment were presented as mean ± S.E.M. and analysed by using Stat Graphics Plus Version 5.1 as described in Chapter 2 (see 2.11).

5.3. Results

5.3.1 Aqueous exposure to titanium dioxide nanoparticles

Aqueous exposure to either bulk or TiO_2 NPs did not cause mortality. Total titanium accumulation in the tissues of trout was seen in the gills of fish from bulk (43.3 ± 12.1 μg g^{-1}) and TiO_2 NPs (31.3 ± 8.1 μg g^{-1}) treatments after 14 days of exposure compared to controls (1.4 ± 0.5 μg g^{-1}) (Means ± SEM, n = 8), with 31 and 22 fold increase in Ti metal in/on the gill tissue compared to the control (ANOVA, $P < 0.001$). There was no indication of increasing Ti concentrations in the intestine, liver, kidney, spleen and brain in the fish from the TiO_2 treatments compared to controls at day 14 (ANOVA, $P > 0.05$). The the levels in the intestine were (mean ± S.E.M., n =7/8) 4.8 ± 2.5, 11.4 ± 3.8, 8.3 ± 3.8 in control, bulk and TiO_2 NP treatments, respectively. In the liver, the Ti levels were 1.9 ± 0.4 in control, 0.8 ± 0.2 in bulk, 2.3 ± 0.2 in TiO_2 NPs. The Ti levels in the kidney were 3.6 ±

1.2, 2.3 ± 0.6, 4.4 ± 1.8 in control, bulk and TiO_2 NP treatments, respectively. In the spleen, the Ti concentrations were 8.2 ± 3.3 in control, 5.4 ± 1.6 in bulk, 11.1 ± 4.7 in TiO_2 NPs. The Ti levels in the brain were 0.9 ± 0.3, 2.0 ± 0.8, 1.9 ± 0.6 in control, bulk and TiO_2 NP treatments, respectively. The full data are available on Ti concentrations and other metals are presented in Boyle *et al.* (2013).

5.3.2 Histological observation on the gill

5.3.2.1 Using light microscopy

Gills from the control groups showed normal histology and the absence of lesions at the end of the experiment (Fig. 5.2). Waterborne exposure to both forms of TiO_2 caused similar types of gill pathology. After 14 days of exposure, all 8 fish examined from each of the bulk and nano TiO_2 treatments showed pathologies included hyperplasia of the primary and secondary lamellae, evidence of oedema and clubbed tips in the secondary lamella, as well as swollen mucocytes in the gill epithelium (Fig. 5.2). A quantitative analysis of the incidence of gill injuries confirmed these observations with a statistically significant increase in the incidence of hyperplasia (means ± S.E.M., $n = 8$) in the TiO_2 NP treatment (7.4 ± 0.6 %), and the bulk TiO_2 treatment (6.5 ± 0.6 %) compared to the unexposed control (0.7 ± 0.3 %) at day 14 (ANOVA, $P < 0.001$, Fig. 5.2). The incidence of clubbed tips on the lamellae increased from (means ± S.E.M., $n = 8$) 2.2 ± 0.7 % in the controls to 11.1 ± 1.0 and 18.1 ± 1.4 % in the bulk and TiO_2 NPs treatments respectively, and with a statistically significant material-type effect (greater in fish exposed to TiO_2 NPs, $P < 0.001$). Similarly, the incidence of swollen mucocytes increased from (means ± S.E.M., $n = 8$) 2.7 ± 0.7 % in

the controls to 17.9 ± 2.0 and 11.9 ± 2.0 % in the bulk and TiO_2 NPs treatments respectively, also with a statistically significant material-type effect (worse with bulk TiO_2, $P < 0.001$). There were also statistically significant increases in the incidence of aneurisms on the lamellae, fusion of lamellae and necrotic cells in the gill epithelium with both the bulk and nano treatments, but these all remained below a 5 % incidence.

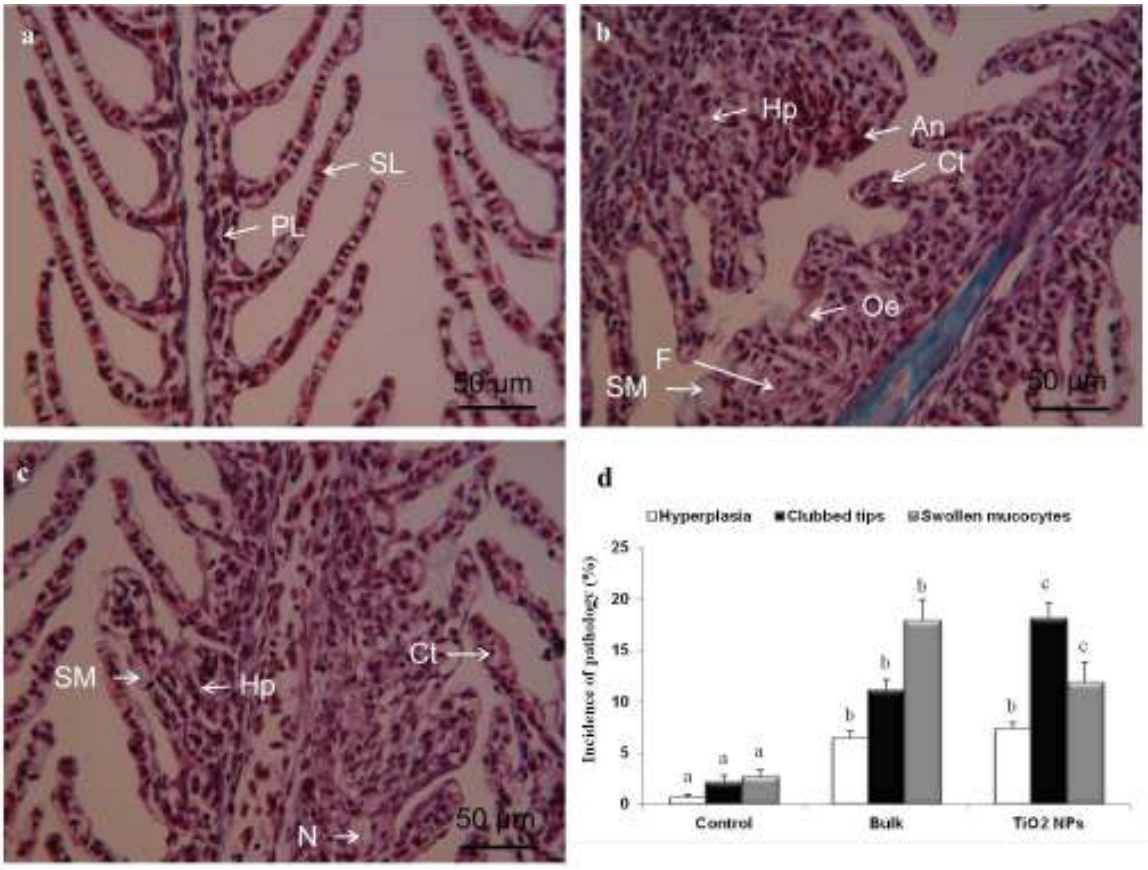

Figure 5.2 Gill morphology in rainbow trout after 14 days of exposure to (a) control, (b) 1 mg l^{-1} bulk TiO_2 (c) 1 mg l^{-1} TiO_2 NPs, using a semi-static exposure method. The gills of control fish showed normal primary (PL) and secondary (SL) lamellae structure, while exposure to 1 mg l^{-1} of either bulk or TiO_2 NPs exhibited similar types of lesion. These lesions included hyperplasia (Hp), aneurisms (An), clubbed tips (Ct), fusion (F), swollen mucocytes (SM), oedema (Oe), and necrosis (N). Some types of lesion (lifting epithelium, aneurisms and swollen mucocytes) were greater with bulk than TiO_2 NPs, but other types (clubbed tips) were opposite. Scale bar indicates magnification. Sections were 7 µm thick and stained with Mallory's trichrome. Panel d) % secondary gill lamellae showing hyperplasia (Hp, white bars), clubbed tips (CT, black bars) and swollen mucocytes (SM, grey bars). Data are means ± S.E.M. (n = 8). Different lower case letters denote significant difference between treatments ($P < 0.05$).

5.3.2.2 Using TEM

For the TEM study, gills samples from control groups exhibited typical regular structures with no lesions. This included ordered and parallel arrangements of cells in the epithelium, normal blood sinuses separated by pillar cells (supporting cells), as well as, a thick stratified squamous epithelium that contained chloride cells at the base of the secondary lamellae (Fig. 5.3 a-d). Exposure to bulk and TiO_2 NP treatments showed similar changes in the structure of gill lamellae that included disorganisation of lamellae, deformation of red blood cells and disorganisation of pillar cells (5.3 b & c). The organelles morphology of control groups such as the mitochondria and endoplasmic reticulum showed normal structures (Fig. 5.3 d). Exposure to the bulk material showed 33 % irregular meshes of the endoplasmic reticulum and 67 % of mitochondria rich cells. The mitochondria rich cells exhibited an increase in the number of mitochondria and some of the mitochondria were damaged (Fig. 5.3 b & e). Whereas, lamellae exposed to TiO_2 NPs showed similar changes to that observed with bulk treatment (Fig. 5.3 f).

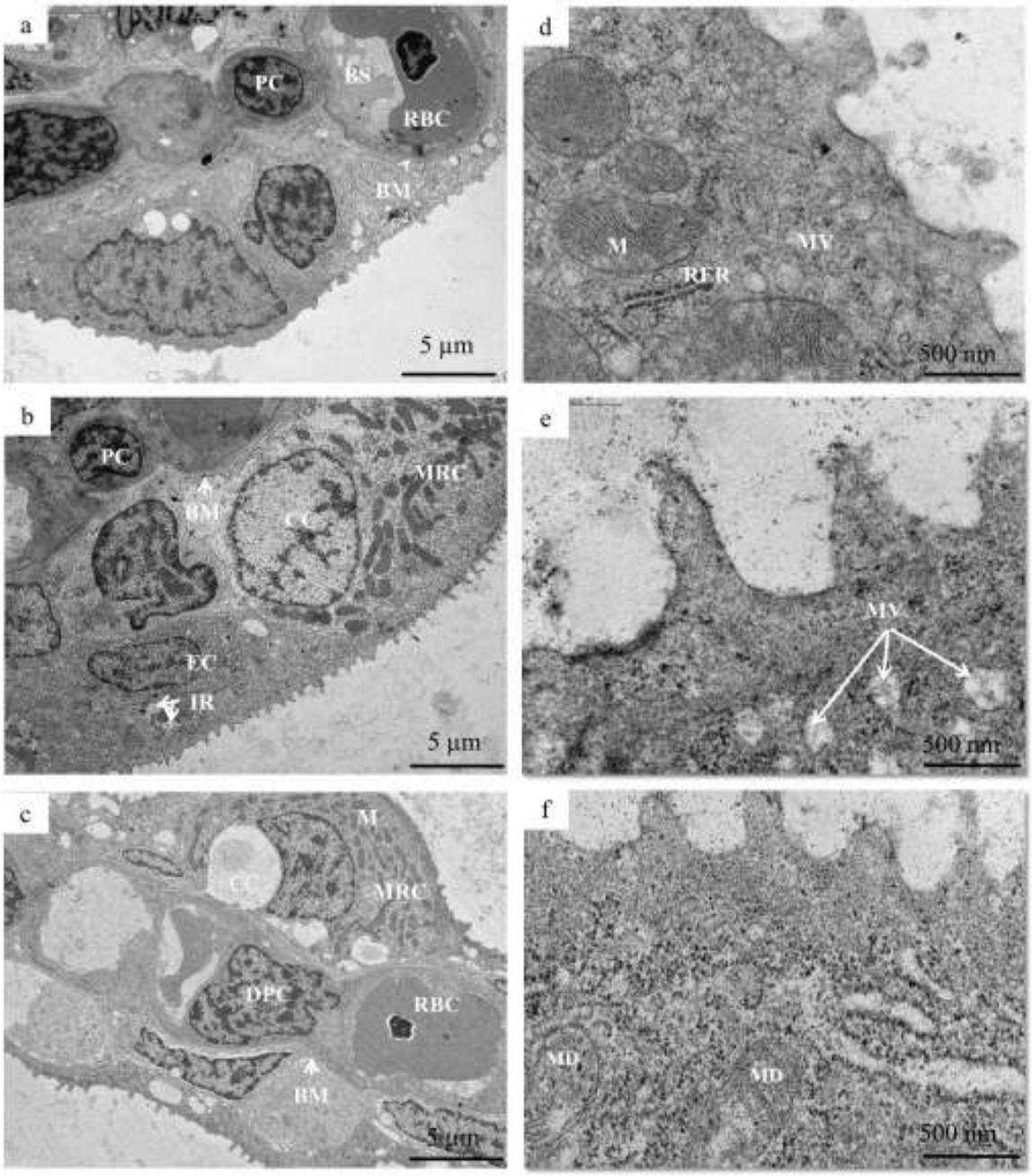

Figure 5.3 Ultrastructural changes in trout gill lamellae viewed by TEM at low magnification (left column) and high magnification (right column). At low magnification the panels include (a) control, (b) 1 mg l^{-1} bulk TiO_2, (c) 1 mg l^{-1} TiO_2 NPs at day 14. At high magnification the panels include (d) control, (e) 1 mg l^{-1} bulk TiO_2, (f) 1 mg l^{-1} TiO_2 NPs at the same time point. Basement membrane (BM), pillar cell (PC), blood space (BS), red blood cell (RBC), mitochondria rich cell (MRC), mitochondria (M), rough endoplasmic

reticulum (RER), epithelial cell (EC), chloride cell (CC), irregular meshes of tubular reticulum (IR), mitochondria damage (MD), disorganisation of pillar cells and deformation of red blood cells (DPC), (MV) micro vesicles.

5.3.3 Histological observation on the Gut

The hind gut of fish from the freshwater control showed normal histology, but minor injuries were noted in fish exposed to either bulk or TiO_2 NPs. The exposure to 1 mg l^{-1} of bulk or TiO_2 NPs showed evidence of columnar epithelial cells with pyknotic nuclei, vacuole formation in the cells, and abnormalities in the shape of the nucleus in the cells in all eight fish from both TiO_2 treatments (Fig. 5.4). Lifting of the gut epithelium, necrosis, and erosion of the intestinal villi tips were observed in 5/8 fish examined per each TiO_2 treatment. A quantitative analysis of gut mucosa dimensions showed a statistically significant increase in the height of villi with both TiO_2 treatments relative to the control. Values were (μm, mean ± S.E.M., n = 8); control, 318.9 ± 6.1; bulk 354.8 ± 6.9; TiO_2 NP 351.3 ± 8.6. There was also a small but statistically significant decrease in the thickness of muscularis layer with both TiO_2 treatments compared to controls (ANOVA, $P < 0.05$), with a 3 % difference between treatments (Table 5.1).

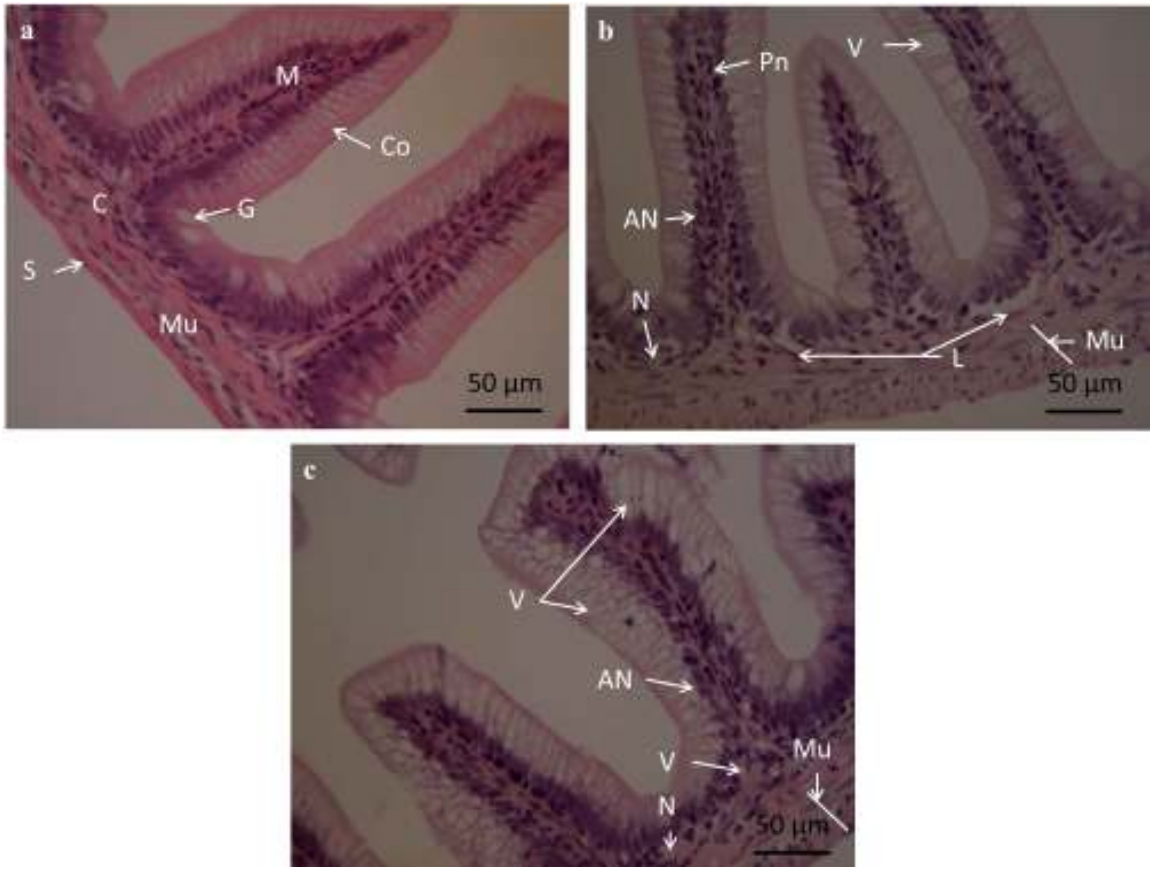

Figure 5.4 Gut morphology in rainbow trout after 14 days of exposure to (a) control, (b) 1 mg l^{-1} bulk TiO_2, (c) 1 mg l^{-1} TiO_2 NPs. The gut of control showed normal histology of columnar cell (Co) and goblet cell (G) in the mucosa layer (M), compactum layer (C), muscular layer (Mu) and serosa layer (S). Both treatments showed similar injuries included evidence necrosis in the epithelium of mucosa (N), lifting epithelium (L), vacuolation in the mucosa layer (V), pyknotic nuclei (Pn), and alteration in the nuclear shape (AN). Scale bar indicates magnification. Sections were 7 µm thick and stained with H&E.

Table 5. 1 Effects of waterborne exposure to 1 mg l^{-1} of either bulk or TiO_2 NPs after 14 days on the morphometrics of the hind gut of rainbow trout.

Parameters		Time/ Days	Controls	1 mg l^{-1} Bulk	1 mg l^{-1} TiO_2 NPs
Dimension of the villi (μm)	Height	0	307.1 ± 2.5		
		14	318.9 ± 6.1	354.8 ± 6.9 *[a]	351.3 ± 8.6 *[a]
	Width	0	107.2 ± 3.2		
		14	106.2 ± 3.4	99.0 ± 1.9	105.0 ± 2.5
Dimension of the columnar cells and cell nuclei (μm)	Columnar cells	0	47.8 ± 0.7		
		14	48.2 ± 1.0	50.2 ± 1.0	50.2 ± 0.5
	Columnar cells nuclei	0	14.8 ± 0.2		
		14	14.7 ± 0.1	14.1 ± 0.0 *[a]	14.2 ± 0.1 *[a]
Thickness of hind gut layers (μm)	Mucosa and sub-mucosa	0	81.7 ± 0.9		
		14	82.4 ± 1.2	85.0 ± 1.8	85.7 ± 1.0 *
	Compactum	0	3.6 ± 0.3		
		14	3.4 ± 0.4	3.8 ± 0.2	3.4 ± 0.3
	Muscular	0	12.7 ± 0.6		
		14	12.5 ± 1.2	9.5 ± 1.5 *	9.1 ± 0.8 *[a]
	Serosa	0	2.0 ± 0.2		
		14	1.7 ± 0.1	1.7 ± 0.2	1.7 ± 0.1

Data are means ± S.E.M. (*n* = fish/treatment). (*), significant different from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). (a), significant difference between control (14 days) within row (ANOVA, $P < 0.05$).

5.3.4 Histological observation on the liver

Histological examination of trout livers from the control showed normal histology and lesions were absent (Fig. 5.5). The exposure to either bulk or TiO_2 NPs showed moderate changes compared to the control with occasional necrotic cell, lipidosis, pyknotic nuclei, inflammation like hepatitis, degeneration of hepatocytes and increased the number of melanomacrophage deposits in 6/8 fish from each TiO_2 treatments (Fig. 5.5). For the latter changes, the number of deposits (means ± S.E.M, counts/field of view at x 400 magnification, 53,200 μm^2, n = 8) increased from 6.5 ± 1.3 (control) to 17.0 ± 0.7 (bulk) and 18.0 ± 3.0 (TiO_2 NPs). The difference from the control were statistically significant, but with no material-type effect (Table 5.2).

A quantitative analysis confirmed changes in the liver of trout which showed that both treatments (bulk or TiO_2 NPs) caused a small but statistically significant increase (ANOVA, $P < 0.05$) in the diameter of hepatocytes and hepatocytes nuclei compared to the control (Table 5.2). The proportion of the hepatic area and sinusoid space showed a minor, but statistically significant changes that included a decrease in the proportion of hepatic area and an increase in the proportion of sinusoid space with bulk and TiO_2 NPs treatments compared to unexposed controls (ANOVA, $P < 0.05$; Table 5.2). A minor material-type effect was observed in the proportion of hepatic area and sinusoid space (worse with bulk than TiO_2 NPs).

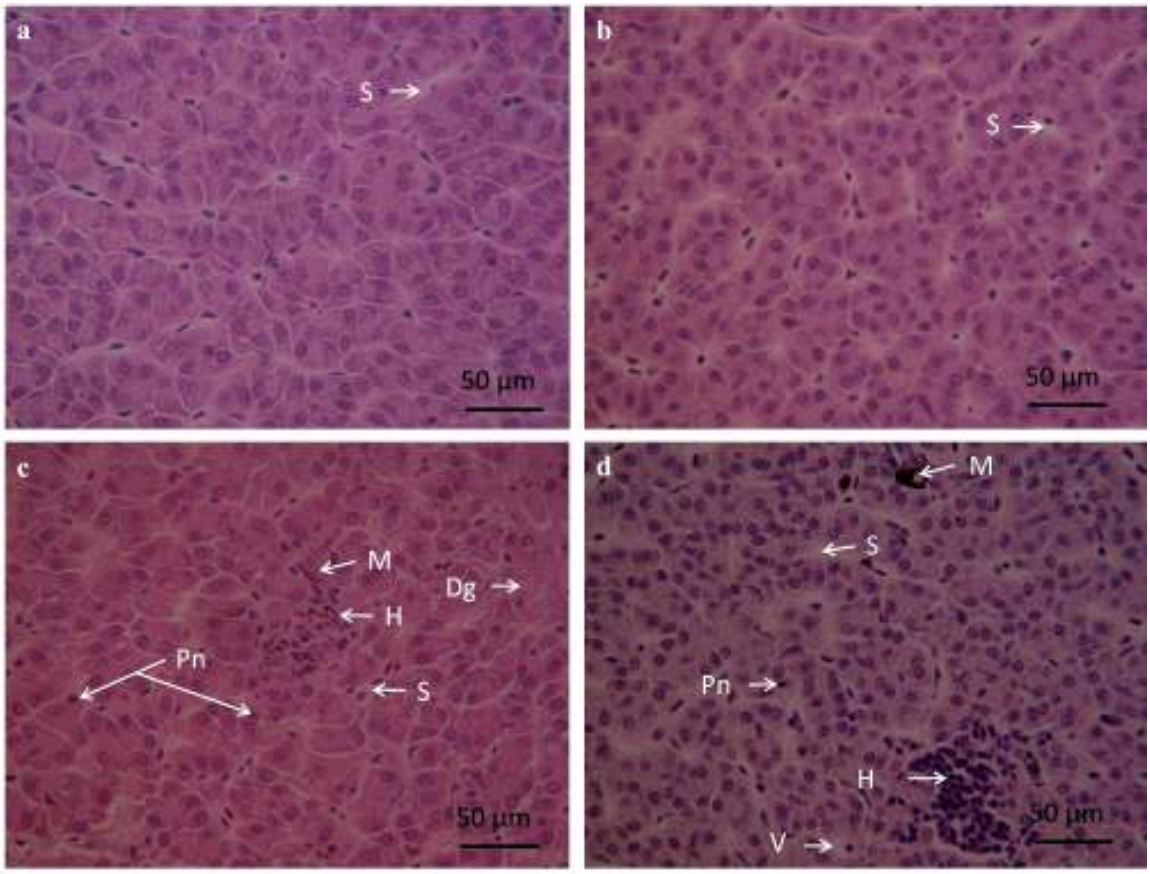

Figure 5.5 Liver morphology of rainbow trout after 14 days of exposure to (a) control day 0 (initial fishes), (b) control, (c) 1 mg l^{-1} of bulk and (d) 1 mg l^{-1} of TiO_2 NPs. The liver of control showed normal histology with normal sinusoid space (S). Both TiO_2 treatments showed injuries included vacuole formation (V), pyknotic nuclei (Pn), melanomacrophage deposition (M), hepatitis-like injury (H), and degeneration of hepatocytes (Dg). Scale bar indicates magnification. Sections were 7 µm thick and stained with H&E.

Table 5.2 Quantitative analysis of changes in the liver of rainbow trout after 14 days of waterborne exposure to 1 mg l^{-1} of either bulk or TiO_2 NPs.

Parameters	Time/ Days	Control	1 mg l^{-1} Bulk	1 mg l^{-1} TiO_2 NPs
Diameter of hepatocytes (µm)	0	21.9 ± 0.3		
	14	22.9 ± 0.1 *	26.1 ± 0.2 *[a]	26.4 ± 0.2 *[a]
Diameter of hepatocytes nuclei (µm)	0	8.8 ± 0.1		
	14	8.9 ± 0.0	9.6 ± 0.1 *[a]	9.7 ± 0.0 *[a]
Ratio of nuclei to hepatocytes diameter	0	0.40 ± 0.00		
	14	0.39 ± 0.00 *	0.37 ± 0.00 *[a]	0.37 ± 0.00 *[a]
Percentage of hepatic area	0	94.3 ± 0.2		
	14	94.3 ± 0.3	91.7 ± 0.4 *[a]	92.6 ± 0.2 *[ac]
Percentage of sinusoid space	0	5.7 ± 0.2		
	14	5.7 ± 0.3	8.3 ± 0.4 *[a]	7.4 ± 0.2 *[ac]
Number of melanomacrophage deposits	0	5.0 ± 1.1		
	14	7.0 ± 1.3	17.0 ± 0.8 *[a]	18.0 ± 3.1 *[a]

Data are means of proportional areas, or means ± S.E.M. n = 8 fish/ treatments. (*), significantly different initial fish (stock fish at time zero, ANOVA, $P < 0.05$). (a), significant difference from control within row (ANOVA, $P < 0.05$). (c), significantly different between bulk and TiO_2 NPs (ANOVA, $P < 0.05$).

5.3.5 Histological observation on the spleen

Spleens of trout from control groups were shown normal histology with normal proportion of red and white pulp and small deposits of melanomacrophage (Fig. 5.6). The exposure to 1 mg l^{-1} of either bulk or TiO_2 NPs for 14 days showed similar injury types that include alteration in the proportion of red and white pulp, occasional necrotic cell, depletion of lymphocytes, vacuole formation and elevated the number of melanomacrophage deposits in 7/8 fish examined per each treatment (Fig. 5.6). There was generally no material-type effect in the spleen structure with both bulk and nano forms causing similar changes in the spleen. The fractional volume of red pulp was significantly decreased in trout exposed to either bulk or TiO_2 NPs with a concomitant increase in sinusoid space and white pulp when compared to controls (Fig. 5.7). Notably, there was also a statistically significant increase in the number of melanomacrophage (means ± S.E.M, counts/field of view at x 400 magnification, 53,200 μm^2, n = 8) from 58.8 ± 3.2 (control) to 138.2 ± 5.8 (bulk) and 195.0 ± 9.0 (TiO_2 NPs) (ANOVA, $P < 0.001$). This melanomacrophage infiltrate was greater in the nano than bulk treatment (statistically significant material-type effect) (Fig. 5.7).

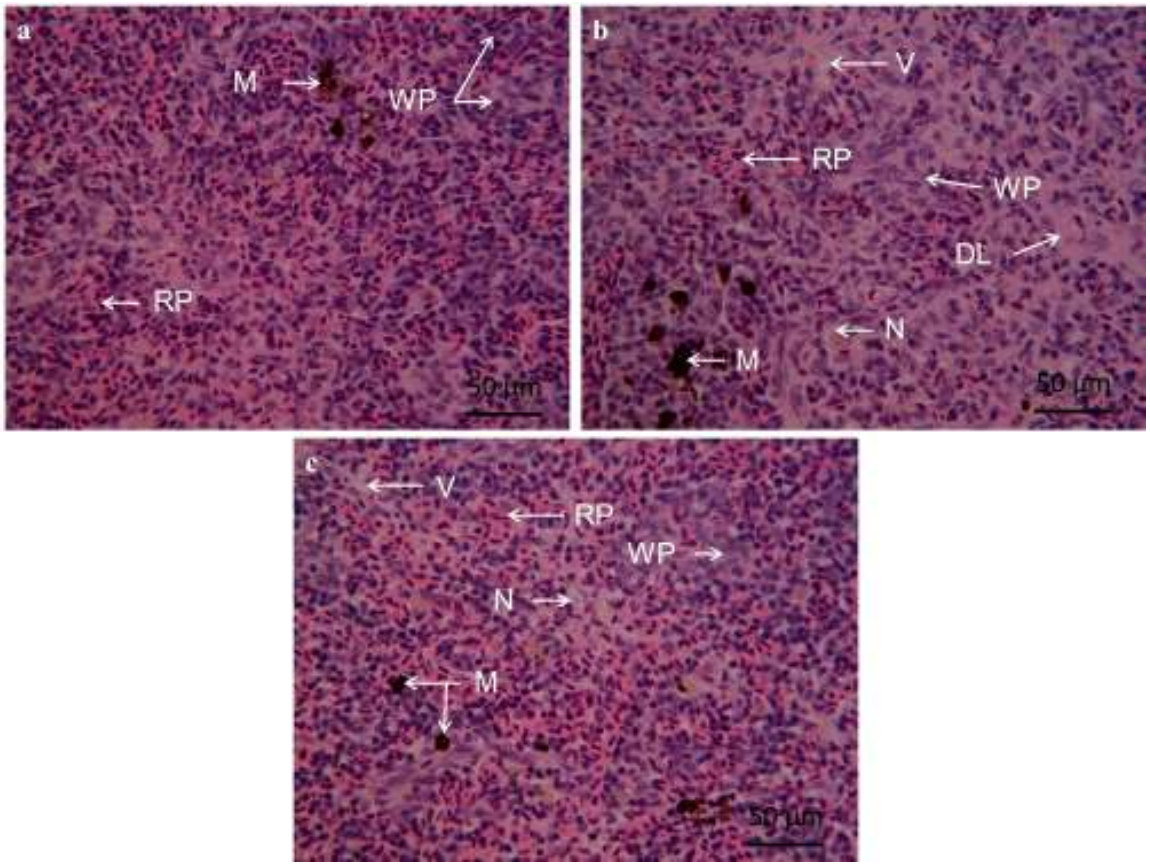

Figure 5.6 Spleen morphology in rainbow trout after 14 days of exposure to (a) control, (b) 1 mg l^{-1} of bulk and (c) 1 mg l^{-1} of TiO_2 NPs. The spleen from control fish showed normal histology, with defined red (RP) and white pulp (WP) and small deposits of melanomacrophage (M). Both treatments showed injuries included a decrease in the proportion of red pulp, necrosis (N), depletion of lymphocyte (DL), vacuole formation (V) and increased the number of melanomacrophage deposits. These injuries were similar with TiO_2 NPs and bulk form. Scale bar indicates magnification. Sections were 7 µm thick and stained with H&E.

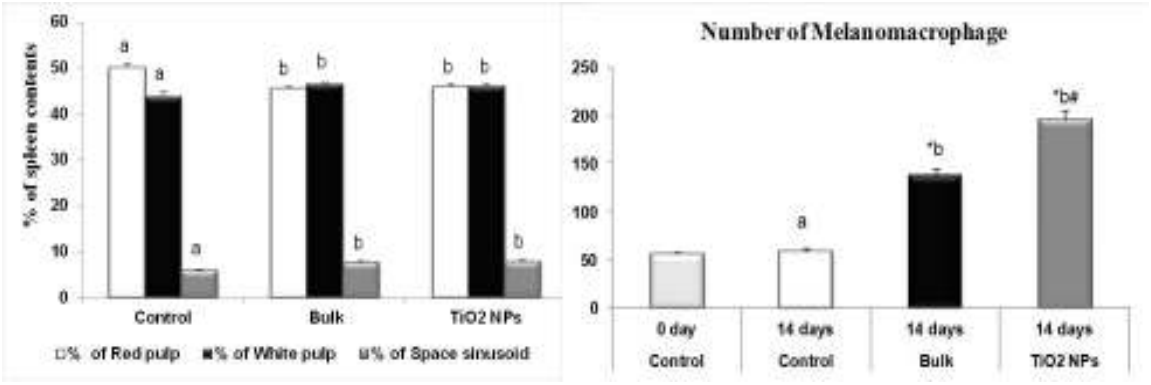

Figure 5.7 Alteration observed in the proportion of red, white pulp and sinusoid space as well as the number of melanomacrophage deposits in the spleen of trout after 14 days of exposure to control, 1 mg l^{-1} of either bulk or TiO_2 NPs. Data are means of proportional areas, or means ± S.E.M., $n = 8$. (*), indicates significantly different than initial fish (stock fish day 0, ANOVA, $P < 0.05$). Different letters indicate a significant difference from the control within treatments (ANOVA, $P < 0.05$). (#), Significant difference between bulk and TiO_2 NPs (t - test, $P < 0.05$).

5.3.6 Spleen print

Spleen prints of trout from control groups showed normal haematopoietic cells, including the presence of immature (haemocytoblast and erythroblast) and mature cells (erythrocytes and neutrophils) (Fig. 5.8). An analysis of haematopoietic cells in the spleen prints showed both Ti treatments (bulk and TiO_2 NPs) caused a minor increase in the proportion of haemocytoblast, erythroblast, and progranulocytes compared to controls (statistically significant, ANOVA, $P < 0.05$; Table 5.3). The proportion of erythrocytes showed a statistically significant decrease (ANOVA, $P < 0.05$) with both TiO_2 treatments, but bulk forms caused a greater decrease in the proportion of erythrocytes than TiO_2 NPs. Immune cells (neutrophils) showed a small but statistically significant increase with bulk

treatment when compared to the control and TiO_2 NP treatment (ANOVA, $P < 0.05$; Table 5.3).

Both TiO_2 treatments also showed erythrocyte abnormalities compared to control images of the spleen prints (Fig. 5.8). A quantitative analysis of red blood cell morphology was consistent with the observation on the proportion of red pulp in the spleen. Both bulk and TiO_2 NPs treatments showed a few more swollen red blood cells and blood cells with dividing nuclei (< 5 %, all statistically significant compared to the control; Table 5.3). The proportion of cells with membrane abnormalities in the spleen showed a statistically significant increase with both TiO_2 treatments compared to controls (Table 5.3); and with a material-type effect (more effect with bulk than NPs).

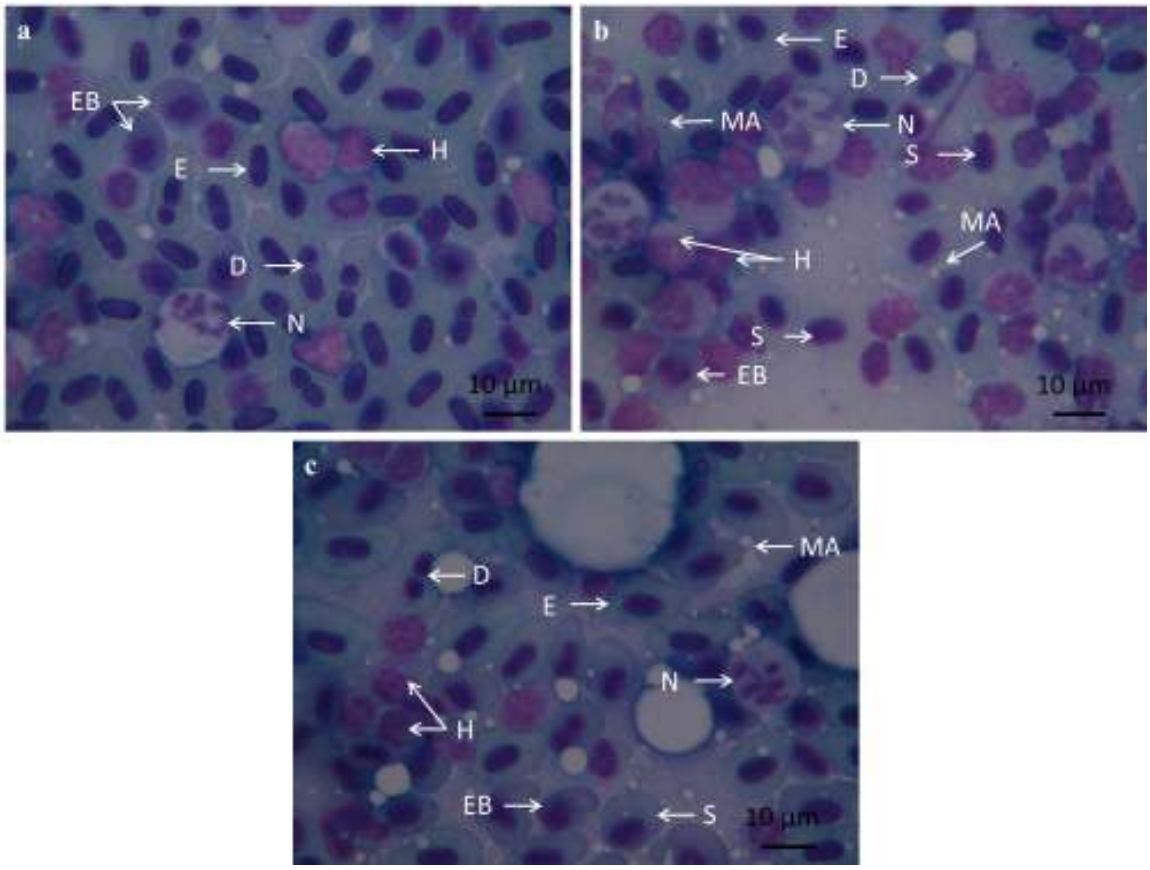

Figure 5.8 Spleen prints from rainbow trout after 14 days of exposure to (a) control, (b) 1 mg l^{-1} of bulk, and (c) 1 mg l^{-1} of TiO_2 NPs. All treatments showed alteration in the proportion of haematopoietic cell and erythrocytes abnormalities. Erythrocytes (E), erythroblast (EB), neutrophil (N), haemocytoblast (H), red blood cells with a dividing nucleus (D), membrane abnormalities of erythrocytes (MA), swollen erythrocytes (S). Scale bar indicates magnification. Smear stained with May- Grünwald-Giemsa.

Table 5.3 Alteration in the proportion of erythrocytes abnormality and haematopoietic cells in the spleen prints following 14 days of waterborne exposure to 1 mg l^{-1} of bulk TiO_2 or TiO_2 NPs.

Parameters		Time/ days	Control	1 mg l^{-1} of Bulk TiO_2	1 mg l^{-1} of TiO_2 NPs
% of haematopoietic cells	Haemocytoblast	0	7.9 ± 0.4		
		14	10.4 ± 0.2 *	13.4 ± 0.4 *+	12.2 ± 0.5 *+a
	Erythroblast	0	13.2 ± 1.3		
		14	11.4 ± 0.4	14.1 ± 0.3 +	14.8 ± 0.5 +
	Progranulocytes	0	1.5 ± 0.1		
		14	1.4 ± 0.2	2.3 ± 0.1 *+	2.4 ± 0.2 *+
	Erythrocytes	0	72.8 ± 1.6		
		14	72.1 ± 0.5	63.3 ± 0.4 *+	65.9 ± 0.6 *+a
	Neutrophils	0	4.6 ± 0.2		
		14	4.7 ± 0.2	6.9 ± 0.2 *+	4.8 ± 0.5 a
% of erythrocyte abnormalities	Swollen cells	0	Not observed		
		14	Not observed	2.5 ± 0.2 *+	2.7 ± 0.4 *+
	Cells with dividing nuclei	0	8.8 ± 1.1		
		14	14.4 ± 1.3 *	19.5 ± 0.8 *+	19.1 ± 1.2 *+
	Cells with membrane abnormalities	0	1.2 ± 0.2		
		14	1.5 ± 0.2	13.2 ± 0.3 *+	11.9 ± 0.2 *+a

Data are means of proportional cells ± S.E.M. (n = 8 fish/treatment). (*), significantly different from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). (+), significantly different from control within row (ANOVA, $P < 0.05$). (a), significantly different between bulk and TiO_2 NPs (t-test, $P < 0.05$).

5.3.7 Histological observation on the kidney

The hind kidney from control fish showed normal structure of renal corpuscle (glomerulus and Bowman's capsule) renal tubules (proximal and distal tubule) and the haematopoietic tissue amongst the nephrons (Fig. 5.9). Pathological observation showed that exposure to 1 mg l^{-1} of either bulk or TiO_2 NPs for 14 days caused similar injury types including moderate necrosis in the haematopoietic tissue, individual renal tubules with separation of the epithelium, enlargement of the Bowman's space, and increased melanomacrophage deposits, which were seen in 6/8 fish examined from each treatment (Fig. 5.9). Oedema was also seen in 3/8 fish from the bulk treatment and in 1/8 fish from the TiO_2 NPs treatment.

A quantitative analysis revealed that both TiO_2 treatments did not cause alteration in the proportion of the renal corpuscle, proximal and distal tubule, and the haematopoietic tissue compared to controls (Table 5.4), but at the same time both treatments caused small but statistically significant alteration in the dimension of renal corpuscles, and proximal tubules when compared to the control (Table 5.4). The kidney showed melanomacrophage activity, with a statistically significant increase in the number of melanomacrophages (means ± S.E.M., counts/field of view of 212,400 μm^2 at x 200 magnification, $n = 8$) from 230.3 ± 18.0 (control) to 295.3 ± 15.3 (bulk) and 295.5 ± 13.2 (TiO_2 NPs) (ANOVA, $P < 0.001$). There was no material-type effect in the melanomacrophage infiltrate in the kidney.

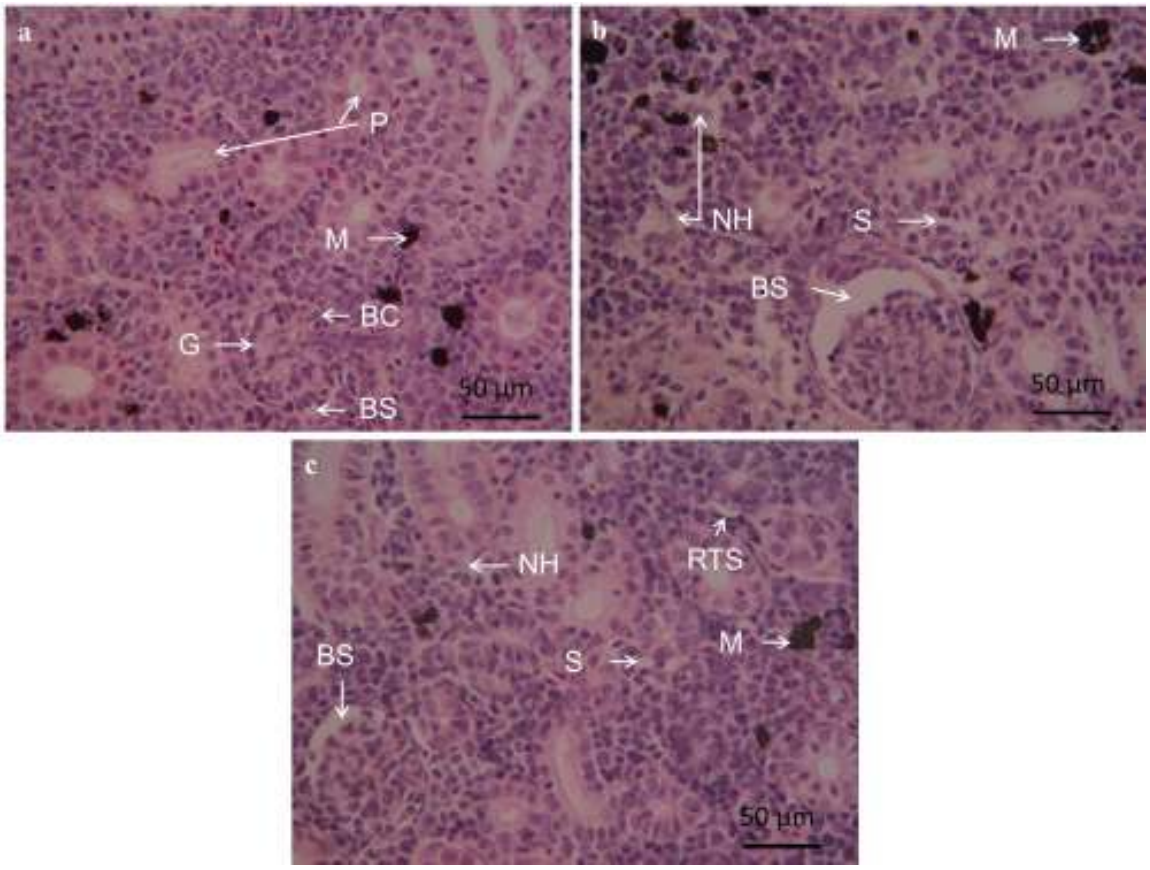

Figure 5.9 Kidney morphology in rainbow trout after 14 days of exposure to (a) control (b) 1 mg l^{-1} bulk TiO_2 and (c) TiO_2 NPs. Kidney of control groups showed the normal structure of proximal tubules (P), glomerulus (G), Bowman's capsule (BC), Bowman's space (BS) and melanomacrophage deposits (M). Both treatments showed injuries included an increase in the number of melanomacrophage deposits (M), necrosis in haematopoietic tissues (NH), increased Bowman's space (BSI), renal tubular separation (RTS) and sinusoid were enlarged (S). Scale bar indicates magnification. Sections were 7 µm thick and stained with H&E.

Table 5.4 Effect of waterborne exposure to 1 mg l^{-1} of either bulk or TiO_2 NPs for 14 days on the hind kidney of rainbow trout.

Parameters		Time /Days	Control	1 mg l^{-1} Bulk TiO_2	1 mg l^{-1} TiO_2 NPs
Proportion of renal corpuscles		0	4.5 ± 0.5		
		14	3.6 ± 0.5	4.4 ± 0.5	4.0 ± 0.3
Proportion of proximal tubules		0	23.0 ± 1.2		
		14	28.8 ± 2.3	23.6 ± 0.7	24.3 ± 0.6
Proportion of distal tubules		0	5.4 ± 0.9		
		14	4.6 ± 0.5	4.8 ± 0.6	5.4 ± 0.5
Proportion of haematopoietic and connective tissues		0	67.2 ± 1.5		
		14	63.0 ± 2.3	67.1 ± 1.4	66.3 ± 1.0
Number of melanomacrophage deposits		0	136.0± 28.0		
		14	230.0 ± 18.0 *	295.0 ± 15.0 *[a]	296.0 ± 13.0 *[a]
Dimension of renal corpuscle (μm)	Total dimension	0	77.8 ± 0.6		
		14	76.7 ± 2.0	89.8 ± 2.7 *[a]	86.3 ± 1.2 *[a]
	Glomerulus	0	70.2 ± 1.4		
		14	71.2 ± 1.5	77.2 ± 2.7 *[a]	75.2 ± 1.3
	Bowman's space	0	7.6 ± 1.6		
		14	5.5 ± 0.6	12.6 ± 1.5 *[a]	11.1 ± 1.5 [a]

Continued

Table 5.4

Dimension of proximal tubules (µm)	Total width	0	58.7 ± 1.1		
		14	58.1 ± 0.7	56.8 ± 0.6	56.7 ± 0.5
	Lumen	0	18.8 ± 0.4		
		14	19.6 ± 0.2	20.7 ± 0.5 *[a]	20.7 ± 0.2 *[a]
	Epithelial cell	0	39.9 ± 1.2		
		14	38.5 ± 0.8	36.1 ± 0.6 *[a]	36.0 ± 0.5 *[a]
Dimension of distal tubules (µm)	Total width	0	50.3 ± 0.5		
		14	49.6 ± 0.3	48.7 ± 0.6 *	48.6 ± 0.5 *
	Lumen	0	12.1 ± 0.4		
		14	12.7 ± 0.3	13.3 ± 0.3 *	13.3 ± 0.1 *
	Epithelial cell	0	38.2 ± 0.6		
		14	36.9 ± 0.3	35.4 ± 0.7 *	35.3 ± 0.5 *[a]

Data are means of proportional areas, or means ± S.E.M. (n = 8 fish/treatment). (*), significant difference from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). (a), significant difference between control within row (ANOVA, $P < 0.05$).

5.3.8 Histological observation on the brain

Trout brains were removed whole, and the histology of the major region of the brain control groups (fore, mid, and hindbrain) showed a normal architecture. The histology of telencephalon (forebrain) showed normal nerve cells in control groups (Fig. 5.10). The exposure to 1 mg l^{-1} of either bulk or TiO_2 NPs caused similar types of injury that include occasional nerve cell body with pyknotic nuclei, swollen nerve cells, necrotic nerve cells and vacuole formation in 6/8 fish examined from each TiO_2 treatments. A quantitative analysis observed in the forebrain showed both TiO_2 treatments showed a small but statistically significant (ANOVA, $P < 0.05$) decrease in the proportion of nerve cell, with a small material-type effects (Table 5.5) (worse with bulk than TiO_2 NPs).

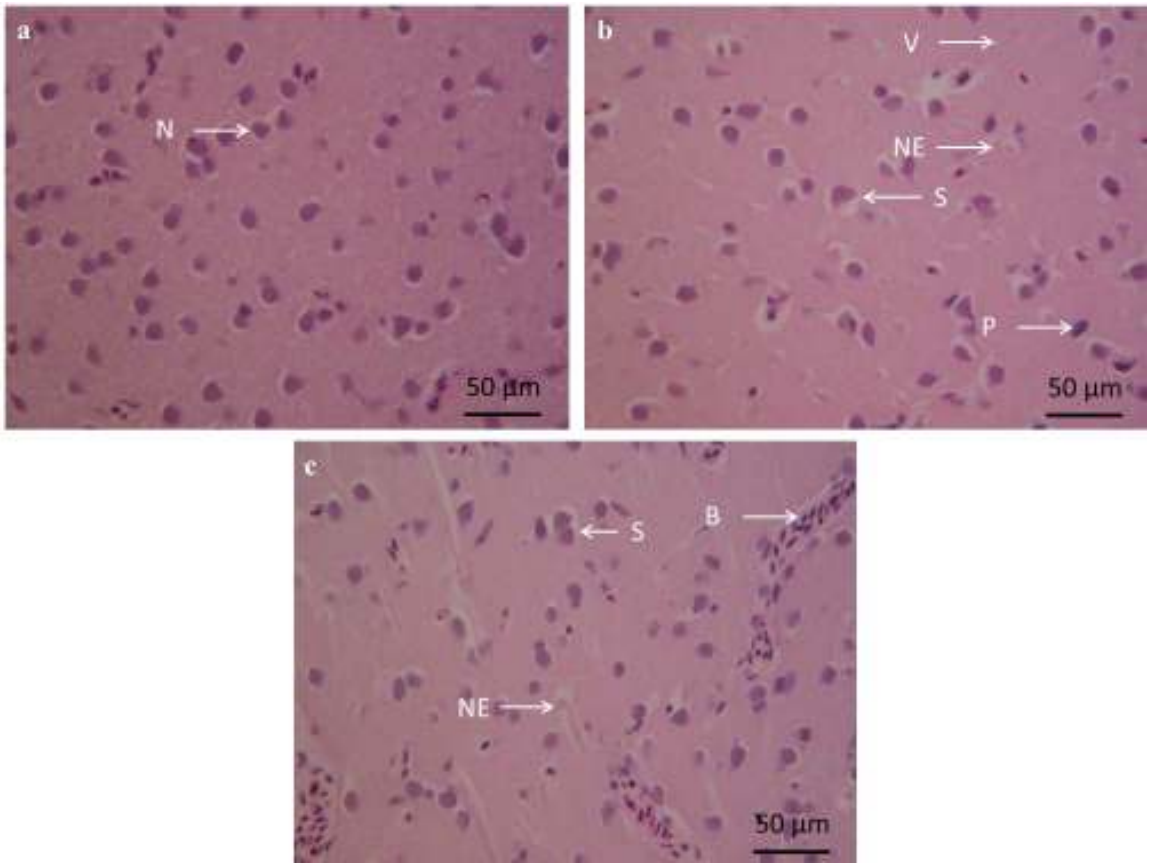

Figure 5.10 Telencephalon morphology in rainbow trout after 14 days of exposure to (a) control (b) 1 mg l^{-1} bulk, (c) 1 mg l^{-1} TiO_2 NPs. All control groups showed the normal structure of neuron (N) in telencephalon. Exposure to bulk and TiO_2 NPs showed injuries included a pyknotic nuclei (P), swollen neuron (S), vacuolisation (V), appeared a lot of blood cell (B) and necrosis (NE). These injuries were a slight severe with bulk than TiO_2 NPs. Scale bar indicates magnification. Sections were 7 µm thick and stained with H&E.

The mesencephalon region of the control group showed normal structure (Fig. 5.11). Both bulk and TiO_2 NPs treatments caused a minor alteration in the thickness of mesencephalon layers (Fig. 5.11). A quantitative analysis confirmed the results for histology that showed a statistically significant alteration in the thickness of stratum opticum and stratum periventriculare layers after 14 days of exposure to TiO_2 NPs. Whilst, the exposure to bulk treatment caused a small alteration in the thickness of capsule layer

compared to other treatment (control and TiO_2 NPs) (Table 5.5). Nano effects showed greater decrease in the thickness of stratum periventriculare layers with TiO_2 NPs than bulk treatment.

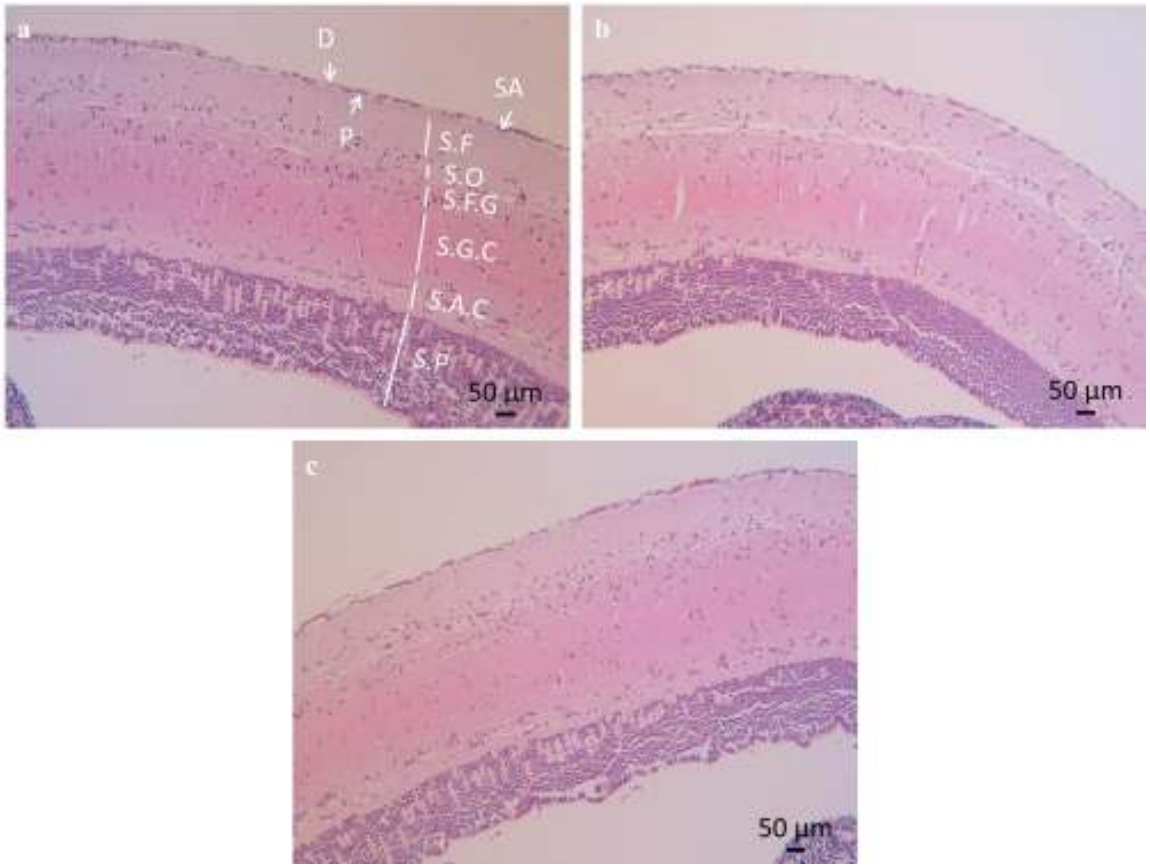

Figure 5.11 Mesencephalon morphology in rainbow trout after 14 days of exposure to (a) control (b) 1 mg l^{-1} bulk, (c) 1 mg l^{-1} TiO_2 NPs. All control groups showed normal structure in mesencephalon layers included dura mater (D), subarachnoid space (SA), pia mater (P), stratum fibrosum marginale (S.F), stratum opticum (S.O), stratum fibrosum et griseum superficiale (S.F.G), stratum griseum centrale (S.G.C), stratum album centrale (S.A.C), stratum periventriculare (S.P). Exposure to bulk and TiO_2 NPs showed a small alteration in the thickness of mesencephalon layers. Scale bar indicates magnification. Sections were 7 µm thick and stained with H&E.

Brain metencephalon (cerebellum) region of control animals showed normal structure of capsule, molecular, purkinje and granular layers (Fig. 5.12). Both treatments (bulk and TiO_2 NPs) caused enlarged blood vessel on the surface of cerebellum which observed in 6/8 fish treatments compared to controls (Fig. 5.12). This injury was worse with bulk than TiO_2 NPs treatments.

A quantitative analysis confirmed the results for histology (Table 5.5), that showed exposure to bulk TiO_2 caused statistically significant increased (ANOVA, $P < 0.05$) in the proportion of the capsule layer (% mean ± S.E.M., n =8) 2.8 ± 0.5 compared to 1.9 ± 0.2 with control and 1.7 ± 0.2 with TiO_2 NPs.

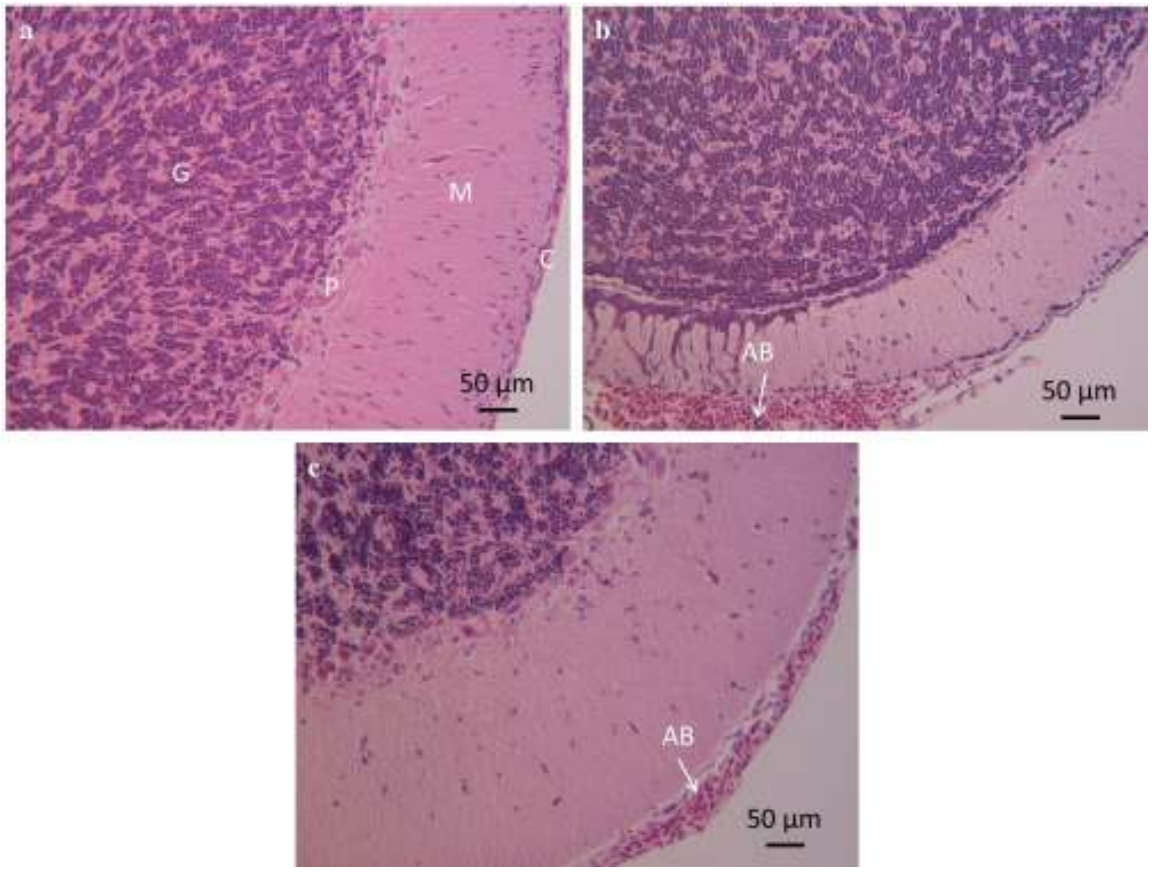

Figure 5.12 Cerebellum morphology in rainbow trout after 14 days of exposure to (a) control (b) 1 mg l^{-1} bulk, (c) 1 mg l^{-1} TiO_2 NPs. All control groups showed normal structure included capsule (C), molecular layer (M), purkinje layer (P), granular layer (G). Exposure to bulk and TiO_2 NPs showed injuries included blood vessel abnormalities on the ventral surface (AB). These injuries were more severe with bulk than TiO_2 NPs. Scale bar indicates magnification. Sections were 7 µm thick and stained with H&E.

Table 5.5 Alteration observed in the brain of rainbow trout following 14 days of exposure to 1 mg l^{-1} of either bulk TiO_2 or TiO_2 NPs.

Parameters		Time / Days	Control	1 mg l^{-1} Bulk	1 mg l^{-1} TiO_2 NPs
Telencephalon	Proportion of nerve cell	0 day	13.0 ± 0.3		
		14 days	13.2 ± 0.3	9.5 ± 0.1 *[a]	11.1 ± 0.3 *[a#]
	Dimension of nerve cells (µm)	0 day	14.5 ± 0.2		
		14 days	14.2 ± 0.1	14.7 ± 0.3	14.2 ± 0.2
	Dimension of nerve cells nuclei (µm)	0 day	11.4 ± 0.2		
		14 days	10.5 ± 0.1 *	11.2 ± 0.2 [a]	10.7 ± 0.2 *[#]
	Ratio of nuclei to nerve cell (µm)	0 day	78.9 ± 0.0		
		14 days	74.3 ± 0.8 *	76.3 ± 1.1	75.2 ± 1.3 *
% of Mesencephalon layers (µm)	Dura mater	0 day	0.7 ± 0.2		
		14 days	0.8 ± 0.0 *	0.7 ± 0.0 *[a]	0.8 ± 0.0 *
	Subarachnoid space	0 day	1.2 ± 0.1		
		14 days	2.1 ± 0.2 *	1.7 ± 0.2 *	1.8 ± 0.2 *
	Pia mater	0 day	0.7 ± 0.0		
		14 days	0.8 ± 0.0 *	0.7 ± 0.0 *[a]	0.8 ± 0.0 *
	St.fibrosummarginale	0 day	15.9 ± 0.7		
		14 days	15.1 ± 0.5	14.5 ± 0.3 *	15.6 ± 0.4
	St. opticum	0 day	5.7 ± 0.0		
		14 days	6.8 ± 0.3	7.5 ± 0.4 *	8.7 ± 0.4 *[a]

Continued

Table 5.5

	St.fibrosum et griseum superficial	0 day 14 days	12.8 ± 0.5 14.6 ± 0.2 *	14.9 ± 0.4 *	15.3 ± 0.2*
	St. griseumcentrale	0 day 14 days	17.4 ± 0.8 16.5 ± 0.8	15.3 ± 0.3 *	17.5 ± 0.5 #
	St. album centrale	0 day 14 days	12.3 ± 0.5 12.0 ± 0.6	12.6 ± 0.8	12.5 ± 0.4
	St.periventriculare	0 day 14 days	33.3 ± 1.2 31.2 ± 1.2	31.9 ±1.0	27.0 ± 1.2 *[a#]
% of Cerebellum layer	Capsule	0 day 14 days	1.6 ± 0.2 1.9 ± 0.2	2.8 ± 0.5 *[a]	1.7 ± 0.2 #
	Molecular layer	0 day 14 days	39.9 ± 3.1 38.2 ± 3.0	41.6 ± 4.9	42.0 ± 4.0
	Purkinje layer	0 day 14 days	5.6 ± 0.4 4.1 ± 0.2 *	4.7 ± 0.3 *	4.4 ± 0.2 *
	Granular layer	0 day 14 days	53.8 ± 3.4 55.8 ± 2.9	50.9 ± 5.0	51.9 ± 4.1

Data are means of proportional areas, or means ± S.E.M., (n = 8 number of fish/treatments). (*), significant difference from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). (a), significantly different between control (14 days) within row (ANOVA, $P < 0.05$). (#), significant difference between bulk and TiO_2 NPs (t- test, $P < 0.05$).

5.3.9 Histological observation on the muscles

The skeletal muscle structure of the unexposed control groups showed normal histology structure (Fig. 5.13). The exposure to either bulk or TiO_2 NPs for 14 days caused increase in the extracellular space around the muscle fibres compared to controls. These lesions were seen in six out of eight fish from both TiO_2 treatments (Fig. 5.13). A quantitative analysis exhibited that both treatments caused statistically significant (ANOVA, $P < 0.05$) decrease in the proportion of muscle fibres and increase in the extracellular space among the muscle fibers compared to the control (Table 5.6). The diameters of muscle fibers were also showed alteration following 14 days of exposure to bulk or TiO_2 NPs compared to controls (Table 5.6).

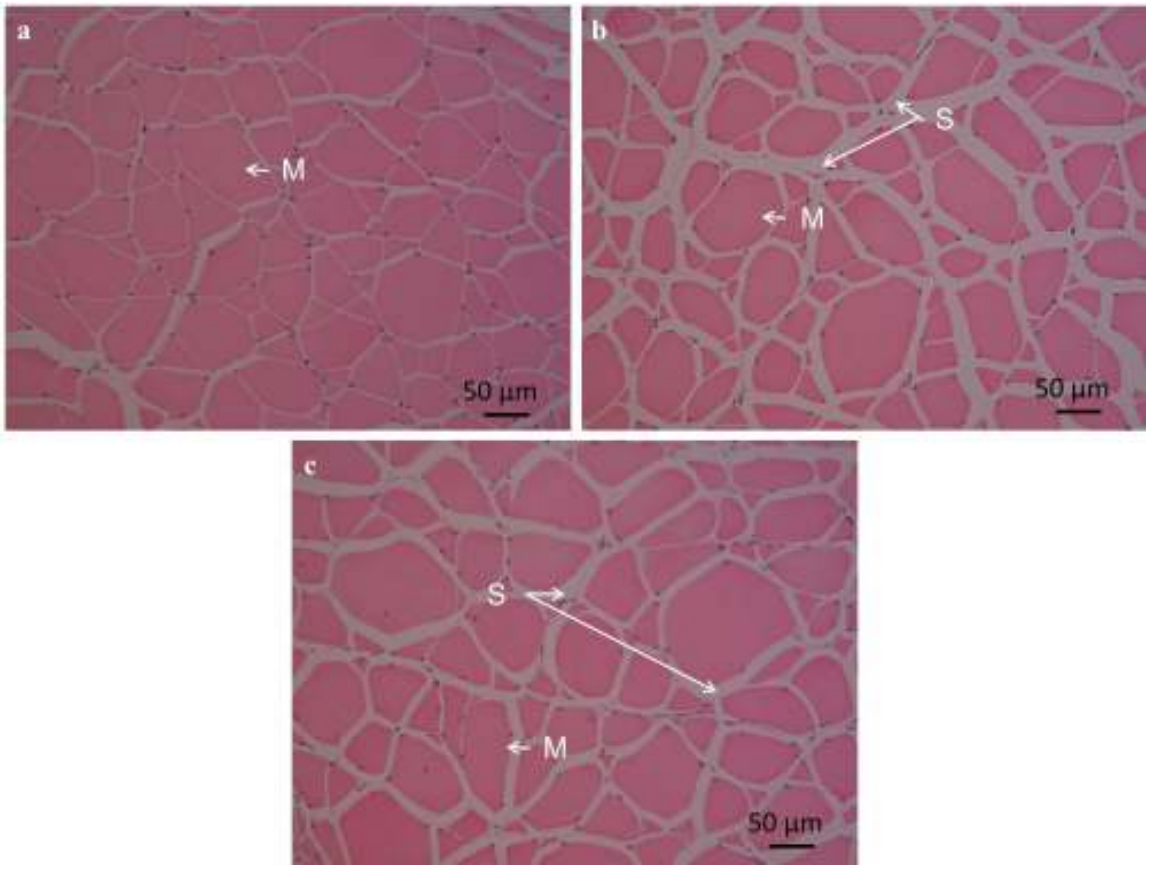

Figure 5.13 Muscle morphology in trout after 14 days of exposure to (a) control (b) 1 mg l^{-1} bulk TiO_2 (c) 1 mg l^{-1} TiO_2 NPs. Muscles of control groups showed normal structure of muscle fibers (M). Both treatments showed increased space among muscles fibers (S). Scale bar indicates magnification. Sections were 7 µm thick and stained with H&E.

Table 5.6 Quantitative analysis of the alteration observed in the muscle of rainbow trout following exposure to 1 mg l^{-1} of either bulk or nano scale TiO_2 for 14 days.

Parameters	Time/ Days	Control	1 mg l^{-1} bulk	1 mg l^{-1} TiO_2 NPs
Proportion of muscle fibres	0	69.2 ± 0.8		
	14	71.4 ± 1.3	60.4 ± 2.6 *[a]	61.0 ± 2.3 *[a]
Proportion of extracellular space	0	30.8 ± 0.8		
	14	28.6 ± 1.3	39.6 ± 2.6 *[a]	39.0 ± 2.3 *[a]
The height of muscle fibres (µm)	0	115.1 ± 2.4		
	14	108.9 ± 2.0 *	93.4 ± 1.5 *[a]	90.3 ± 1.8 *[a]
The width of muscle fibres (µm)	0	70.9 ± 0.8		
	14	71.3 ± 1.1	60.5 ± 0.6 *[a]	63.2 ± 0.2 *[ab]

Data are means of proportional areas, or means ± S.E.M. (n = 8 fish/treatments). (*), significant difference from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). (a), significant difference from control within row (ANOVA, $P < 0.05$). (b), significantly different between bulk and TiO_2 NPs (t -test, $P < 0.05$).

5.4. Discussion

Few studies have so far investigated the effects of waterborne TiO_2 NPs on trout, and here we present a comparison with an equal mass concentration exposure to the bulk TiO_2 on organ integrity by using histological alteration on these organs as well as the alteration on the haematopoietic system. The results of this study showed that exposure to bulk or TiO_2 NPs causes similar organ pathologies, but bulk TiO_2 generally caused greater effects than TiO_2 NPs on some organs (same types of lesion in the gill, liver, brain and skeletal muscles).

5.4.1 Gill histopathology

Titanium dioxide nanoparticle is a well-known respiratory distress in fish (Federici *et al.*, 2007), and there is some evidence that water borne exposure to TiO_2 NPs may result in particle accumulation in or on the epithelial cells (Moger *et al.*, 2008). The current study showed that bulk and TiO_2 NPs caused similar types of gill injuries, but some types of pathologies (clubbed tips) were greater with NPs than bulk form. Whereas, other pathology types like swollen mucocytes were greater with bulk than NPs material. The gills of bulk and TiO_2 NPs treated fish also had Ti accumulation (likely as TiO_2) at the end of the experiment (Boyle *et al.*, 2013). The first line of the defence to metal exposure in the gills, which can temporarily protect the underlying epithelium from injury, is mucus secretion (Handy and Maunder, 2009). Mucus secretion and swollen mucocytes were observed in this study, and has been noted in rainbow trout with the same and other nanomaterials (SWCNT, Smith *et al.*, 2007; TiO_2 NPs, Federici *et al.*, 2007; Cu-NPs, Al-Bairuty *et al.*, 2013). However, in the current study mucus secretion was not sufficient to protect the gills from the pathology arising from either bulk or TiO_2 NPs exposure. Gill pathologies that observed in the present study for TiO_2 NPs have also been reported for waterborne exposures with the same and other types of nanomaterials in trout (TiO_2 NPs, Federici *et al.*, 2007; SWCNT, Smith *et al.*, 2007; Cu-NPs, Al-Bairuty *et al.*, 2013) and for TiO_2 NPs in carp (Hao *et al.*, 2009). The branchial aneurism that found in this study (with bulk material) and the study by Federici *et al.* (2007) would suggest some interruption of gill perfusion that might contribute to a systemic hypoxia. However, the hyperplasia, fusion and lifting of the gill epithelium suggest that bulk material like TiO_2 NPs, could cause to disturb in oxygen uptake mechanism or change in the metabolic rate in respiration.

For ultrastructural studies, whilst the gill lamellae of control groups showed normal regular structure, there were distinct morphological changes in bulk and TiO_2 NPs treatments. Gill cells from both bulk and TiO_2 NP treatments showed similar changes that include an increase in the number of mitochondria, mitochondrial damage, as well as disorganisation of pillar and red blood cells. Structural damaging to the mitochondria observed here could attribute to the particles or nanoparticles may be transported within cells and be taken by cell mitochondria (Geiser *et al.*, 2005). Deformation of red blood cells was noticed in this study and this could be due to reduced vascular space. The restricted lumen of the blood vessels could force the red blood cell (RBC) to move into a reduced space giving an increase to cellular deformation. However, Nilsson *et al.* (1995) have suggested that this deformation may play a role in oxygen uptake by declining the diffusion boundary layer of fluid around the RBC and mixing of intracellular haemoglobin molecules. Due to restricted passage, though, cells have to pass slowly through the lamella favouring oxygen uptake. However, the changes observed here with bulk and nano forms could impair respiratory functions (gas exchange) resulting an internal hypoxia which may stimulate the RBC to be released from the haematopoietic system (*i.e.* spleen) into the blood stream.

5.4.2 Gut histopathology

In the gut of fish, there is a little information about the pathological effects of the bulk metal oxide. Previous studies explained TiO_2 NPs can cause stress-induced drinking response that resulted in ingestion of TiO_2 and pathologies of the mucosa (Federici *et al.*, 2007). The current study showed a similar type of pathologies in the gut with both TiO_2

treatments and the quantitative analysis confirmed this observation. The quantitative analysis in this study showed that both TiO_2 treatments caused changes in the villi height and the dimension of columnar cell nuclei compared to the control, and this could suggest induce irritative effects in the gut. The thickness of gut muscularis was also changed in this study with both TiO_2 treatments and this could induce disturbance in the gut function, such as gut motility. Recently, study by Al-Bairuty *et al.* (2013) showed that waterborne exposure to $CuSO_4$ or Cu-NPs caused similar types of gut pathologies, and the severe pathology was observed with NPs than dissolved Cu. However, with only a few materials investigated so far, more studies on the uptake mechanism and biological effects of NPs in the gut are needed.

5.4.3 Liver histopathology

The livers of fish are an important organ of active metabolism and detoxification and are very sensitive to pollutants. The types of lesions reported here for TiO_2 NPs are known (Federici *et al.*, 2007; Hao *et al.*, 2009) and the elevation of lipid peroxidation in the liver of carp suggest that the liver might be the most susceptible organ to TiO_2 NPs (Hao *et al.*, 2009). Exposure to bulk TiO_2 produced the same types of pathologies in the liver, with a small difference. For example, the exposure to bulk treatment causes small more alteration in the proportion of hepatic area and sinusoid space than NPs treatment, even though the livers from both TiO_2 treatments did not show Ti accumulation. This could suggest that bulk or NPs had an ability to produce similar liver pathology due to defects in energy metabolism. The increase in hepatocyte diameter that associated with the decrease in hepatocyte diameter relative to nuclei diameter that observed with both bulk and NPs

treatment suggests that liver cells are more metabolically active, and the increased presence of melanomacrophage deposits support this opinion as they are known to be implicated with detoxification and recycling of endogenous materials from damaged cells (Haaparanta *et al.*, 1996).

5.4.4 Spleen histopathology

Much less information is known about the effects of bulk particles or NPs on the fish immune system, although the immune system is considered one of the defence mechanisms to counteract the effects of toxicant. The spleen is an essential organ of the haematopoietic system in fish, which is important in removing damaged blood cells and foreign materials from the circulation, and to give normal circulating blood cells. For that reason, the injuries found in the cells contained in the spleen with both bulk or nano forms of TiO_2 suggests a hardworking of the spleen trying to remove foreign particulate matter (bulk or TiO_2 NPs), and/or damaged cells from the circulation. The latter may be a direct particle effect or due to a secondary respiratory distress (gill injuries that mentioned above). In mice, Li *et al.* (2010) found that TiO_2 NPs induced spleen pathology and apoptosis which lead to a decline of immunity. Whereas, injections of different doses of TiO_2 NPs in mice caused spleen injuries due to direct entry of some of TiO_2 NPs in the spleen (Chen *et al.*, 2009). Little is known about the spleen injuries in fish but an alteration in the proportion of red and white pulp (in fish, Handy *et al.*, 2002c) and the presence of lesions are typical during oral exposure to chemicals (*e.g.*, pesticides in mice, Handy *et al.*, 2002a). Spleen injuries in this study were associated with a decrease in the proportion of red pulp and an increase in the proportion of white pulp and sinusoid space as well as increased the

number of melanomacrophage deposits (showing a material-type effect) with both TiO_2 treatments. A decrease in the proportion of red pulp could occur due to the release of red blood cells from the spleen into the circulatory system, whilst an increase in the white pulp (immune cells) and the number of melanomacrophage could suggest an immunological stress response. An increase in the proportion of sinusoid space would suggest the spleen is working to supply red blood cell to the bloodstream after removing damaged cells. However, the findings of the current study do not support the previous research. Handy *et al.* (2011) reviewed *O. mykiss* from Federici *et al.* (2007) and showed no changes in the proportion of red and white pulp and sinusoid space as well as no increases in the granular deposits in the spleen of trout exposed to 1 mg l^{-1} of TiO_2 NPs for 14 days. This difference between studies at the same exposure concentration can be explained by the approach of preparing the stock dispersion of TiO_2 NPs or due to the Ti accumulations that were observed in the gill of the current study.

It is not clear whether sub-types of blood cells are affected by NPs in mammals or fish, therefore, spleen prints are good indicators of immunological response. Studies of the effects of NPs on fish spleen prints are rare. The spleen prints of trout that used in the TiO_2 NPs study of Federici *et al.* (2007) showed a few more alterations in the proportion of haematopoietic cells and the proportion of erythrocytes abnormalities (Review, Handy *et al.*, 2011). Haematopoietic cells in the current study (different stages and types of red and white blood cells) in the spleen showed only a minor increase in the proportion of immature cells (haemocytoblast, erythroblast and progranulocytes) and decrease in the proportion of mature cell (erythrocytes) with both Ti treatments. An increase in the proportion of

immature cells could suggest the exposure to bulk or TiO_2 NPs may stimulate erythropoiesis to produce new cells, while a decrease in the proportion of erythrocytes could suggest the spleen was working to supply red blood cell into the circulation. Also, immune cells (neutrophils) were only increased with bulk treatment. This could suggest the spleen was responding by inducing immune cell to protect the body against the bulk material. However, why only the bulk material might be immunogenic is unclear, and with only one cell-type changing by a small amount, this may be just incidental. In the present study, a quantitative analysis of red cell morphology was consistent with observations on the proportions of red pulp. Both treatments (bulk and TiO_2 NPs) caused swollen red blood cell, a dividing nucleus cell, or membrane abnormalities. These abnormalities could occur due to several factors such as intercellular osmotic disorders, stress (handling fish, exposure to bulk or TiO_2 NPs) and hypoxia (due to gill injuries). For example, swollen red blood cell could occur due to increase in the acidity of the blood as results to hypoxia that would lead to increased anaerobic respiration (producing a higher carbon dioxide concentration in their blood).

5.4.5 Kidney histopathology

There are few reports of renal pathology from waterborne exposure to bulk or TiO_2 NPs in rainbow trout, but the pathologies reported here seems broadly similar to recent reports on fish with metal or metal-NPs. For example, in the *O. mykiss* following 10 days of exposure to 20 μg l^{-1} of either dissolved Cu or Cu-NPs and 100 μg l^{-1} of Cu-NPs pathologies included some damage to the epithelial cells of the renal tubules, changes in the Bowman's space and elevated in the number of melanomacrophage deposits (Al-Bairuty *et*

al., 2013). The present study also showed lipid peroxidation in the kidney with both bulk and TiO_2 NPs treatments, although TiO_2 was not accumulated in the kidney (Boyle *et al.*, 2013). This may indicate the kidney suffered from oxidative stress (creating hydroxyl and superoxide radicals) which could result in lipid peroxidation of the unsaturated lipid of the cell membranes. In mice, one study has shown nephritis and oxidative stress in the kidney that led to disruption of the kidney function, accumulation of Ti in the kidney, and alteration in the activity of antioxidant enzymes following abdominal exposure of high dose of anatase TiO_2 for 14 days (Zhao *et al.*, 2010). The enlarged of Bowman's space with either bulk or TiO_2 NPs treatments observed in the current study could suggest the osmoregulatory strategy may also be affected. One explanation of this observation is that water is being drawn osmotically into the space, presumably from glomerular filtration. A study by Scown *et al.* (2009) found that maintenance of creatinine clearance in trout injected to mg levels of TiO_2 NPs, implying that the glomerular filtration rate can be maintained in some situations. An increase in the number of melanomacrophage deposits in the kidney that observed in the current study with both Ti treatment and with other type of NPs (Cu-NPs, Al-Bairuty *et al.*, 2013) would suggest the metallic particles can produce an immunological response, but without appreciable metal accumulation in the kidney this would seem to be more likely related to inflammation and secondary hypoxia.

5.4.6 Brain histopathology

Recently, biological studies have suggested that the brain may be an important target organ for NPs in fish. In this study, the exposure to either bulk or TiO_2 NPs caused similar types of injury in the main regions of the brain and the quantitative analysis

confirmed these results. The current study also showed lipid peroxidation (TBARS, with NPs only), small elevation of total GSH (with both TiO_2 treatments, worse with NPs than bulk) and perturbation of electrolytes in the brain after 14 days of exposure to either bulk or TiO_2 NPs (Boyle *et al.*, 2013), indicating that these materials could cause severe oxidative stress in the brain. These findings of the current study are consistent with Ma *et al.* (2010) who found that injection of anatase TiO_2 NPs or bulk TiO_2 into the abdominal cavity of mice caused greater alteration with TiO_2 NPs than bulk TiO_2 such as generation of ROS and lipid peroxidation, the decline of the total activities of antioxidant enzymes, and brain pathologies. A study by Federici *et al.* (2007) also showed that waterborne exposure to 1 mg l^{-1} TiO_2 NPs caused minor brain injuries (necrotic cell bodies, small foci of vacuolation) in trout. The formation of vacuole in the brain may have been due to glycolysis which leading to microsomal and mitochondrial dysfunction (Sarma *et al.,* 2010). Several studies have been reported that TiO_2 NPs could be phagocytized by neurons and microglia, which then released ROS (•OH and O_2^-) (Long *et al.*, 2006; Long *et al.*, 2007). Therefore, the injuries observed in the brain could be produced, partly, by systemic hypoxia or blood perfusion injury problem. Vascular injury on the surface of trout brain, necrotic cell bodies and small foci of vacuoles in the optic lobe and cerebrum have also been found with SWCNT (Smith *et al.*, 2007). Bulk and TiO_2 NPs caused some changes in the relative thickness of the mesencephalon layers. These changes cannot be due to differential tissue growth over 14 days and is almost certainly related to oedema or to the cut orientation.

5.4.7 Skeletal muscle histopathology

In fish, there is a gap in the information about muscle pathology following exposure to NPs. This is the first study showed that exposure to bulk or TiO_2 NPs caused similar muscle injuries which include an increase in the extracellular space in the muscle. A quantitative histological analysis of trout muscle fibre dimensions appears not to have been done before, but measurements confirm a statistically significant increase in the proportion of extracellular space relative to the muscle fibre area in the bundle with both bulk and TiO_2 NPs, and this effect appears to be related to an increase in the dorso-ventral thickness of the fibre. A measurement also confirmed a decrease in the height and width of muscle fibre, with a small material-type effect on the width of muscle was observed (worse with bulk than the NP form). Boyle *et al.* (2013) also found a subtle decrease in time spent swimming at high speed in juvenile rainbow trout that exposed to 1 mg l^{-1} TiO_2 NPs for 14 days compared to controls; whereas, no difference in the swimming activity was observed when compared to fish exposed to bulk TiO_2. This suggests there may be locomotor consequences of muscle injury. However, the muscle injuries observed in this study could be attributed to the deficiency of oxygen as resulting from gill injuries, disorder in the activity of metabolic enzymes (ATPases necessary for muscle contraction), or nervous disorder (innervation of the muscle) due to the brain injuries. Similar muscle pathologies were observed in gold fish following exposure to copper (Vogel, 1959), and in trout fish following exposure to $CuSO_4$ or Cu-NPs (Al-Bairuty *et al.*, 2013).

Conclusions

The present study provides details of pathological effects, target organs, and immunological response following waterborne exposure to bulk or TiO_2 NPs in rainbow trout. Overall, the current data demonstrate that the target organs for TiO_2 NPs are similar to those of bulk TiO_2. It has also demonstrated that the types of pathologies caused by TiO_2 NPs are similar to those for bulk form in the gill, gut, liver, spleen, kidney, brain and muscle, but these injuries were slightly greater effects with bulk TiO_2 than TiO_2 NPs in some organs. Additionally, the spleen print method has been demonstrated that both forms of TiO_2 induce stimuli to release red blood cells in the systemic circulation and also to produce an immunological response. The finding of the present histopathological study demonstrates a direct correlation between both waterborne bulk or TiO_2 NPs exposure and histopathological disorders in several tissues. The toxic effects of bulk or TiO_2 NPs are consistent with the idea of hypoxia and/or oxidative stress that could lead to organ pathologies. The pathological effects can occur without appreciable Ti accumulation in the internal organs of fish; therefore further investigation is needed on the aspect of direct target organ toxicity.

Chapter 6

Histopathological and haematopoietic studies after intravenous injection of bulk titanium dioxide or titanium dioxide nanoparticles in rainbow trout (Oncorhynchus mykiss)

Hypotheses: Intravenous injection of either bulk or TiO_2 NPs in trout leads to different pathological effects on different target organs due to particle size or crystallinity. The effects of injection may be more severe than waterborne exposure as protective epithelial barriers are by-passed with direct systemic administration of particles.

Abstract

Little is known about the body distribution and toxicity of bulk TiO_2 or titanium dioxide nanoparticles (TiO_2 NPs) in fish. The aim of this study was to investigate the distribution and histopathological effects of intravenous injection of either control (Cortland's saline, 50 µl) or 50 µg TiO_2 in 50 µl saline as either bulk TiO_2 or TiO_2 NPs to the main body system of juvenile rainbow trout (14 fish/treatment). Fish were sampled after 4 days of injection for histology. Both bulk TiO_2 and TiO_2 NPs showed similar types of organ injuries, except the spleen and kidney that showed a material-type effect. These injuries included small incidences of oedema and clubbed tips in the gill; occasional necrotic cells and epithelial lifting in the gut; elevation of the number of melanomacrophage deposits and oedema in the liver; enlargement of the Bowman's space and degeneration of the renal tubules in the kidney; occasional necrotic cells and an increase in the number of melanomacrophage deposits in the spleen. The spleen also showed a decrease in the proportion of red pulp from (mean % ± S.E.M., n = 6) 52.8 ± 1.9 in the control to 46.8 ± 0.9 and 41.4 ± 0.7 for bulk and TiO_2 NPs respectively; while the proportion of white pulp was increased from 40.0 ± 1.8 in the control to 46.4 ± 0.8 and 52.2 ± 0.8 with bulk and TiO_2 NPs respectively. The main region of the brain showed minor

injuries including a pyknotic nuclei and enlarged nerve cells in the telencephalon area; minor alterations in the thickness of mesencephalon layers; and necrotic cells as well as enlargement of blood vessel on the surface of the cerebellum. Overall, we conclude that either bulk TiO_2 or TiO_2 NPs caused similar type of injuries in most organs except the spleen and kidney that showed nano-effects due to the particle size and/or crystallinity.

6.1. Introduction

Recently, increasing production and use of engineered nanoparticles have raised concern about the released of these materials into the aquatic environment and toxic effects on the different body systems of fishes have been partly identified (Handy *et al.*, 2011). However, laboratory studies have been confounded by the difficulty of maintaining the exposure concentrations of NPs (Handy *et al.*, 2012), also by uncertainty about the bioavailability (Shaw and Handy, 2011). This relating exposure with effect and identifying target organs is problematic. So far, little is known about the fate and behaviour of NPs that entering into the body (distribution) of aquatic animals, although this has been discussed from a theoretical viewpoint (Handy *et al.*, 2008a).

There are almost no injection studies with NPs on aquatic species that allow the identification of the body distribution, and subsequent excretion mechanisms. One study only by Scown *et al.* (2009) showed that a single high dose of intravenously injected of TiO_2 NPs (100 $\mu g\ l^{-1}$, 1 ml) in trout caused accumulation of Ti in the kidney with minimal effects on kidney function . In mice, the injection of different dosages of TiO_2 NPs (1944 - 2592 mg kg^{-1}) for 7 days exhibited the highest accumulation of Ti in the spleen and then in

the liver, kidney and lung, as well as causing pathologies in the liver (hepatocellular necrosis, hepatic fibrosis), spleen (many neutrophilic cells, entry of TiO_2 particles into the spleen), and kidney (renal glomerulus swelling) (Chen *et al.*, 2009). Additionally, Ma *et al.* (2009) found that intraperitoneal injection of higher doses of anatase TiO_2 NPs for 14 days caused accumulation of Ti in the liver, liver pathologies, thus leading to the impaired of liver function. Whilst the other routes of exposure, for example, the intragastric administration of TiO_2 NPs caused liver function disorder, which associated with the damage of blood system haemostasis and the reduction of immunity (Duan *et al.,* 2010). Additionally, the single oral gavage of different size of TiO_2 NPs suspension (80 nm & fine 155 nm) caused pathologies in the liver (degeneration around the central vein and necrosis of hepatocytes), kidney (swelling in the renal glomerulus and filled renal tubule with proteinic liguids) and alteration in the serum biochemical parameters (ALT/AST, LDH) in mice (Wang *et al.*, 2007).

Spleen injuries and apoptosis in the mouse splenocytes leading to reduction of immunity has been shown following intraperitoneal injection of TiO_2 NPs (50 – 150 mg kg^{-1}) in the mice after 45 days (Li *et al.,* 2010). Brains of mice also showed increased Ti accumulation, oxidative stress and injuries (neuron cleavage into filamentous and the presence of inflammatory cells in local area) following abdominal injection of anatase TiO_2 NPs (100 -150 mg kg^{-1}) and bulk (150 mg kg^{-1}) for 14 days (Ma *et al.*, 2010). The intranasal inhalation of TiO_2 NPs in mice for 30 days has been shown to directly enter the brain through the olfactory bulb and then caused an increase Ti accumulation in the hippocampus that lead to irregular arrangement and loss of neuron in this region as well as

oxidative stress (Wang *et al.*, 2008). Whilst, Wang *et al.* (2007) showed slight brain injury (increased the number of vacuoles in the neurons of hippocampus) after a single oral gavage of different size of TiO_2 NPs suspension (80 nm & fine 155 nm) in mice. These examples clearly show that direct injection can result in the appearance of NPs in the internal target organs, and sometimes with pathology.

The present study was conducted to investigate the distribution of bulk or TiO_2 NPs via the bloodstream of rainbow trout into the target organs and then identify the major pathological effect that occurs due to the injection of these materials. The aim was to documenting organ pathologies during caudal vasculature injection of either bulk or TiO_2 NPs and if there was any difference between the effects of TiO_2 NPs and ordinary form of bulk TiO_2 which lead to induce specific pathologies. We therefore measured a range of endpoints including organ pathologies, and immunological responses (spleen prints) that presented here. Other endpoints such as haematology and plasma ion concentration, biochemical measurements as indicator to physiological function as well as trace element profiles were presented in Boyle *et al.*, (unpublished).

6.2. Materials and Methods

6.2.1 Rainbow trout husbandry

Juvenile rainbow trout were obtained from a local supplier (Torre fishery, Watchet, Somerset) with total body weight of 38.6 ± 8.6 g (Mean ± Standard deviation, n = 49). Stock fish were maintained in aerated, dechlorinated and recirculating Plymouth tap water (means ± S.D., n = 5; Ca^{2+}, 0.342 ± 0.004 mmol l^{-1}; Mg^{2+} , 0.046 ± 0.001 mmol l^{-1}; Na^{+}

0.422 ± 0.008 mmol l^{-1}; K^+, 0.027 ± 0.003 mmol l^{-1}; pH 6.8 ± 0.3; temperature 16.2 ± 0.5 °C; DO 100 ± 1 % saturation; total NH_3, 0.1 ± 0.1 mg l^{-1}) for 4 weeks prior to experimentation. During this acclimation period fish were fed twice daily to satiation state with a commercial trout diet (EWOS, Westfield, UK) until 48 h prior to injection. Then, fish were not fed during the 96 h exposure period.

6.2.2 Materials

All chemicals were bought from Sigma-Aldrich, UK, unless otherwise stated. Trout control's saline (Cortland's saline, 50 µl) was made up in ultrapure water (Milli-Q®, Millipore Corporation) with the following ionic strength (in mmol l^{-1}): 137 NaCl, 2.7 KCl, 4.3 Na_2HPO_4, and 1.4 NaH_2PO_4. The final pH of the saline was 7.8.

6.2.3 Titanium dioxide stock suspensions characterisation

Nano particle and bulk forms of TiO_2 were obtained from DeGussa AG ("Aeroxide" P25, supplied by Lawrence Industries, Tamworth, UK) and ACROS (New jersey, USA) respectively and were the same dry powders as used by Federici *et al.* (2007), Ramsden *et al.* (2009) and Boyle *et al.* (2013). Manufacturer's information indicated that TiO_2 NPs have a crystal structure of 25 % rutile and 75 % anatase TiO_2, with > 99 % purity (as TiO_2, maximum impurity stated was 1 % Si). Analysis of the crystal structure of bulk TiO_2 by transmission electron microscopy (TEM, JEOL 12000EXII, Tokyo, Japan) as described in Boyle *et al.* (2013) and in chapter 6. Briefly, the crystal structure of particles was (means ± S.D., n = 10 micrographs analysed) 25 ± 5 % rutile and 75 ± 5 % anatase (Boyle *et al.*, 2013). Whereas, the primary particle sizes of 1 g l^{-1} dilution with Milli-Q water of the

stocks (10 g l^{-1}) was (means ± S.D., *n* = 100) 134 ± 42 nm and 24 ± 10 nm of the bulk TiO_2 and TiO_2 NPs respectively (see Boyle *et al.*, 2013). Stock suspensions of 1 g l^{-1} of either bulk TiO_2 or TiO_2 NPs (nominal concentrations) were prepared in Cortland's saline (as described above) and sonicated in sonicator bath for 6 h (35 kHz frequency, Fisherbrand FB 11010, Germany) before injection. The particle size distributions of the stock saline solution were not measured because of the high ionic strength of the saline that made particles to settled out too fast. However, the actual concentrations of TiO_2 in stock saline suspensions were measured by ICP (Means ± S.D., *n* = 3): 0.98 ± 0.02 g l^{-1} and 1.08 ± 0.03 g l^{-1} for bulk and NPs respectively.

6.2.4 Titanium dioxide injection study

Juvenile rainbow trout (*n* = 14 per treatment) were anaesthetised in buffered MS222 (50 mg l^{-1}, pH 7.1) and injected into the caudal blood vessels with Cortland's saline (50 µl, control), bulk TiO_2 or TiO_2 NPs (both 50 µg TiO_2 in 50 µl saline) with a 1 ml Terumo insulin syringe with a 29 gauge needle (Terumo Medical Corporation, Somerset, NJ, USA). After injection, fish were resuscitated in oxygen saturated water and returned to tanks in a recirculating semi-closed system containing Plymouth tap water (water quality as above). All trout exhibited no signs of poor health following injection, and recovered quickly from the procedure. Trout sampled at time zero were not injected with Cortland's saline. At 0, 24 and 96 hours post injection, fish were sampled for haematology and plasma ions, tissue metal, and biochemical analysis (Bolye *et al.*, unpublished). To investigate the pathological effects on target organs, fish were sampled at 0 and 96 h post injections (presented here). Spleen prints samples were collected at 24 and 96 h post injections (also presented here).

6.2.5 Histological analysis

Briefly, after 96 h of injection, fish (six fish/treatment, two fish/tanks) were anaesthetised with MS222 and organs (gill, gut, liver, spleen, kidney and whole brain) collected into buffered formal saline and then the specimens were processed for histopathological examination as described in Chapter 2 (see 2.5- 2.9).

6.2.6 Spleen print

Spleen prints were collected from the trout after 24 and 96 h of injection, then stained with May-Grünwald Giemsa stain and scored the cellular pathologies and haematopoietic cells as described in Chapter 2 (see 2.10).

6.2.7 Statistical analysis

All resulting data from the experiment were presented as mean ± S.E.M. and analysed by using Stat Graphics Plus Version 5.1 as described in Chapter 2 (see 2.11).

6.3. Results

6.3.1 Distribution of Ti into the tissues

The tissues Ti analysis showed that the kidney was the principle site of accumulation of both bulk TiO_2 and TiO_2 NPs injected into the caudal vein (Fig. 6.1). Measured concentrations of Ti in the kidney after 96 h post injection were (means ± S.E.M.): 0.8 ± 0.1 μg g^{-1} in controls and 154.8 ± 40.3 μg g^{-1} and 273.0 ± 33.0 μg g^{-1}, bulk and NP exposed fish respectively. Measured Ti burdens in the kidney of fish exposed to

TiO_2 NPs also increased over time, increasing from (means ± S.E.M.) 0.9 ± 0.1 μg g^{-1} to 170.5 ± 8.5 μg g^{-1} and 273.0 ± 33.0 μg g^{-1} at 0, 24 and 96 hours respectively and a similar trend was evident in fish exposed to bulk TiO_2 (Fig. 6.1). The spleen was also a site of accumulation of TiO_2 NPs, but not bulk TiO_2. After 96 h of injection, the concentration of Ti in spleens of fish increased from (means ± S.E.M.) 1.5 ± 0.3 μg g^{-1} in controls and 2.4 ± 0.5 μg g^{-1} in the bulk exposed fish to 26.2 ± 6.2 μg g^{-1} in fish injected with NPs. Neither bulk TiO_2 nor NPs were observed to accumulate in the gill, intestine, liver, heart, muscle or brains of fish (Fig. 6.1).

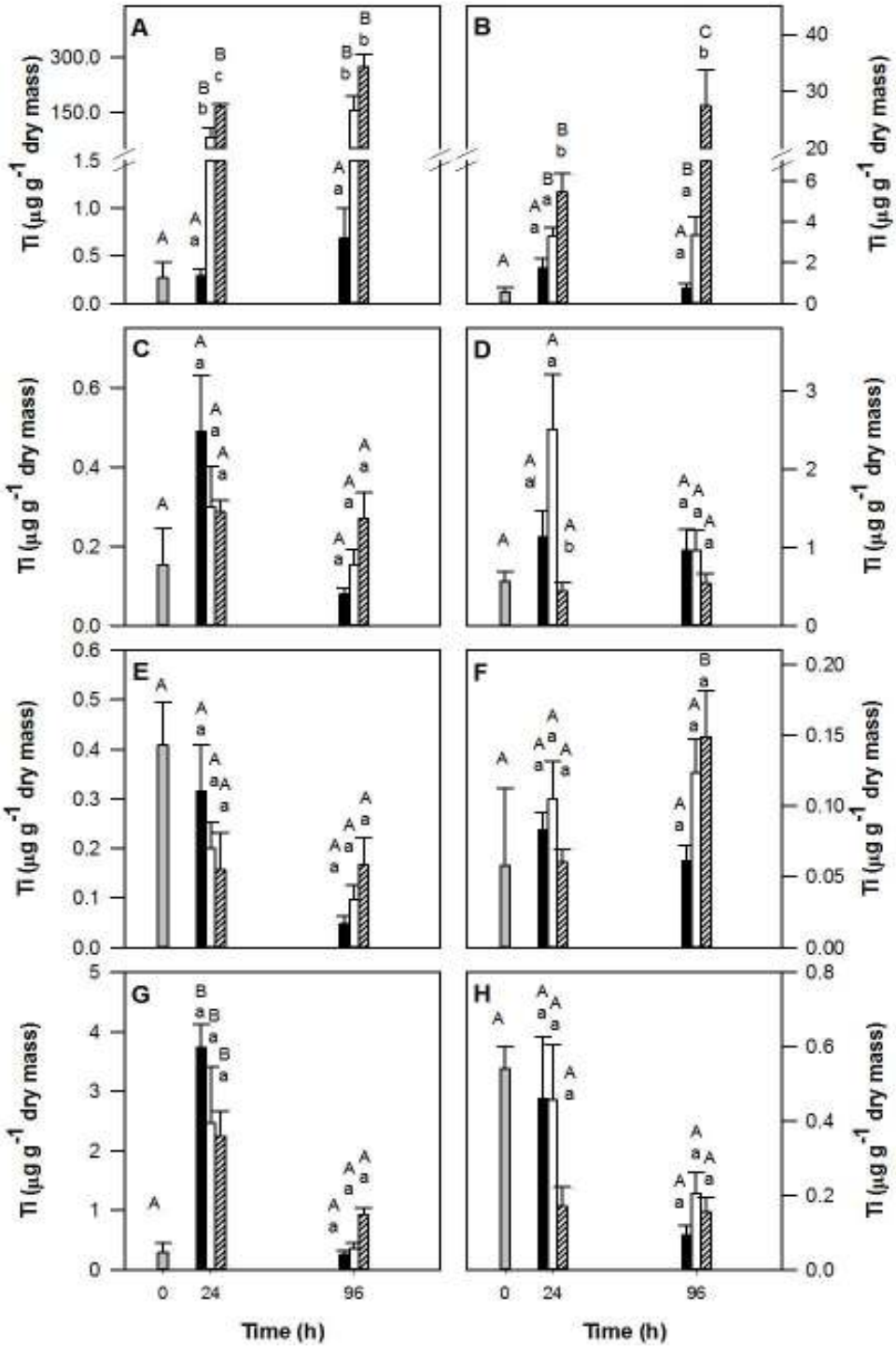
A
B
C
D
E
F
G
H
Tl (µg g-1 dry mass)
Time (h)
0
24
96

Figure 6.1 Titanium concentration in the (A) kidney, (B) spleen, (C) liver, (D) heart, (E) brain, (F) muscle, (G) gill, (H) intestine of rainbow trout after 0, 24, and 96 h injection of, 50 µl ml^{-1} Cortland's saline (black bars), 50 µg of bulk TiO_2 (white bars) in 50 µl saline, 50 µg of TiO_2 NPs (hatched bars) in 50 µl saline. The highest Ti accumulation was observed in the kidney followed by the spleen of fish injected with TiO_2 NPs treatment, whilst bulk TiO_2 treatment showed Ti accumulation in the kidney. Other organs did not show Ti accumulation. Time 0 fish (grey bars) were not injected. Data are means ± S.E.M., dry weight tissue, n = 6 fish/treatment. Different lower case letters denote a statistically significant difference within time points (ANOVA, $P < 0.05$). Different upper case letters denote a statistically significant difference between time-points (ANOVA, $P < 0.05$).

6.3.2 Gill histopathology

Histological examination of the gills in freshwater controls (Cortland's saline injected) showed normal structure and absence of lesions (Fig. 6.2). After 96 h injection of either bulk or TiO_2 NPs, the gills showed minor lesions compared to controls (Fig. 6.2). These lesions included foci of aneurism, hyperplasia, occasional evidence of necrotic cells in the primary lamellae, evidence of oedema and clubbed tips in the secondary lamellae, observed in 4/6 fish examined per treatment. A quantitative analysis of the incidence of gill injury confirmed these observations with a statistically significant increase in the incidence of clubbed tips with both TiO_2 treatments compared to the control (ANOVA, $P < 0.05$, Table 6.1), with no material-type effect. There were also statistically significant increased in the incidence of aneurism, hyperplasia, fusion, atrophy and occasional necrotic cells in the gill epithelium with both bulk and nano treatments, but these all remained below a 4 % incidence rate (Table 6.1).

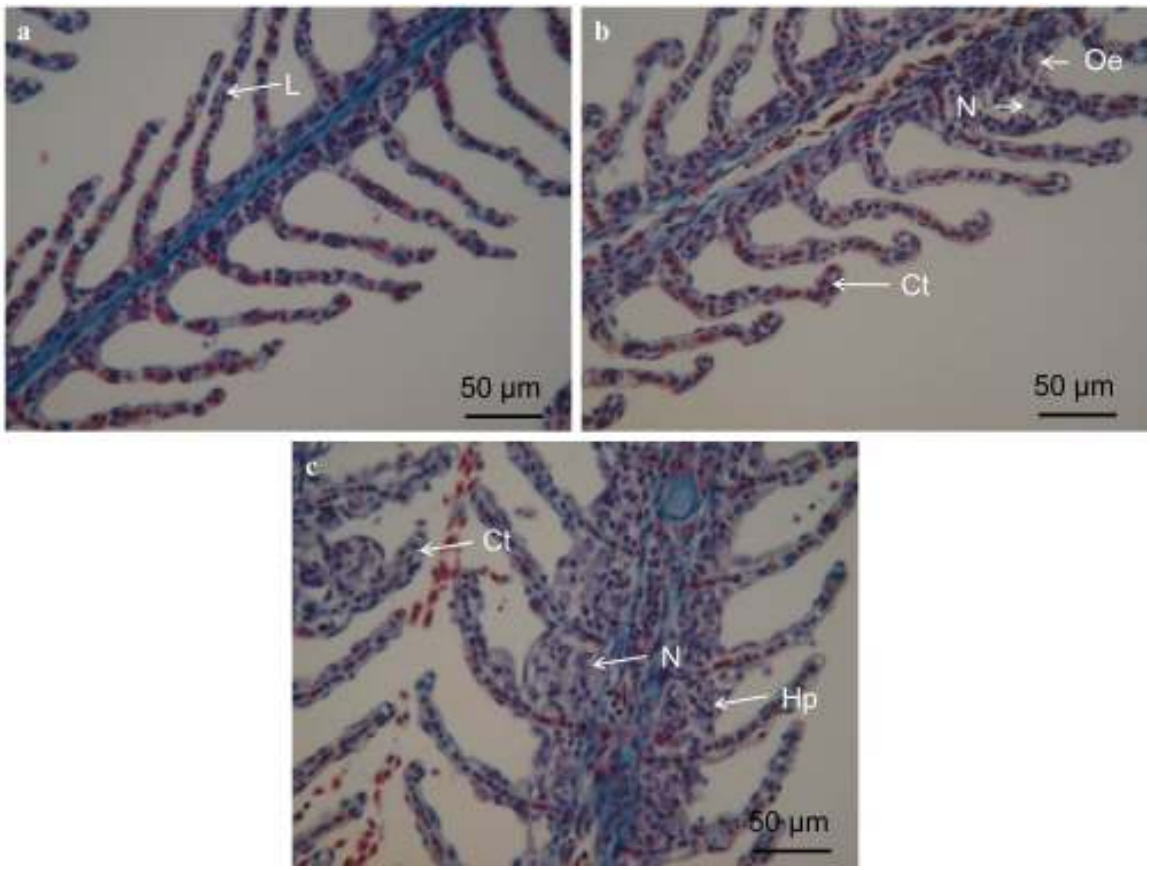

Figure 6.2 Gill morphology of rainbow trout after 96 h injection of (a) 50 µl ml^{-1} Cortland's saline (control), (b) 50 µg bulk in 50 µl of saline, (c) 50 µg TiO_2 NPs in 50 µl of saline. Gills from saline groups showed normal appearance of gill lamellae (L). Both TiO_2 treatments showed incidence of hyperplasia (Hp), clubbed tips (Ct), oedema (Oe) and necrosis cells (N). Scale bar indicates magnification. Sections were 7 µm thick and stained with Mallory's trichrome.

Table 6.1 The proportion of gill lesions in rainbow trout after 96 h injection of 50 µl ml^{-1} Cortland's saline (control), 50 µg of either bulk or TiO_2 NPs in 50 µl of saline.

Proportion of incidence gill lesions	Control	Bulk	TiO_2 NPs
Hyperplasia	Not observed	1.8 ± 0.3 *	1.3 ± 0.3 *
Fusion	Not observed	1.7 ± 0.6 *	2.8 ± 0.6 *
Aneurism	Not observed	2.2 ± 0.8 *	3.3 ± 0.3 *
Clubbed tips	3.5 ± 0.2	14.5 ± 0.3 *	13.5 ± 0.4 *
Atrophy	Not observed	2.0 ± 0.5	3.7 ± 1.3 *
Necrosis	Not observed	1.5 ± 0.3 *	1.0 ± 0.3 *

Data are means of proportional cells ± S.E.M (n = 6 number of fish/treatments). (*), significant different from control group within treatments (ANOVA, $P < 0.05$). Not observed, the lesion was absent from the gills examined from all the fish (a mean of zero).

6.3.3 Gut histopathology

The gut of trout injected with Cortland's saline showed normal histology (Fig. 6.3). After 96 h injection of either bulk or NPs, the gut mucosa showed minor injuries, with a material-type effect (worse with bulk than NPs). These injuries included occasional areas of epithelial lifting associated with necrotic cells, occasional erosion of the intestinal villi tips, as well as occasional incidence of swollen goblet cell in the mucosa layer, which were observed in 3/6 fish examined per treatment (Fig. 6.3). A quantitative analysis of the gut mucosa confirmed a small material-type effect with bulk than NP form. For example,

injection of bulk to trout caused a small alteration in the width of villi, the dimension of columnar cell and the columnar cell nuclei than TiO_2 NPs (Fig. 6.4).

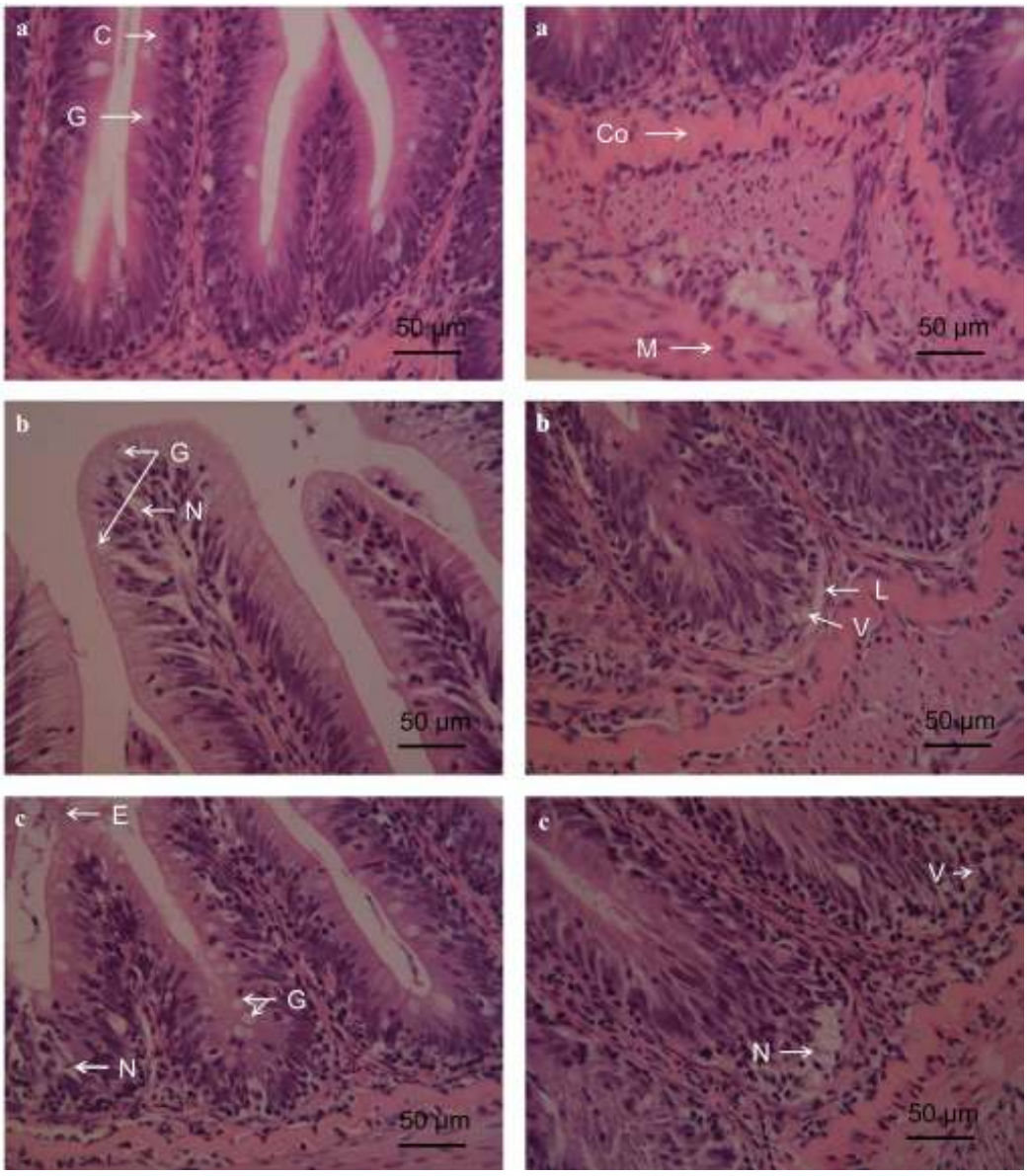

Figure 6.3 Gut morphology in rainbow trout after 96 h injection of (a) 50 µl ml^{-1} Cortland's saline (control) (b) 50 µg bulk in 50 µl of saline (c) 50 µg TiO_2 NPs in 50 µl of saline. The left column shows the shape of villi tips, while the right column shows the base of villi. The gut of controls showed normal histology of the villi (V), columnar cell (C), goblet cell (G), compactum layer (Co) and muscular layer (M). After 96 h post injection of bulk or TiO_2

NPs, the gut showed similar injuries, but the bulk material caused greater effects than TiO_2 NPs. These injuries include incidence of swelling of goblet cells, necrosis in the epithelial of mucosa (N), lifting epithelium (L), vacuole formation in the mucosa epithelium (V), and erosion of villi (E). Scale bar indicates magnification. Sections were 7 µm thick and stained with H&E.

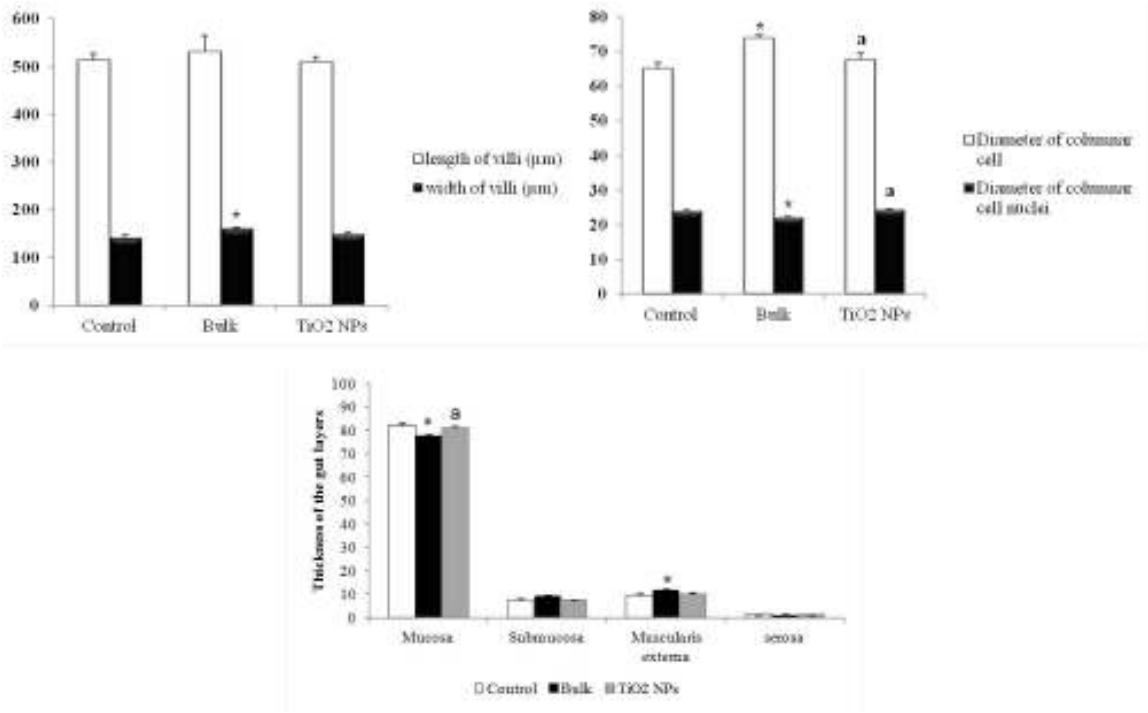

Figure 6.4 Alteration observed in the gut of trout after 96 h injection of 50 µl ml^{-1} of Cortland's saline (control), 50 µg of either bulk or TiO_2 NPs in 50 µl of saline. Data are means ± S.E.M., n = 6 fish per treatment. (*), significant differences between control groups and treatments (ANOVA, $P < 0.05$). (a), significant difference between bulk and TiO_2 NPs (ANOVA or t-test, $P < 0.05$).

6.3.4 Liver histopathology

Livers from control animals that were injected with Cortland's saline showed normal architecture with typical hepatocytes morphology and sinusoid space that contained red blood cells. Liver pathology was absent in control animals with no evidence of oedema,

hepatitis and a few deposits or the absence of melanomacrophages (Fig. 6.5). After 96 h injection of bulk TiO_2, the livers showed lesions (Fig. 6.5). All six fish showed occasional pyknotic nuclei and hepatitis like cell injury. Cytoplasmic vacuolisation, oedema and increases in the number of melanomacrophage deposits were seen in 5/6 fish examined per treatment. The intravenous injection of TiO_2 NPs treated-animals exhibited similar types of lesions to that observed with bulk, in 3/6 fish examined. Quantitative histological analysis of the liver confirmed that both TiO_2 treatments caused alteration in the liver, but the bulk was more effective than TiO_2 NPs treatment. Pathological observation from this study showed a small but statistically significant change in the proportion of hepatic area and sinusoid space with NPs treatment compared to either the bulk or control groups (Table 6.2). Both bulk and NPs treatment exhibited a statistically significant decrease in the diameter of hepatic cells that was associated with an increase in the ratio of the nucleus: hepatocytes diameter compared to unexposed controls (Table 6.2). The number of melanomacrophage deposits was also increased with both TiO_2 treatments, with a material-type effect (statistically significant ANOVA, $P < 0.05$; worse in the bulk than NPs treatment) (Table 6.2).

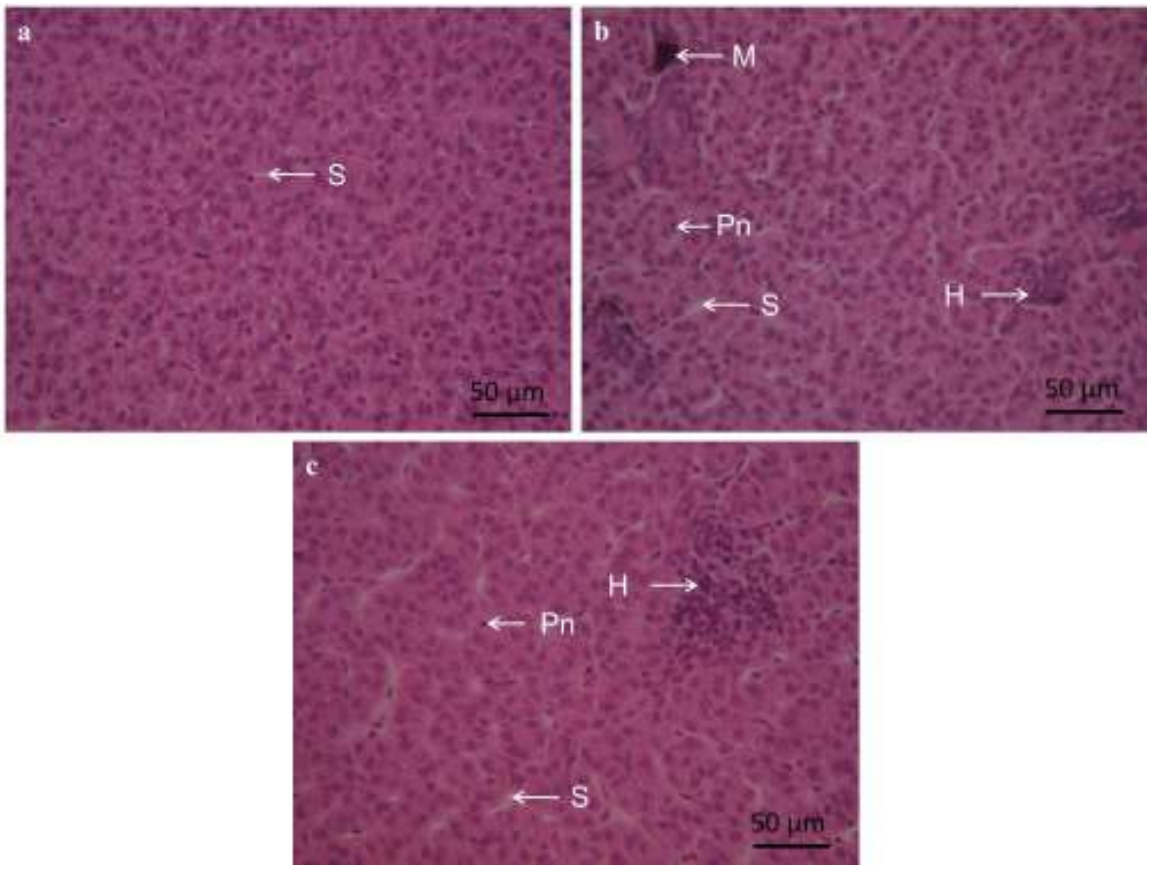

Figure 6.5 Liver morphology after 96 h injection of (a) 50 µl ml^{-1} Cortland's saline (control), (b) 50 µg bulk in 50 µl of saline, (c) 50 µg TiO_2 NPs in 50 µl of saline. The livers of the control group showed normal histology with normal sinusoid space (S). All TiO_2 treatments showed lesions that include foci of melanomacrophage deposits (M); cells with pyknotic nuclei (Pn); and hepatitis-like injuries (H). These lesions were slightly more severe with bulk than TiO_2 NPs. Scale bar indicates magnification. Sections were 7 µm thick and stained with H&E.

Table 6.2 Alteration observed in the liver of rainbow trout after 96 h injection of 50 µl ml^{-1} Cortland's saline (control), 50 µg of either bulk or TiO_2 NPs in 50 µl of saline.

Parameters	Cortland's (saline)	Bulk	TiO_2 NPs
Proportion of hepatic area	96.1 ± 0.2	95.6 ± 0.1	95.2 ± 0.2 *
Proportion of sinusoid space	3.9 ± 0.2	4.4 ± 0.1	4.9 ± 0.2 *
Diameter of the hepatic cells (µm)	18.6 ± 0.1	16.0 ± 0.0 *	16.6 ± 0.2 *[a]
Diameter of the hepatic cell nuclei (µm)	7.8 ± 0.1	7.6 ± 0.1	7.7 ± 0.1
Ratio of hepatic cell nuclei to hepatic cells	0.42 ± 0.01	0.48 ± 0.01 *	0.46 ± 0.01 *
Number of melanomacrophage deposits	6.0 ± 0.6	12.0 ± 0.7 *	10.0 ± 1.0 *[a]

Data are means of proportional areas, or means of dimensions, ± S.E.M. (n = 6 fish/treatments). (*), significant difference between control within row (ANOVA, $P < 0.05$). (a), significant difference between bulk and TiO_2 NPs (t- test, $P < 0.05$). Note: the number of melanomacrophage was counted /field of view at x 400 magnification (53,200 μm^2).

6.3.5 Spleen pathology

Histological observation in the spleens of control fish (injected with Cortland's saline) showed normal histology (Fig. 6.6). The fish showed similar types of lesion after 96 h of injected with either bulk or TiO_2 NPs. These lesions included occasional foci of necrosis, vacuole formation, and elevation in the number of melanomacrophage deposits throughout the spleen in 5/6 fish examined from each treatment (Fig. 6.6). The fractional

volume of red pulp was significantly decreased in trout after injection of either bulk or NPs with a concomitant increased in the white pulp when compared to controls (ANOVA, $P < 0.05$; Fig. 6.7); with a material-types effect (severe with NPs treatment than bulk). The proportion of sinusoid space showed a small decrease with the NP treatment compared to the control or bulk treatments. Notably, there was also a statistically significant increase in the number of melanomacrophage deposits with both TiO_2 treatments compared to the control (Fig. 6.7), but no material-type effect was observed between bulk and NP treatments in this parameter.

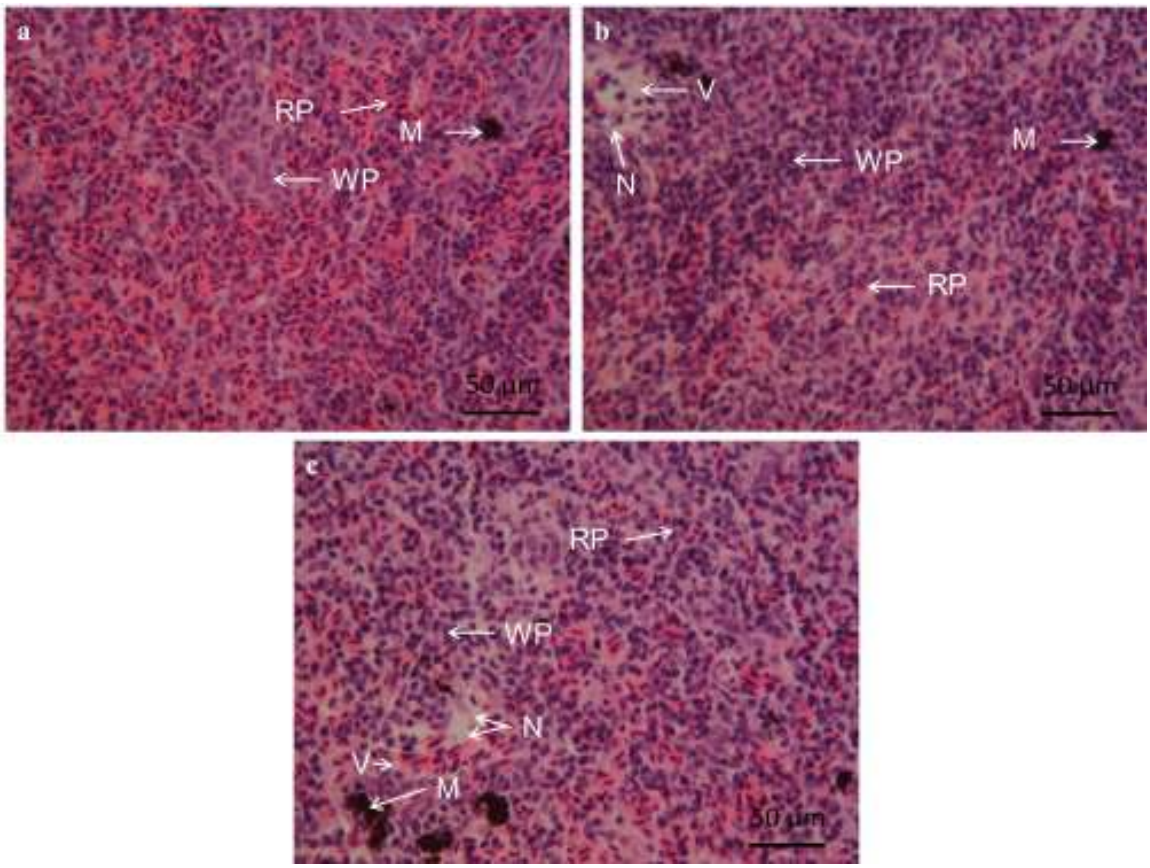

Figure 6.6 Spleen morphology in rainbow trout after 96 h injection of (a) 50 µl ml^{-1} Cortland's saline (control), (b) 50 µg bulk in 50 µl of saline, (c) 50 µg TiO_2 NPs in 50 µl of saline. The spleens from control fish showed normal histology, with defined red (RP) and white pulp (WP) and a small number of melanomacrophage deposits (M). Both TiO_2 treatments showed similar injuries that include alteration in the proportion of red pulp (RP), occasional foci of necrotic cells (N), vacuole formation (V) and increased the number of melanomacrophage deposits (IM). Scale bar indicates magnification. Sections were 7 µm thick and stained with H&E.

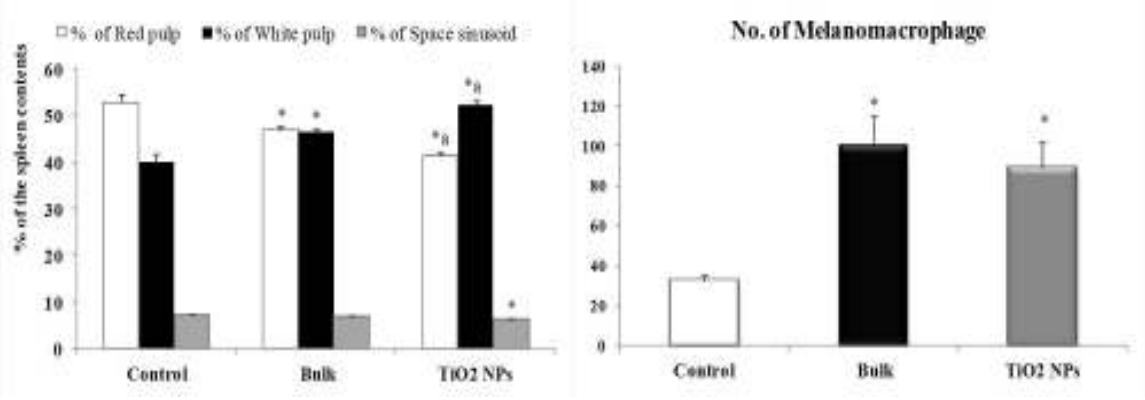

Figure 6.7 Alteration observed in the proportion of red, white pulp, sinusoid space and the number of melanomacrophage deposits after 96 h injection of 50 µl ml^{-1} Cortland's saline (as control), 50 µg of either bulk or TiO_2 NPs in 50 µl of saline. Data are means of proportional area, or means ± S.E.M., n = 6 fish per treatments. (*), significant difference between controls and treatments (ANOVA, $P < 0.05$). (a), significant difference between bulk and TiO_2 NPs (t- test, $P < 0.05$).

6.3.6 Spleen print

Spleen prints of control trout (Cortland's saline injected) showed normal presence of haematopoietic cells (Fig. 6.8). These cells included immature (haemocytoblast, erythroblast) and mature cells (erythrocytes and neutrophils). After 24 and 96 h injection of either bulk or TiO_2 NPs, the proportion of haematopoietic cells showed changes when compared to controls (Table 6.3). These changes included a small but statistically significant decrease in the proportion of immature (haemocytoblast) and mature cells (erythrocytes) compared to the control (ANOVA, $P < 0.05$; Table 6.3). The proportion of immune cells (neutrophils) also showed a statistically significant increase after 24 and 96 h of injection with both TiO_2 treatments compared to controls (ANOVA, $P < 0.05$; Table 6.3). A material-type (greater effects with NPs than bulk) and time effects (worse effects at

24 h than 96 h) were observed in the proportion of erythroblast, erythrocytes and neutrophils (all statistically significant ANOVA, $P < 0.05$; Table 6.3)

The erythrocytes morphology of control animals (Cortland's saline injected) showed normal structure (Fig. 6.8). After 24 and 96 h injection of either bulk or NP treatments, the spleen prints showed a statistically significant (ANOVA, $P < 0.05$) increase in the proportion of swollen erythrocytes, cells with a dividing nuclei, and cells with membrane abnormalities compared to the control (Table 6.3). The proportion of cells with a dividing nucleus and cells with membrane abnormalities showed a material-type effect (worse in NPs treatment than bulk) and time effect (greater changes at 24 h than 96 h from NPs treatment only) (all statistically significant, ANOVA or *t*-test, $P < 0.05$).

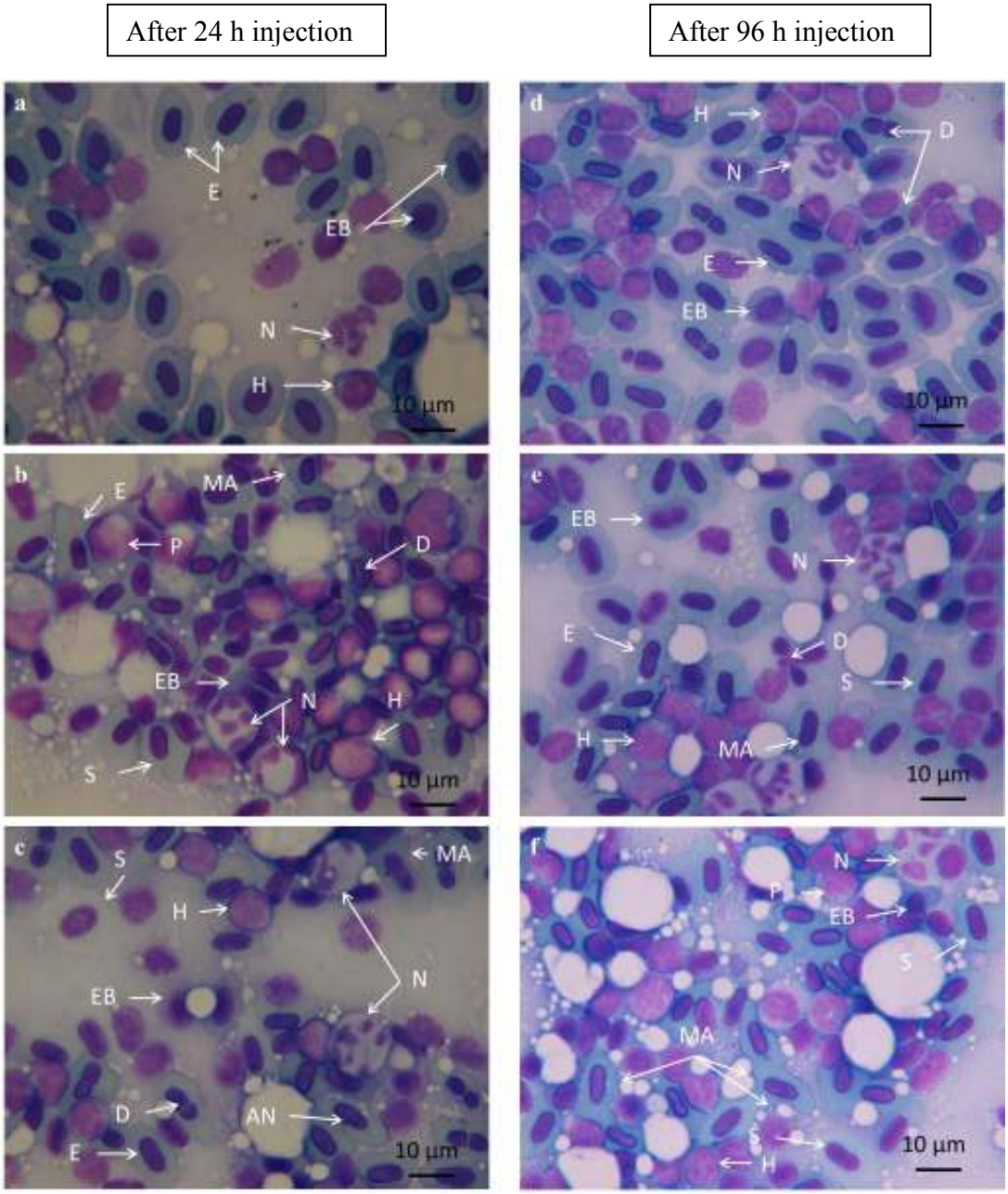

Figure 6.8 Spleen prints of rainbow trout after 24 h (left column) and 96 h (right column) of Ti injection. For 24 h, the panels included (a) 50 µl ml^{-1} Cortland's saline (as control), (b) 50 µg of bulk in 50 µl of saline, (c) 50 µg TiO_2 NPs in 50 µl of saline. For 96 h, the panels included (d) 50 µl ml^{-1} Cortland's saline (as control), (e) 50 µg of bulk in 50 µl of saline, (f) 50 µg TiO_2 NPs in 50 µl of saline. Both TiO_2 treatments showed alteration in the proportion of haematopoietic cells and erythrocytes abnormalities. Erythrocytes (E), erythroblast (EB), neutrophil (N), haemocytoblast (H), progranulocyte (P), red blood cells

with a dividing nucleus (D), membrane abnormalities of erythrocytes (MA), swollen erythrocyte (S), nucleus abnormality (AN). Scale bar indicates magnification. Smear stained with May- Grunwald-Giemsa.

Table 6.3 Alteration in the percentage of haematopoietic cells and the percentage of erythrocytes abnormality in the spleen prints of rainbow trout after 24 and 96 h injection of 50 µl ml^{-1} of Cortland's saline (control), 50 µg of either bulk or TiO_2 NPs in 50 µl of saline. Day 0 fish were not injected with saline.

Parameters		Time/ hours	Controls	Bulk	TiO_2 NPs
% of the haematopoietic cells	Haemocytoblast	0	16.1 ± 0.2		
		24	15.1 ± 0.1 *	13.7 ± 0.2 *$^{+}$	12.8 ± 0.5 *$^{+}$
		96	14.6 ± 0.3 *^{a}	12.9 ± 0.1 *$^{+a}$	12.9 ± 0.1 *$^{+}$
	Erythroblast	0	15.9 ± 0.2		
		24	16.5 ± 0.3	17.4 ± 0.37 *$^{+}$	17.6 ± 0.2 *$^{+}$
		96	14.8 ± 0.1 *^{a}	13.5 ± 0.2 *$^{+a}$	12.5 ± 0.2 *$^{+ab}$
	Progranulocytes	0	2.8 ± 0.2		
		24	3.2 ± 0.2	2.9 ± 0.1	2.6 ± 0.1 $^{+}$
		96	1.7 ± 0.1 *^{a}	3.7 ± 0.1*$^{+a}$	3.9 ± 0.1 *$^{+a}$
	Erythrocytes	0	59.9 ± 0.3		
		24	60.1 ± 0.3	56.6 ± 0.1 *$^{+}$	55.4 ± 0.3 *$^{+b}$
		96	66.7 ± 0.2 *^{a}	62.3 ± 0.1 *$^{+a}$	63.4 ± 0.3 *$^{+ab}$
	Neutrophils	0	5.4 ± 0.3		
		24	5.0 ± 0.1	7.8 ± 0.4 *$^{+}$	11.3 ± 0.7 *$^{+b}$
		96	2.2 ± 0.2 *^{a}	7.0 ± 0.2 *$^{+}$	7.1 ± 0.1 *$^{+ a}$
% of erythrocytes abnormalities	Swollen cells	0	Not observed		
		24	Not observed	6.4 ± 0.4 *$^{+}$	7.1 ± 0.2 *$^{+}$
		96	Not observed	7.1 ± 0.3 *$^{+}$	7.6 ± 0.2 *$^{+}$
	Cells with a dividing nucleus	0	8.4 ± 0.2		
		24	9.2 ± 0.3	11.6 ± 0.4 *$^{+}$	13.5 ± 0.3 *$^{+b}$
		96	10.9 ± 0.3 *^{a}	12.6 ± 0.3 *$^{+}$	9.6 ± 0.5 *$^{+ab}$
	Cells with membrane abnormalities	0	1.9 ± 0.2		
		24	2.0 ± 0.2	11.6 ± 0.6 *$^{+}$	13.3 ± 0.4 *$^{+b}$
		96	4.1 ± 0.2 *^{a}	11.1 ± 0.3 *$^{+}$	11.7 ± 0.3 *$^{+a}$

Data are means of proportional cells ± S.E.M. (n = 6 fish/treatment). (*), significantly different from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). (+), significantly different from control within row in each time point of injection (ANOVA, $P < 0.05$). (a),

significant difference between 24 and 96 h within a treatment (time effect, ANOVA, $P < 0.05$). (b), significant difference between bulk and TiO_2 NPs (*t* - test, $P < 0.05$).

6.3.7 Kidney histopathology

Injection with Cortland's solution showed normal histology of kidney with the absence of lesions (Fig. 6.9). After 96 h injection of either bulk or TiO_2 NPs, the kidney showed injuries. These injuries included occasional areas of necrosis in the haematopoietic tissue, renal tubules separation, cytoplasmic vacuolisation was seen in 3/6 fish examined from each treatment. Increases of the Bowman's space capacity was observed in 4/6 fish examined from both TiO_2 treatments compared to controls (Fig. 6.9).

Quantitative analysis of the kidney histology illustrated that both TiO_2 treatments caused a small but statistically significant increase in the proportional area of the specimen as renal corpuscle, and lumen of proximal and distal tubules compared to controls (ANOVA, $P < 0.05$; Table 6.4). The dimension of Bowman's space was statistically significantly increased with both TiO_2 treatments compared to the control (ANOVA, $P < 0.05$; mean ± S.E.M., $n = 6$; 5.8 ± 1.1 in control, 10.1 ± 0.8 in bulk, 8.5 ± 0.7 in TiO_2 NPs), but with no material-type effect. The number of melanomacrophage deposits did not show alteration with bulk or TiO_2 NPs treatments compared to controls (Table 6.4).

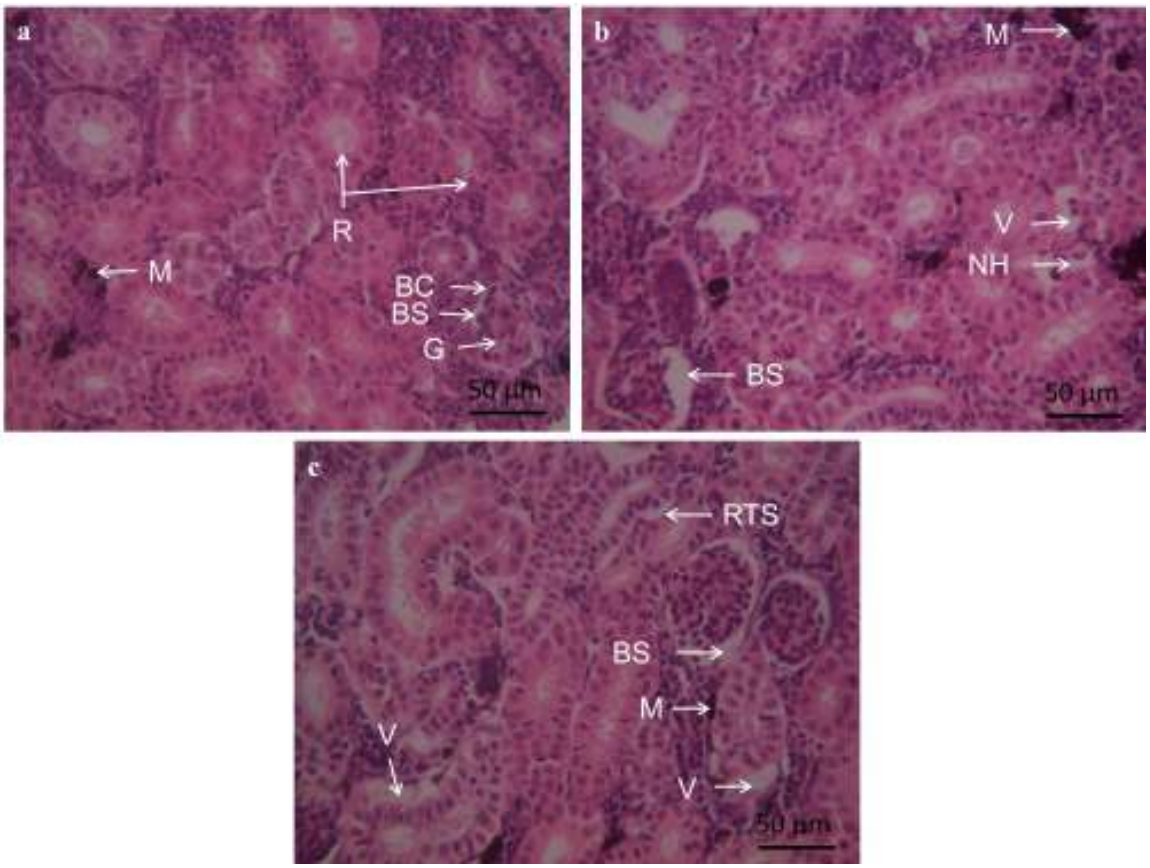

Figure 6.9 Kidney morphology in rainbow trout after 96 h injection of (a) 50 µl ml^{-1} Cortland's saline (control), (b) 50 µg bulk in 50 µl of saline, (c) 50 µg TiO_2 NPs in 50 µl of saline. The kidneys of control groups showed normal structure of the renal tubules (R), glomerulus (G), Bowman's capsule (BC), Bowman's space (BS) and melanomacrophage deposits. Both TiO_2 treatments showed occasional necrosis in the haematopoietic tissues (NH); increased the Bowman's space (BS); renal tubular separation (RTS) and vacuolisation (V). Scale bar indicates magnification. Sections were 7 µm thick and stained with H&E.

Table 6.4 Alteration observed in the kidney of rainbow trout after 96 h injection of 50 µl ml^{-1} Cortland's saline (control), 50 µg of either bulk or TiO_2 NPs in 50 µl of saline.

Kidney Parameters		Control	Bulk TiO_2	TiO_2 NPs
Proportional of kidney areas	% Renal corpuscle	1.2 ± 0.0	2.1 ± 0.3 *	2.7 ± 0.3 *
	% Proximal tubules	34.9 ± 0.9	29.2 ± 1.2 *	36.2 ± 1.7 [a]
	% Distal tubules	8.6 ± 0.2	8.7 ± 1.1	5.1 ± 0.2 *[a]
	% Haematopoietic tissues	55.3 ± 0.9	60.0 ± 1.0 *	56.1 ± 1.6
Number of melanomacrophage deposits		242.8 ± 13.9	295.3 ± 42.4	286.2 ± 31.5
Dimension of renal corpuscle (µm)	Total dimension	57.9 ± 2.5	58.1 ± 1.8	60.0 ± 2.8
	Glomerulus	52.1 ± 2.9	48.0 ± 1.4	51.5 ± 2.6
	Bowman's space	5.8 ± 1.1	10.1 ± 0.8 *	8.5 ± 0.7 *
Dimension of proximal tubules (µm)	Total width	51.2 ± 0.5	53.0 ± 0.3 *	51.2 ± 0.1 [a]
	Lumen	15.1 ± 0.3	17.6 ± 0.2 *	17.3 ± 0.1 *
	Epithelial cell	36.1 ± 0.5	35.3 ± 0.3	33.9 ± 0.2 *[a]
Dimension of distal tubules (µm)	Total width	38.6 ± 0.5	38.9 ± 0.4	38.6 ± 0.2
	Lumen	9.0 ± 0.4	10.3 ± 0.2 *	10.2 ± 0.2 *
	Epithelial cell	29.6 ± 0.5	28.6 ± 0.5	28.4 ± 0.3

Data are means of proportional areas, or means ± S.E.M. (n = 6 fish/treatments). (*), significant difference between control within row (ANOVA, $P < 0.05$). (a), Significant difference between bulk and TiO_2 NPs (t- test or ANOVA, $P < 0.05$). Note: the number of melanomacrophage deposits was counted/field of view at x 200 magnification of 212,400 μm^2.

6.3.8 Brain histopathology

The brains of control animals showed normal structure of telencephalon, mesencephalon and metencephalon (Fig. 6.10, 6.11 & 6.12), and the gross morphology of trout brains injected with either bulk or TiO_2 NPs was mainly normal, although subtle structural changes in the brain were observed. In the cerebrum or fore brain (telencephalon), there was evidence of the occasional nerve cell body with pyknotic nuclei, or necrotic nerve cells, and enlarged nerve cells, but the occasional incidence was the same in both bulk and TiO_2 NPs treatments (no clear material-type effect, Fig. 6.10). These lesions were observed in 3/6 fish examined from each TiO_2 treatments. The diameter of nerve cells in the forebrain showed a statistically significant increase with both bulk and TiO_2 NPs treatments compared to controls (Table 6.5), with a material-type effect (worse with bulk than NPs treatments). The types of forebrain lesions were not observed in the mid-brain (mesencephalon) or in the hind brain (cerebellum).

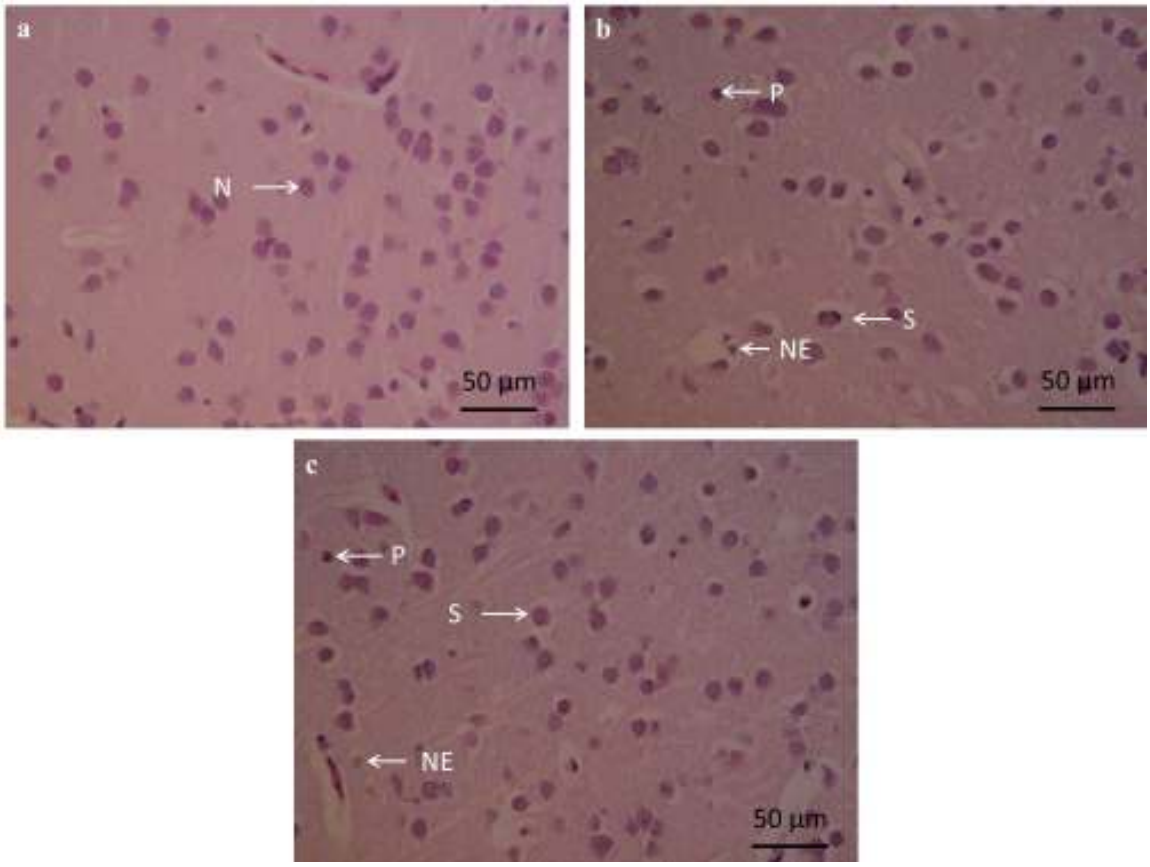

Figure 6.10 Telencephalon morphology in rainbow trout after 96 h injection of (a) 50 µl ml^{-1} Cortland's saline (control) (b) 50 µg bulk in 50 µl of saline (c) 50 µg TiO_2 NPs in 50 µl of saline. All control groups showed normal structures of neuron (N) in the telencephalon. Injection of bulk or TiO_2 NPs showed injuries that include occasional pyknotic nuclei (P), swollen neurons (S), and evidence of necrotic nerve cells (NE). These injuries were slightly severe with bulk than TiO_2 NPs. Scale bar indicates magnification. Sections were 7 µm thick and stained with H&E.

The mesencephalon was normal in fish from control groups and both TiO_2 treatments (Fig. 6.11), with small changes in the proportion of mesencephalon thickness layers. The thicknesses of st. fibrosum marginale, st. fibrosum et griseum superficial and st. griseum central in the mesencephalon were changed with both TiO_2 treatments compared to controls (difference less than 3 %, Table 6.5).

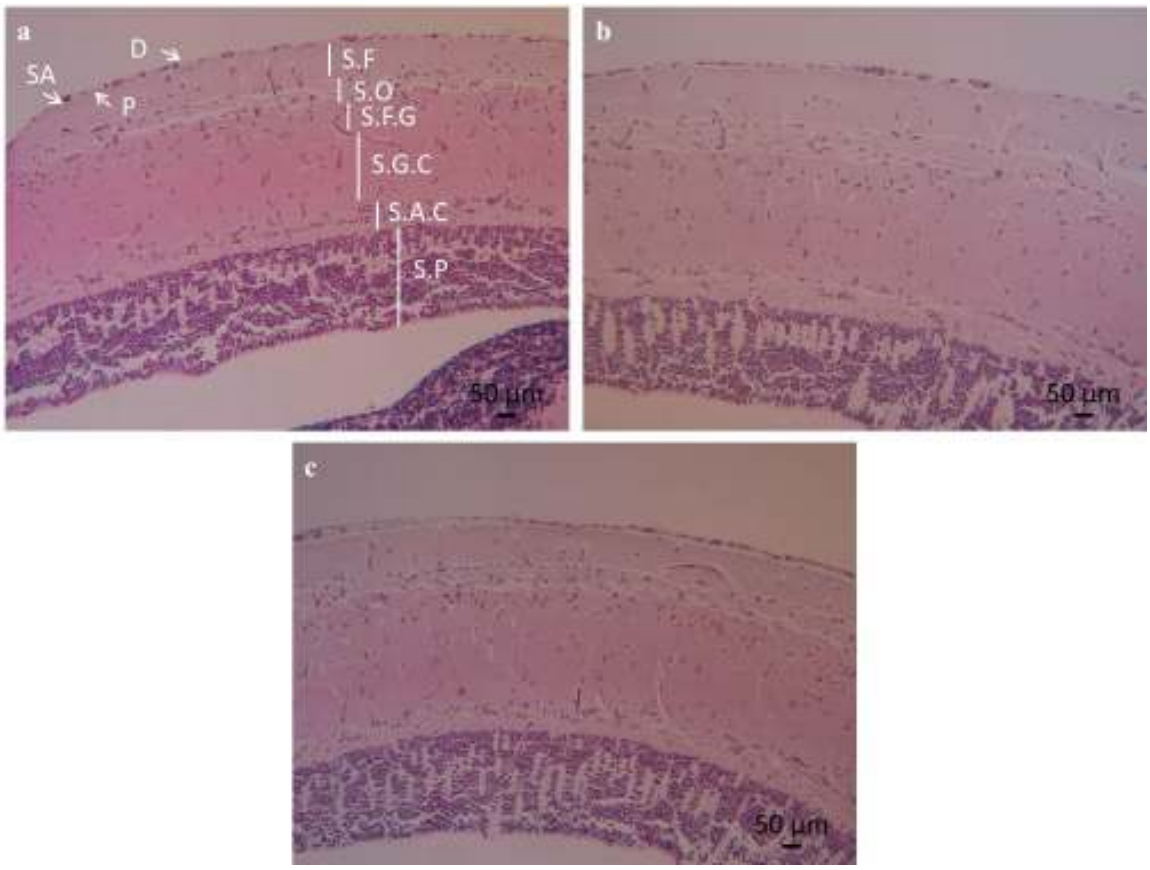

Figure 6.11 Mesencephalon morphology in rainbow trout after 96 h injection of (a) 50 µl ml^{-1} Cortland's saline (control), (b) 50 µg bulk in 50 µl of saline, (c) 50 µg TiO_2 NPs in 50 µl of saline. All control groups showed normal structure in the mesencephalon layers that include dura mater (D), subarachnoid space (SA), pia mater (P), stratum fibrosum marginale (S.F), stratum opticum (S.O), stratum fibrosum et griseum superficiale (S.F.G), stratum griseum centrale (S.G.C), stratum album centrale (S.A.C), stratum periventriculare (S.P). Both treatments showed a small alteration in the thickness of mesencephalon layers. Scale bar indicates magnification. Sections were 7 µm thick and stained with H&E.

The metencephalon regions showed normal structure in fish that were injected with Cortland's saline (Fig. 6.12). The fish injected with bulk or TiO_2 NPs treatments showed similar changes included enlarged blood vessels on the surface of cerebellum, which was observed in 5/6 fish examined from each treatment. Occasional evidence of necrosis

between the molecular and granular layers were seen in 2/6 fish examined per treatment (Fig. 6.12). A quantitative analysis confirmed these results and showed both TiO_2 treatments caused a statistically significant increase in the thickness of the capsule layer (the space between capsule and molecular layer) in the cerebellum compared to the control (ANOVA, $P < 0.05$, Table 6.5). Both TiO_2 treatments did not show any statistically significant differences (ANOVA, $P > 0.05$; Table 6.5) in the thickness of cerebellum layers (molecular, purkinje and granular) when compared to the control.

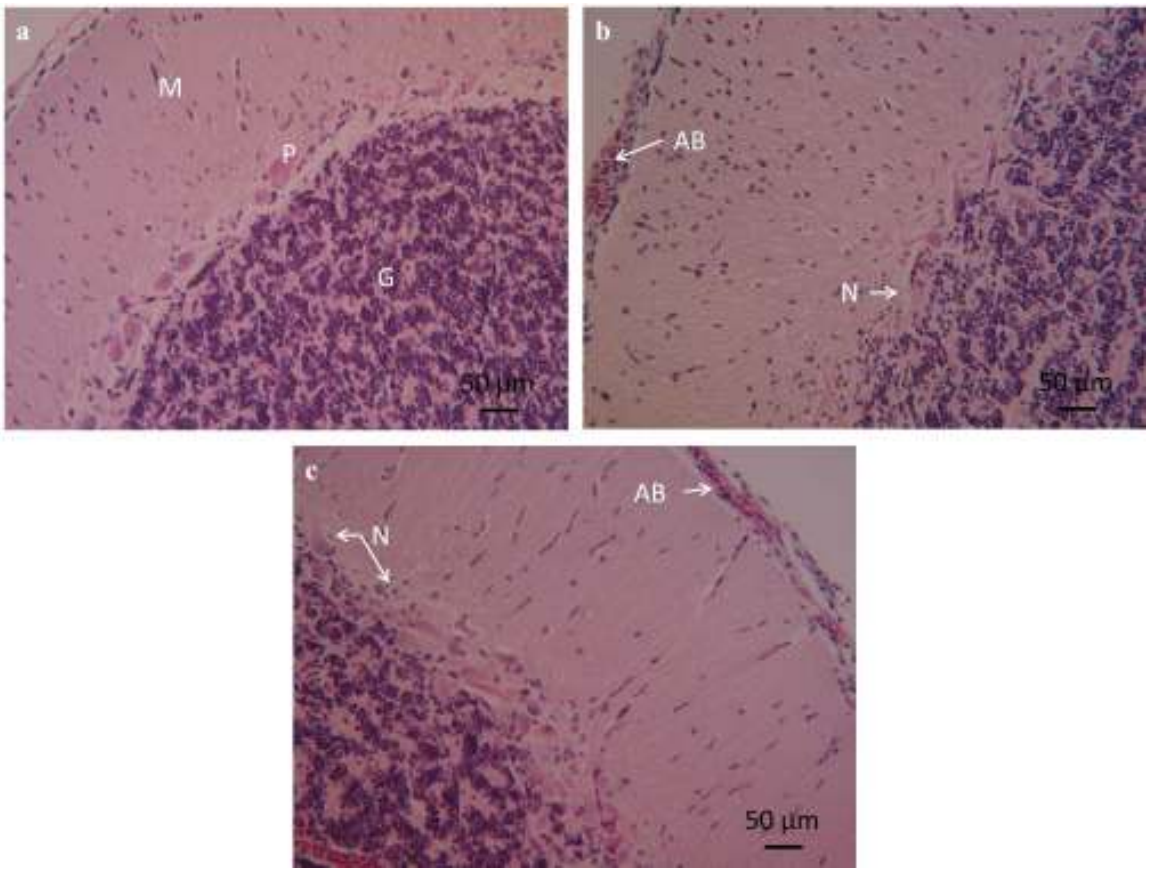

Figure 6.12 Cerebellum morphology in rainbow trout after 96 h injection of (a) 50 µl ml^{-1} Cortland's saline (control), (b) 50 µg bulk in 50 µl of saline, (c) 50 µg TiO_2 NPs in 50 µl of saline. All control groups showed normal structure that include molecular layer (M), purkinje layer (P) and granular layer (G). Both TiO_2 treatments showed injuries that included enlarged blood vessels on the surface (AB) and evidence of necrotic cells (N) between molecular and granular layer. Scale bar indicates magnification. Sections were 7 µm thick and stained with H&E.

Table 6.5 Alteration in the main regions of rainbow trout brain after 96 h injection of 50 µl ml^{-1} of Cortland's saline (control), and 50 µg of either bulk or TiO_2 NPs in 50 µl of saline

Parameters		Control	Bulk	TiO_2 NPs
Telencephalon	Dimension of nerve cells (µm)	14.8 ± 0.4 (5)	22.1 ± 0.5 (6) *	18.4 ± 0.4 (6) *#
	Dimension of nerve cells nuclei (µm)	10.8 ± 0.4 (5)	11.6 ± 0.6 (6)	11.4 ± 0.4 (6)
Thickness of Mesencephalon layers (µm)	Dura mater	0.7 ± 0.0 (6)	0.7 ± 0.1 (6)	0.6 ± 0.0 (6)
	Subarachnoid space	0.9 ± 0.2 (6)	1.4 ± 0.1 (6)	1.1 ± 0.2 (6)
	Pia mater	0.7 ± 0.0 (6)	0.6 ± 0.0 (6) *	0.6 ± 0.0 (6) *
	St. fibrosum marginale	12.6 ± 0.4 (6)	14.4 ± 0.5 (6) *	14.3 ± 0.5 (6) *
	St. opticum	9.7 ± 0.3 (6)	10.3 ± 0.3 (6)	9.8 ± 0.2 (6)
	St. fibrosum et griseum superficial	13.8 ± 0.3 (6)	16.1 ± 0.2 (6) *	15.5 ± 0.3 (6) *
	St. griseum centrale	21.4 ± 0.6 (6)	18.3 ± 0.4 (6) *	17.2 ± 0.3 (6) *
	St. album centrale	10.9 ± 0.2 (6)	9.5 ± 0.2 (6) *	10.4 ± 0.4 (6) #
	St. periventriculare	29.3 ± 0.6 (6)	28.9 ±0.6 (6)	30.4 ± 0.7 (6)
% of Cerebellum cortex layer	Capsule	2.7 ± 0.3 (5)	4.6 ± 0.8 (6) *	4.6 ± 0.2 (6) *
	Molecular layer	42.9 ± 0.3 (5)	42.5 ± 0.7 (6)	42.6 ± 0.7 (6)
	Purkinje layer	7.9 ± 0.1 (5)	7.5 ± 0.2 (6)	7.4 ± 0.1 (6)
	Granular layer	46.5 ± 0.4 (5)	45.4 ± 0.7 (6)	45.4 ± 0.7 (6)

Data are means of proportional areas, or means ± S.E.M., (n = number of fish). * Significant difference from control within row (ANOVA, $P < 0.05$). # Significant difference between bulk and TiO_2 NPs (t – test or ANOVA, $P < 0.05$).

6.4. Discussion

The current study detailed the effects of intravenous injection of bulk TiO_2 compared to TiO_2 NPs on organ integrity of rainbow trout by using histological changes on these organs as well as the changes on the haematopoietic system. Generally, the injection of either bulk or NP treatments showed Ti accumulation in the kidneys (with both TiO_2 treatments) and spleens (with NP treatment only), and similar types of organ pathologies were observed in all organs. Although, in some organs the injuries observed did not show accumulation of Ti. The propensity of NPs to aggregate or agglomerate in saline conditions (Handy *et al.*, 2011) could suggest that they adhere to blood cells. The nanoparticles also have ability to adsorb onto blood proteins (albumins or other small macromolecules) and forming a protein coat on the surface of the NPs (protein corona on the surface of NMs, Hellstrand *et al.*, 2009). These abilities could transfer NPs to the different organs via the blood stream, resulting in organ pathologies. Fabian *et al.* (2008) showed that intravenously injected of TiO_2 in rats did not show detectable TiO_2 in blood cells or plasma at day 1, 14 and 28, and Fabian *et al.* (2008) suggests that rapid clearance of the substance from the blood into the lung, spleen, kidney and liver. However, more studies need to investigate the effects of NP on the plasma proteins, lipids and the enzymes in blood cells.

Previous studies have done to investigate the effects of TiO_2 NPs on rainbow trout by exposure via the waterborne (Federici *et al.*, 2007; Boyle *et al.*, 2013) or via the diet (Ramsden *et al.*, 2009). In some of this work, the sub-lethal toxicity of TiO_2 NPs included gill injuries that associated with increases the Ti concentrations, decreased area of the red pulp in the spleen, increases the haematocrit and whole blood haemoglobin,

brain injury, shifted in swimming speed distribution (Boyle *et al.*, 2013). Boyle *et al.* (2013) suggest that waterborne exposure could produce hypoxia due to the gill injuries which it led to these alterations. Whereas the dietary exposure to TiO_2 NPs showed normal growth rate and haematology, although the Ti was accumulated in the gill, gut liver, brain and spleen (Ramsden *et al.*, 2009). The latter study identified the spleen and brain as important target organs for the dietary exposure. However, in all these studies TiO_2 was not measured in the blood, making explanation of the finding difficult. Overall, the results of the present study shows that Ti can accumulate in the kidney and spleen of TiO_2 NPs treatments, and in the kidney of animals exposed to bulk TiO_2. This could indicate of a particle size effect on the tissue distribution of TiO_2. Moreover, exposure to TiO_2 NPs cause similar types of pathologies in all the target organs (gill, gut, liver, spleen, kidney and the brain) like the bulk form. Although, material-type effects were observed in the kidney and spleen. However, these pathologies in general from the injection study were less severe than that observed with waterborne exposure to these materials, indicating that the route of exposure is important.

6.4.1 Histopathological observation on the gill

Gills are considered the main organ for respiration and have been sensitive to waterborne exposure to nanoparticles. In this study, although Ti was not accumulated in the gills after injection of either bulk or TiO_2 NPs via the caudal vein, they showed similar minor incidence of gill injuries. These injuries include aneurism and evidence of necrotic cells with oedema in the primary lamellae. Additionally, the quantitative analysis of the gills confirmed these results. The minor injuries observed in this study could suggest that injection of either bulk or TiO_2 NPs into the caudal vein may reach to

the gill via the bloodstream, perhaps enabling some minor respiratory disorder, but nothing as severe as a waterborne exposure. Scown *et al.* (2009) found very low levels of Ti in the blood of some of the rainbow trout sampled at 6 and 12 h after injection 100 μg TiO_2 in 1 ml of trout saline, and high levels of Ti was detected in the blood at day 90 in two out of 8 fish, post-injection.

6.4.2 Histopathological observation on the gut

The hind gut of trout also showed similar minor injuries with both TiO_2 treatments that include occasional areas of epithelial lifting associated with necrotic cells, as well as occasional incidence of swollen goblet cell in the mucosa layer. These injuries were worse with bulk than TiO_2 NPs treatment and the quantitative analysis confirmed these results. This might suggest that bulk TiO_2 worked as ions that was easy to transfer and penetrate into the gut via the active transport while TiO_2 NPs might aggregate or deposits on the blood vessel walls during its transfer, and then they reached a small amount into the gut (*e.g.*, via endocytosis), which was caused these minor injuries.

6.4.3 Histopathological observation on the liver

The liver is generally the main organ for detoxification of toxic materials. High doses of intravenously injected of TiO_2 NPs recorded low levels of Ti accumulated in the liver (about 15 time lower than that in the kidney) (Scown *et al.*, 2009). In rats, the intravenous injection of 5 mg kg^{-1} body weight of TiO_2 in serum caused the highest TiO_2 levels in the liver followed in the spleen, lung and kidney after one day of injection (Fabian *et al.*, 2008). So far, there are no reports about the pathological effects of bulk or TiO_2 NPs in fish liver after injection of these materials via the caudal vein.

Although Ti accumulation in the liver was not observed in the current study, but both TiO_2 treatments showed similar types of injuries (occasional pyknotic nuclei, hepatitis-like injury, and increases in the number of melanomacrophage deposits) in the liver. The quantitative liver analysis confirmed that the bulk was a slightly greater effect than TiO_2 NPs. Ma *et al.* (2009) showed that intraperitoneal injection of higher doses of nano-anatase TiO_2 NPs and bulk TiO_2 in mice caused accumulation of Ti in the liver and liver injuries (congestion of the central veins, vacuole formation, and apoptosis) leading to the loss of liver function. Ma *et al.* (2009) also showed that the bulk material caused less toxicity than TiO_2 NPs. However, the findings of the current study do not support the previous studies. It is difficult to explain this difference, but it might be related to the fish liver structure is not the same as in mammals. The decrease in hepatocytes diameter was observed here with both TiO_2 treatments, with greater alteration with bulk than TiO_2 NPs. This could suggest that liver cells are more metabolically active with bulk than NPs. Generally, the liver injuries observed in the current study (injection exposure) were less severe when compared to the waterborne exposure study (see Chapter 5), and this could attribute to reach a small amount of particles into the liver which were led to generate the ROS. However, the severity lesions observed with the waterborne exposure could be due to the systemic hypoxia.

6.4.4 Histopathological observation on the spleen and spleen print

The observation that intraperitoneal injection of titanium dioxide nanoparticles can cause spleen pathology which lead to the reduction of immunity in mice (Li *et al.,* 2010), is consistent with our observations on trout spleens where fish injected with either bulk or TiO_2 NPs showed occasional necrotic cells and increase the presence of

melanomacrophage deposits. The pathologies observed in this study were associated with accumulation of Ti after 24 and 96 h injection of TiO_2 NPs (Boyle *et al.*, unpublished). This suggests that TiO_2 NPs could reach to the spleen through the circulatory system and then caused these injuries that lead to alteration in the spleen contents and caused immunological response by increasing the number of melanomacrophage deposits. In mice, studies by Li *et al.* (2010) and Chen *et al.* (2009) found that intraperitoneal injection of various doses of TiO_2 NPs caused the spleen lesions due to entering of some TiO_2 NPs in the spleen. The latter researchers suggested that TiO_2 NPs could transport to and deposit in other organs after intraperitoneal injection. However, the quantitative spleen analysis of the current study showed also a decrease in the proportion of red pulp and an increase in the proportion of white pulp with both TiO_2 treatments, and these alterations were severe with NPs than bulk treatment. Additionally, the spleen contents also showed an increase in the proportion of erythrocyte abnormalities, a decrease in the proportion of immature (haemocytoblast) and mature cells (erythrocytes) as well as an increase in the proportion of neutrophils. These changes were consistent with the observation on the proportion of red pulp and white pulp and the number of melanomacrophage deposits in the spleen. The spleen is considered part of the reticuloendothelial system that is NPs could bind to antibodies in the plasma and be subsequently recognized by phagocytic cells (macrophages and neutrophils) in the spleen (review, Garnett and Kallinteri, 2006). This could explain the severity effects of NPs when compared to bulk form by causing greater changes in the spleen contents with NP than the bulk form. However, this could indicate the spleen was working to remove these particles from the circulation.

The erythrocyte abnormalities observed here could be due to several factors such as a stress (due to the injection) and intercellular osmotic disorder (Brucka-Jastrzebska and Protasowicki, 2005; Noyes *et al.*, 1991; Houston and Murad, 1995). The alterations observed in the spleen contents (% red and white pulp) of the current study were similar to that with waterborne exposure. This could indicate the spleen was working to supply red blood cells to the circulatory system, and removing the particulate matter from the circulation by producing immune cells. The blood samples of the current study showed that injection of either bulk or TiO_2 NPs in trout did not show changes in the concentrations of haemoglobins and red blood cells after 96 h post injection (Boyle *et al.*, unpublished).

6.4.5 Histopathological observation on the kidney

There is one report of renal pathology from an injection of TiO_2 NPs in rainbow trout (Scown *et al.*, 2009). The pathologies reported here include the occasional area of necrosis in the haematopoietic tissues and renal tubules separation, which were associated with the Ti accumulation in the kidney after 96 h post injection of both TiO_2 treatments. The results of the current study are consistent with Scown *et al.* (2009) that found the kidneys were the main target organs for Ti accumulation after intravenous injected with a single high dose of TiO_2 NP. Scown *et al.* (2009) also found kidney injuries with a very limited impairment of the renal function or oxidative stress in the blood, and the TEM images of the kidney showed clusters of TiO_2 NPs in the haematopoietic cells surrounding the kidney tubules after 21 days injection of 100 μg TiO_2 NPs in 1 ml of trout ringer. The pathologies observed here also were similar to previous reports on mice. For example, Chen *et a*l. (2009) found that injection of a high

dose of TiO_2 NPs in mice caused high Ti accumulation in the kidneys with kidney pathologies (renal glomerulus swelling, dilatation and proteinic liquids in the renal tubular). The quantitative kidney analysis of the current study confirmed the results for histology which showed a minor increase in the proportion of renal corpuscles, dimension of the Bowman's space, lumen of proximal and distal tubules with both TiO_2 treatments. These observations suggest that accumulation of either bulk TiO_2 or TiO_2 NPs could effect on glomerular filtration rate (increased Bowman's space) and re-absorption or excretion of various substances in the renal tubules. Therefore, more studies are required to investigate the effects of the NP on the renal functions.

6.4.6 Histopathological observation on the brain

Nanoparticles can enter the blood brain barrier and accumulate in the brain (Panyala *et al.*, 2008). In mice, Ma *et al.* (2010) found that injected anatase TiO_2 NPs into the abdominal cavity daily for 14 days caused oxidative stress and brain injuries, and they suggests that TiO_2 NPs could be translocated into the brain and then caused brain injuries. In the current study, histological examination of the main regions of the brain after 96 h injection of either bulk or TiO_2 NPs showed minor injuries in the telencephalon cells; minor changes in the thickness of mesencephalon layers; and enlarged of a blood vessel on the surface of the cerebellum, although the brain did not show Ti accumulation. However, the alterations observed in the brain of this study were less severe when compared to the waterborne exposure study (see Chapter 5), although both studies did not show detectable Ti accumulation in the brain. This could indicate that injected of bulk or NPs could reach the brain in a small amount and subsequently caused brain injuries. However, the brain in this study also did not show alterations in

the activity of Na^+/K^+-ATPase and TBARS level with NPs and bulk form after 96 h of injection (Boyle *et al.*, unpublished). These biochemical results confirm the minor alteration observed in the brain of the current study. The changed observed in the mesencephalon layer in the current study could produce due to the cut orientation or due to treatments. The enlarged blood vessels on the surface of the cerebellum may not be pathological changes, but simply due to the supply of oxygen and nutrients to the brain. Brain pathologies were observed after 14 days of waterborne exposure to bulk or TiO_2 NP in trout (Boyle *et al*., 2013). Therefore, further studies required to investigate the effects of bulk and TiO_2 NPs on the neurotransmitter (acetylcholine) in the brain of rainbow trout.

Conclusion

The current study provides detailed of the pathological effects, target organs, and immunological response after injection of either bulk or TiO_2 NPs in juvenile rainbow trout. Overall, this study has demonstrated that the liver, kidney, spleen and brain are the target organs after injected of either bulk or TiO_2 NPs, and both TiO_2 treatments showed similar types of injuries in all organs. The spleen and kidney of this study showed severe lesions when compared to the waterborne exposure, and these lesions were associated with Ti accumulation. The material-type effects showed greater effects with TiO_2 NPs than bulk forms in the spleens and kidneys after injection. Additionally, the spleen prints also showed both TiO_2 treatments caused changes in the erythropoiesis and immune cells, and greater effects observed with NP than bulk forms. This could indicate to the role of kidney and spleen to remove these materials from the circulation. The livers and brains also showed similar type of injuries, and these injuries may be

related to its capacity for catalyzing oxidative reactions, leading to the formation of reactive oxygen species which lead to induce similar organ pathologies or disturbance in the metabolic activity. The gills and gut of the present study also showed lesions, but these lesions were less severe when compared to the waterborne exposure to bulk or TiO_2 NPs. This could indicate that bulk or NPs forms have systemic effects on these organs, probably from direct effects of these particles on the blood stream.

Chapter 7

General discussion

General discussion

Currently, there is very limited information on the pathological and immunological effects, and the major target organs of metal NP compared to the traditional forms of metal. Additionally, less is known about the material-type effects (*e.g.*, differences between dissolved metal and metal nanoparticles), and the effects of low water pH on the sub-lethal toxicity of either form of metal to the main body system of fish. Therefore, this thesis aimed to give a better understanding of the pathological and immunological effects of either metal nanoparticle (as Cu-NPs or TiO_2 NP) or metals (as $CuSO_4$ or bulk TiO_2), and the major target organs of these materials via different routes of exposure (waterborne, dietary, and injection) as well as the effects of acidic pH on the toxicity of Cu-NPs and $CuSO_4$ in fishes. These effects could lead to a better understanding of the hazard and risk assessment of either Cu-NPs or TiO_2 NPs intake via water, food or injection.

The results observed throughout this thesis confirm that metal nanoparticles caused similar types of injuries to the metal forms in the most target organs of fish following either waterborne or injection exposure (Chapter 3-4 and 5-6). Waterborne exposure to $CuSO_4$ or Cu-NPs showed similar types of pathologies in all organs, but there were some material-type effects in severity or incidence of injuries with Cu-NPs causing more injuries in the gut, liver, and brain than the equivalent concentration of dissolved Cu at the end of experiment, but in the gill and muscle dissolved Cu caused more pathologies (Chapter 3). The spleen morphology and spleen prints also showed greater effects with $CuSO_4$ than Cu-NPs (Chapter 3). Some organ injury observed throughout this experiment occurred without the presence of Cu accumulation in the internal organs. This could indicate that a secondary systemic hypoxia or oxidative

stress leads to appear these injuries. The effects of low pH on the sub-lethal toxicity of either dissolved Cu or Cu-NPs on the accumulation, physiology, and pathology of target organs showed greater effects at pH 5 with both Cu treatments than pH 7 (Chapter 4). Both Cu treatments showed more Cu concentration in the gills at both pH values (7 & 5), with a material-type effects (more Cu concentrations with $CuSO_4$ than Cu-NPs), and in the livers (at pH 7 only), as well as more haematological changes were observed at pH 5 than 7. The gills, liver, spleen and kidney were considered target organs of either $CuSO_4$ or Cu-NPs at pH 7 and 5, and more severe injuries were observed in these organs at pH 5 than pH 7. This suggests that acidic pH could produce another stress to the fishes.

The waterborne exposure to either bulk TiO_2 or TiO_2 NPs also showed similar organ pathologies (gill, gut, liver, spleen, kidney, brain and muscles), although the Ti was only accumulated in the gills (Chapter 5). The material type effects were also observed in the gill, liver, spleen prints contents, brain and muscle which showed slightly greater effects with bulk TiO_2 than TiO_2 NP. However, the TEM gill images of both TiO_2 treatments confirmed there were similar changes occurred in the tissues and cells of the gill lamellae with both bulk TiO_2 and TiO_2 NP materials. More investigation required to investigate the precise locations of NPs in tissues and cells of different target organs in fish. After injection of either bulk or TiO_2 NPs, the fish also showed similar types of pathologies in the gill, gut, liver, spleen, kidney, and brains, although the injuries observed in the gill and gut were less severe to that observed following waterborne exposure (Chapter 6). The liver, spleen, kidney and brain were considered the main target organs after injected of either bulk or TiO_2 NP forms. The material-type effects were only observed in the spleen and kidney by causing more Ti accumulation

and greater effects with TiO_2 NPs than bulk form. The effects of dietary and waterborne exposure to TiO_2 NP on spleen prints of fish were used as indicators of the immunological response (Appendix A). Dietary exposure showed fewer changes in the haematopoietic cells and erythrocyte morphology in the spleen compared to the waterborne exposure. This suggests that the routes of exposure could effect on the toxicity of NPs.

7.1 Nanoparticles toxicity on target organs

Throughout this study, the organ pathologies were useful indicators to identify target organs after exposed to metal and metal nanoparticles via different routes of exposure. However, the biological effects may not always be in the same organ that is a target for metal accumulation. In this thesis less difference was noted between the effects of NPs on target organs in fish when compared with the ordinary forms of the metals. The gill, gut, liver, spleen, kidney, brain and muscles were recognised as target organs for waterborne exposure to metals (as $CuSO_4$ or bulk TiO_2) or metal NPs (Cu-NPs or TiO_2 NPs) in this study (Chapter 3 and 5). The Cu accumulations were observed in the gills (with both Cu treatments), livers (with $CuSO_4$ only) and gut (with Cu-NPs only) after exposure to dissolved Cu or Cu-NPs (Chapter 3). Whilst, the accumulation of Ti was observed in the gills only whereas other internal organs did not show detectable Ti accumulated (Chapter 5). The gills are the first target organs that contact directly with the waterborne environment, therefore the accumulation of metals (from $CuSO_4$ or Cu-NPs and bulk or TiO_2 NPs) observed in the gills which induced severe gill injuries. These injuries could produce a systemic hypoxia which leads to changes in the internal organs (liver, kidney, brain, and muscles). The waterborne exposure to

dissolved Cu or bulk TiO_2 showed greater effects on the gills, and muscles of rainbow trout than Cu-NPs or TiO_2 NPs. This suggests that the Cu salt or bulk forms are more bioavailable or bioreactive than nano forms. Griffitt *et al.* (2007) explained that waterborne exposure to $CuSO_4$ and Cu-NPs caused damage to the gill lamellae (proliferation of epithelial cell, oedema of primary and secondary gill filaments) and inhibition of branchial Na^+/ K^+-ATPase activity, but the effects of $CuSO_4$ were greater than Cu-NPs. Some organ-specific material-type effects were also observed with Cu-NPs causing greater injuries in the gut, liver, and brain than the equivalent concentration of the dissolved Cu, and little difference between materials were observed in the kidney. The waterborne exposure to bulk or TiO_2 NP also showed material type effects in the gill, liver, spleen prints contents, brain and muscle which caused slightly greater effects with bulk TiO_2 than TiO_2 NP. This could explain there is a crystal or size effects on the organ pathologies which leads to induce slightly greater effects with bulk than NP forms. Study by Karlsson *et al.* (2009) confirmed this explanation that found the bulk TiO_2 caused more DNA damage compared to TiO_2 NPs, and this damage is likely explained by the crystal structures. Therefore, further studies need to investigate the mechanism toxicity or the effects of particles sizes or crystal structure of either bulk or TiO_2 NPs on all target organs.

After injection of either bulk or TiO_2 NPs, the spleen and kidney were considered as target organs for Ti accumulation (Chapter 6). The intravenous injected study of TiO_2 NPs (100 μg l^{-1}) showed that the Ti was concentrated in the liver and kidney after 6 h, and the highest Ti concentration was found in the kidney (after 12 h), with a minor effect on the kidney functions (Scown *et al.*, 2009). Scown *et al.* (2009) also showed the presence of NPs in the haematopoietic cells of the kidney by using

TEM images. This study confirms our finding by showing the highest Ti accumulation in the kidney, and the lowest Ti concentration was observed in the spleen. A material type effects were observed in the Ti accumulation which showed more Ti accumulation with NP than the bulk form in both organs (kidney and spleen). In this study, the kidney and spleen also showed more changes with TiO_2 NPs than bulk forms. This could be related to the accumulation of Ti in these organs. The liver and brain also showed similar type of injuries with both TiO_2 treatments, and these injuries were less severe when compared to the waterborne exposure. This could be related to the effects of these materials on the blood vessels. The generation of ROS and disturbance in metabolic activity is another reason to produce organ injuries. The abdominal injection of anatase TiO_2 NPs in mice for 14 days (daily) showed oxidative stress and brain injuries (Ma *et al.*, 2010). The latter author suggests that TiO_2 NPs could be translocated into the brain and subsequently caused brain injuries. The gills and gut of the intravenous injection study also showed lesions, but these lesions were less severe when compared to the waterborne exposure. This suggests that bulk or NPs forms can have systemic effects on the gills and gut; probably from direct effects of particles in the blood stream.

7.2 Effects of nanoparticles on some organ system

7.2.1 Immune system (using spleen)

Fish have different defensive mechanism to counteract the effects of toxicants. One is the immune system, where the spleen and kidney are the main organs of the fish immune system. The spleen pathologies and alteration in the spleen contents are good indicators to assess the effects of NP on the immune system. However, the results of this organ have not previously been described. The waterborne exposure to metals (as

$CuSO_4$ or bulk TiO_2) or metal NPs (as Cu-NPs or TiO_2 NPs) showed similar types of spleen injuries and alteration in the proportion of red (decreased), white pulp (increased) and sinusoid space (transient changes) (see Chapter 3 and 5). The alterations observed in the spleen contents were slightly greater with metal salts and bulk forms than NP forms. This could attribute to the bioavailability of metals (metal salts) or due to the systemic hypoxia that associated with gill injuries. A decrease in the proportion of red pulp in the spleen is consistent with the release of red blood cells from the spleen into the circulatory system in response to hypoxia (Handy *et al.*, 2011). An increase in the proportion of white pulp in the spleen is consistent with the stimuli of the haematopoietic system to produce immune cells to counteract the effects of these materials. After intravenous injection of either bulk or TiO_2 NPs, the spleen showed similar changes in the red, white pulp and sinusoids space to that observed with waterborne exposure, but these changes were greater with NPs than bulk forms (Chapter 6). This could attribute to the Ti accumulation in the spleen of the injection study. Chen *et al.* (2009) showed that intraperitonal injection of various doses of TiO_2 NPs in mice caused spleen lesions, and they suggest that NPs could transport to or deposits in other organs.

The spleen also showed an increased in the number of melanomacrophage deposits following waterborne or injection of either metals ($CuSO_4$ or bulk) or NPs (Cu-NPs or TiO_2 NPs) in fishes. The presence of these deposits in the spleen could attribute to the normal phagocytic functions. Spleen prints are considered good indicators to detect the changes in the haematopoietic cells and cellular morphology in the spleen after exposure to a stressor (Peters and Schwarzer, 1985). It is not clear for mammals or fish whether the haematopoietic cells are affected by NP, and this information could be

important for administrating research towards specific areas of immunology. Waterborne exposure to either metals (as $CuSO_4$ or bulk) or metal NPs (as Cu-NPs or TiO_2 NPs) showed similar changes in the proportion of immature cells (increased) and mature erythrocytes (decreased) in the spleen (Chapter 3 and 5). This suggests that waterborne exposure may stimuli the erythropoiesis to produce new cells, and subsequently was supplied to the circulation or these materials may effects directly by causing inhibition in the production of erythrocytes. The post injection of either bulk or TiO_2 NPs showed similar changes in the proportion of immature and mature cells to that observed with the waterborne exposure of the same materials, but more changes observed with NP than bulk forms. The nano-effects observed in the spleen of injection study could be correlated to the highest Ti accumulation in the spleen. Dietary exposure to TiO_2 NPs showed less effect on the proportion of haematopoietic cells than waterborne exposure (Appendix A). This could indicate that the routes of exposure may effects on the toxicity of NPs.

The proportion of immune cells also showed changes following exposure to waterborne, dietary or injection study (Chapter 3, 5, 6, and Appendix A). Increasing the number of immune cells (neutrophils, lymphocytes) following different routes of exposure to metal or metal nanoparticles were observed in the spleen of rainbow trout. This suggests that the spleen was responding by inducing immune cells to protect the body against these materials.

The erythrocyte morphology also showed alteration following exposure to metal (as $CuSO_4$ or bulk TiO_2) or metal NPs (as Cu-NPs or TiO_2 NPs) via different routes of exposure (Chapter 3, 5, 6, Appendix A). Increasing the number of erythrocyte

abnormalities could indicate to the hard working of the spleen to remove damaged blood cells from the circulation.

7.2.2 Brain pathologies

Brain pathologies are good indicators to detect the effects of particles and NPs on the central nervous system via different routes of exposure. However, the pathologies observed in the main regions of the brain following exposure to NP have not previously been described. The waterborne exposure to $CuSO_4$ or Cu-NPs showed injuries in the telencephalon cells, minor alteration in the thickness of mesencephalon with parts of the preventriculare layer being damaged, as well as enlargement of blood vessels on the ventral surface of the cerebellum (Chapter 3; Al-Bairuty *et al.*, 2013). The Cu accumulation in the brain of fish was not detected in this study, and the effects on the brain are best explained by indirect toxicity on neuro-endocrine function or by hypoxia (Handy, 2003). The presences of enlarged blood vessels on the surface of the brain are consistent with an attempt to increase blood flow to offsets of hypoxia (due to gill injury) (Al-Bairuty *et al.*, 2013). The minor alteration in the mesencephalon layers that observed with both Cu treatments was not due to differential tissue growth, but it is related to oedema (AL-Bairuty *et al.*, 2013). The $CuSO_4$ or Cu-NPs seems to show similar brain pathologies, and several studies show swollen blood vessels so if this emerging as a general feature of particle toxicity that we have not seen before for dissolved metals.

The brains of waterborne exposure to bulk or TiO_2 NPs also showed similar injuries to that observed with the $CuSO_4$ or Cu-NPs treatments (Chapter 5; Boyle *et al.*, 2013). After injection of either bulk or TiO_2 NPs, the brain (in particular fore and hind

brain) showed less severe changes when compared to the waterborne exposure of the same materials (Chapter 6). The accumulation of Ti in the brain was not observed in this experiment. However, the brain injuries observed here could attribute to the ROS generated due to the injection. Oxidative stress and brain injuries were observed in the mice that injected with anatase TiO_2 NPs into abdominal cavity daily after 14 days (Ma *et al.*, 2010).

7.2.2 Muscle pathologies

Skeletal muscle tissues are good pointers to determine the effects of NPs on the swimming behaviour in fish. Waterborne exposure to metal (as $CuSO_4$ or bulk) and metal NPs (Cu-NPs or TiO_2 NPs) showed similar changes in the structure of muscle bundles, although there were no metals (Cu or Ti) detectable in the muscles (see Chapter 3 and 5). In fish, the effects of NPs on skeletal muscles have not been previously described. Degeneration of muscle bundle and an increased in the extracellular space were observed in the muscle of rainbow trout with both NPs treatments (Cu-NPs and TiO_2 NPs) (Cu-NPs; Al-Bairuty *et al.*, 2013). These changes may effect on the muscle function which was led to spend more time at lower swimming speed. However, more investigation needs to investigate the effects of NPs on the swimming speed and its relationship with muscle biochemistry.

7.3 Effects of abiotic factors and water quality on nanoparticles toxicity

The effects of abiotic factors and water quality on the bioavailability of metals have been investigated over many years. The main idea is that the free metal ion is the bioavailable metal species, and therefore many researchers have focused on identifying metal speciation in water (Bury and Handy, 2010; Kamunde and Wood, 2004). The

metal speciation is affected by abiotic factors such as pH, dissolved organic matter, anions (*e.g.*, chloride and hydroxyl ions), or cations (*e.g.*, H^+, Ca^{2+}, Mg^{2+}, and Na^+) (*e.g.*, Cusimano *et al.*, 1986; Erickson *et al.*, 2008; Shaw and Handy 2011) (Fig. 7.1). However, there are similarities between metal ions and metal NPs in those abiotic factors (pH, the presence of divalent ions, and ionic strength), which can influence on the behaviour of NPs (aggregation state). Some types of metal NP (*e.g.*, Cu-NPs and Ag NPs) will dissolve by dissolution of metal ions from the surface of the particles. Griffitt *et al.* (2009) showed similar increased in branchial Cu levels with both dissolved and nano particulate forms after 48 h, and the authors interpreted as uptake of dissolved Cu from the Cu-NPs. The branchial Ag levels were much higher in fish exposed to Ag-NPs compared to dissolve Ag, and this could contribute to the branchial Ag burden (Griffitt *et al.*, 2009). Therefore, an experiment was performed to show the effects of pH on the sub-lethal toxicity of Cu-NPs compared to the equivalent concentration of $CuSO_4$ (Chapter 4). Generally, the TEM and the dialysis results showed that the low pH affects on particle size, aggregation, and the rate of Cu dissolution from Cu-NPs (Chapter 4). These effects included a decrease in the aggregation rate of particle size and an increased in the dissolution of Cu from Cu-NPs. Handy *et al.* (2008b) have been reviewed that the pH could effect on the characterisation of NPs such as size and surface charge (*e.g.*, Cu-NPs), and can influence on the aggregation and subsequent bioavailability and toxicity of NPs. The alterations observed in the characterisation of Cu-NPs at the acidic pH of this study could attribute to the surface charges. The presence of H^+ in the solution can change the surface charged of NPs (Handy *et al.*, 2008b), and measuring the zeta potential as a function of pH on particles can provide

good prediction of colloidal NP stability. Therefore, further studies need to measure the zeta potential of Cu-NPs at different pH values.

The effects of acidic pH on the toxicity of NPs in fish showed similar types of physiological, biochemical, and histological changes when compared to the neutral pH value. These changes were greater at acidic pH than neutral pH, although the effects of Cu-NPs were similar or less than $CuSO_4$ (Chapter 4). This suggests that acidic pH could stimulate the dissolution of Cu from Cu-NPs, but the presence of H^+ may compete with metals ions on the binding sites of gill surface. Cusimano *et al.* (1986) reported that the presence of H^+ in water could interference with metal uptake or could stimulate the mucous secretion, and then the resulting free mucus will able to chelate the metals. Therefore, the severity changes observed with both Cu treatments at acidic pH could be attributed to the acidic stress or may be both metal and acidic stress. However, more investigation needs to compare the effects of NPs with the ordinary form at the alkaline pH.

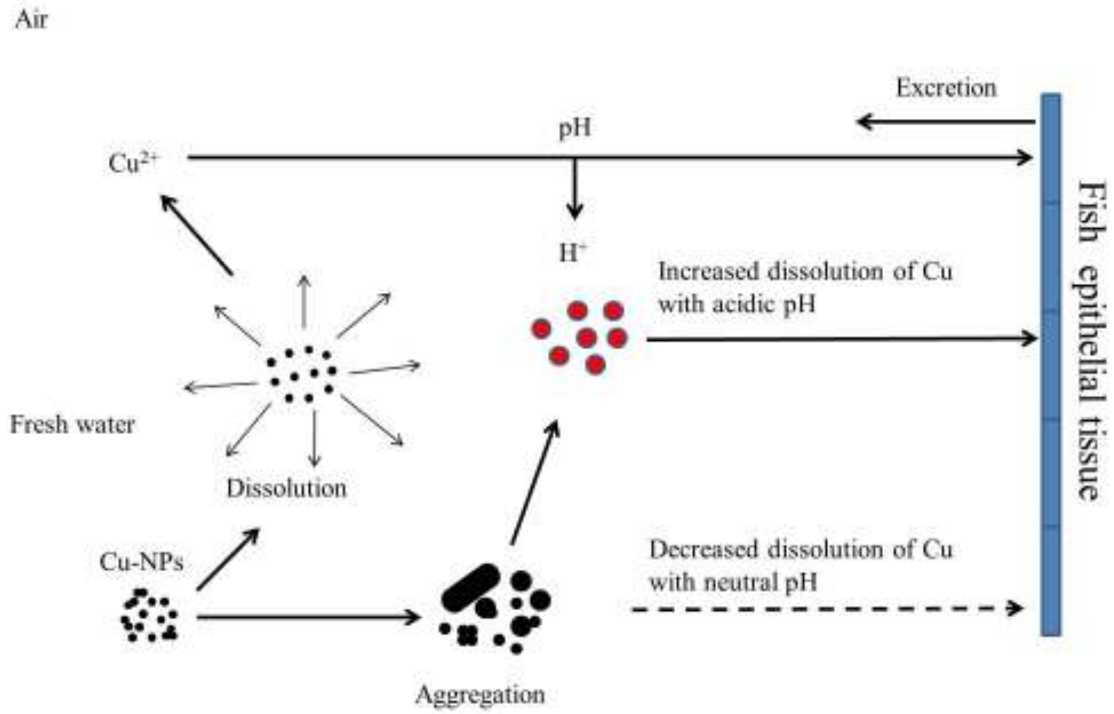

Figure 7. 1 Conceptual diagram following the results obtained throughout this study. The diagram showed the current understanding of the differences in bioavailability of Cu-NPs to fish with change in pH values as a biotic factor. Unbroken lines show high bioavailability or contact with epithelial cell, with broken line indicate limited bioavailability. The theory in the metal literature suggests that acidic pH is protective against toxicity, but bioavailability increases, whilst the opposite is true for neutral pH values. Red filled circles represent hydrogen ions as an indicator of pH effects; joined blue rectangles represent fish epithelial tissue such as the gill or gut epithelia. Single NPs are represented by black small circles with space between, whereas aggregates of NPs are represented by larger, irregular black shapes (rod, circles shapes).

7.4 Exposure to environmental contaminants by metal or metal NPs and fish health

The general health and fitness of the fish could be affected by the exposure to metal or metal NPs. Laboratory evidence of adverse effects in animal models has prompted concern about the health effects of metal and metal nanoparticles in humans. Acute exposure to high mg l^{-1} concentrations of NPs could cause direct mortality of fish populations, but this is unlikely to occur except during an accidental spill of large

qunatities of material. Whereas, chronic exposure to much lower metal or metal NPs concentrations may alter the animal mating behaviour, its ability to catch food or to avoid being eaten, and thus alter population survival. From a bioenergetics perspective, observations of fish health and the presence or absence of pathology could provide valuable information on the probability of population survival, as well as aid management decisions for the fishery. For example, metal or metal NP exposure may damage the gills and increase the costs of respiration, tissue repair and growth at the expense of reproduction, and recruitment. Critically, histopathology parameters are good early indicators of environmental stress as they provide visible biological endpoints that can be related to exposure over time (Myers and Fournie, 2002). Pathologies observed throughout this thesis in the gill, gut, liver, spleen, kidney, brain and muscle with metal and metal NPs indicates that fish health and fitness may be affected in the wild.

7.5 Hazard and risk assessment of NPs in the environment

Little is known about the potential effects of nanoparticle applications on human health and the environment. Metal oxide such as bulk or TiO_2 NPs is widely used in applications that included food, drugs and water treatments. There are also concerns about the possible releases of NPs into the environment through accidental spills during manufacturing and transport, or its presence in waste, sewage, and runoff water. The exposure characterisation part of the risk assessment requires some information on predicted or measured exposure concentration in the environment. The predicted environmental concentrations of TiO_2 NPs in aquatic environments are in the range from 0.7 to 16 $\mu g\ l^{-1}$ (Mueller and Nowack, 2008), depending on the modelling

approach used. The concentration of TiO_2 NPs in surface water was predicted to be 0.02 μg l^{-1}, whereas the concentration in the sewage treatment water was 4 μg l^{-1} (Gottschalk *et al.*, 2009). Therefore, these low concentrations suggest that sub-lethal chronic effects rather than acute effects on human health and wildlife are expected.

In the present studies, the fish successfully tolerated exposure to metal or metal NP for the first few days, but after 2-3 weeks of continuous exposure survival would be unlikely (with the levels of gill injuries that were reported here). Therefore, the risk for mortality would be high (Campbell *et al.*, 2005). Predictions of chronic effects are unclear, but expect changes in the cell proliferation in the gills (hyperplasia), with possibly specific trace element disturbances over a long time of exposure. This will elevate the metabolic costs and food requirements of the animals. However, rainbow trout have the ability to ingest water containing NP (Cu-NPs, Al-Bairuty *et al.*, 2013; TiO_2, NPs, Federici *et al.*, 2007; SWCNTs, Smith *et al.*, 2007). The main findings of this study were the NPs caused similar or less effect than metal on most fish organs and can translocate within the body and the environment.

The hazard potential of the NP on organ integrity (showing organ pathologies) which was observed after waterborne or injection exposure could lead us to reconsider current metals risk assessments that based only on the dissolved form of the metal. The Cu or Ti accumulation observed in the gills from waterborne exposure to Cu-NPs or TiO_2 NPs could suggest the gills are the main target organs for waterborne toxicity of NPs. Whereas, the main target organs after injection of TiO_2 NPs was the kidney and spleen which showed high Ti accumulations. Therefore, the hazard resulting from metal

accumulations in these organs could generate a concern about the risk of these materials on wildlife and to human health.

Once environmental concentrations are established we need to know what factors will alter concentration. For dissolved metals the biotic ligand models and free ions activity models indicate pH and water hardness as critical factors. The acidic pH caused shrinkage to the Cu-NP size and the dissolution of Cu from Cu-NPs was increased. The organ pathologies observed after waterborne exposure to Cu-NPs at pH 5 was similar to that observed with $CuSO_4$, but the effects were greater severity at pH 5 than pH 7. Thus, the effects of pH on the toxicity of NPs which generate the idea of the environmental factor hazard from Cu-NPs exposure.

All the results in this study indicate that the hazard of metal nanoparticles is similar or less than metal forms, and this must be considered in risk assessments and the waterborne or injection hazard exposure to the NP (Cu-NPs or TiO_2 NPs) for both the ecosystem and human health. Therefore, further studies on the effects of these materials on food, water and drugs risk assessment and NPs hazards are advisable.

7.6 Future work and recommendation

There are a number of various ideas about the future work as described in the list below:

- Further investigation is required to investigate the relation between the spleen function and the role of NPs on the spleen content cells using spleen prints. For example, investigate the effects of other NP materials on the spleen

function using spleen prints and spleen pathologies as an indicator of immunological responses.

- Some studies illustrated that the presence of metal-NPs could increase the uptake of other contaminants (for example, Cd and TiO_2 NP, Zhang *et al.*, 2007; arsenate and TiO_2 NPs, Sun *et al.*, 2007; 2009). Therefore, further studies need to investigate if there is any interaction between the toxicity of NPs with other contaminants.
- Dietary administration and injection of Cu-NPs experiments would be a necessary project in order to make comparisons with waterborne Cu-NPs exposure. Sub-lethal concentration of the $CuSO_4$ or Cu-NPs is recommended, to learn more about the target and pathological organs.
- The effect of alkaline pH on the Cu-NPs toxicity is necessary; in order to make comparisons with the acidic and neutral pH results (Chapter 4). This would then give a good understanding of the effects of pH on the dissolution of Cu from Cu-NPs.
- The effect of temperature on the NPs toxicity is needed; in order to investigate whether the temperature effect on the dissolution of Cu from Cu-NPs. Sub-lethal concentrations of Cu-NPs is recommended to investigate if the temperature effects on the toxicity of NPs in organ integrity.
- More studies are required to investigate the effects of particle size on the toxicity of NPs using the filtration method. This would be an interesting way to detect the effects of particle size on the toxicity. In order to avoid further dissolution of Cu from Cu-NPs over time following filtration it may be necessary to limit the exposure time.

- To improve our information about the toxicity of NPs in fish, it will be very useful to study a molecular gene expression changes (*e.g.*, metallothionein gene regulation). This will provide a better understanding on the toxicity of NPs.
- The effect of NPs on digestion, nutrient absorption, and energy balance remains to be investigated.
- In order to assess the effect of Ca^{+} on the NPs toxicity, more studies are required to explore whether the NPs toxicity affected by the cations. Calcium was used as a competing for biological uptake with trace metal. Therefore, the Ca^{+} may change the toxicity of NPs.
- Mammalian studies (in mice, rats) are required to achieve our findings from the fish which represent a comparative study between vertebrates and mammals. In order to gain more information about the pathological and immunological effects of Cu-NPs or TiO_2 NPs on target organs.

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Appendixes:

Appendix A: Supplementary chapter

This Appendix contains an analysis of archived samples from the dietary study of Ramsden et al. (2009) and the waterborne study of Federici et al. (2007). The staining and analysis of the spleen prints was conducted by Genan Al-Bairuty.

Haematopoietic observation on spleen prints from rainbow trout (Oncorhynchus mykiss) following dietary or waterborne exposure to TiO_2 nanoparticles

Hypotheses: Dietary and waterborne exposure of TiO2 NPs in trout leads to different effects on the haematopoietic cells in the spleen due to the route of exposure.

Abstract

Little is known about the immunotoxicity of dietary and waterborne exposure to nanoparticles in aquatic organisms. In this study, cellular pathologies and haematopoietic characteristics were examined in spleen prints of juvenile rainbow trout that were exposed to TiO_2 NPs. Dietary exposures were TiO_2 (10-100 mg kg^{-1} for up to 8 weeks and followed by 2 weeks recoveries), and waterborne exposures were TiO_2 (0.1-1 mg l^{-1} for up to 14 days). At least 200 cells were counted on each slide of spleen prints from each fish per treatment at each time point (6 fish/treatment). Dietary exposure to TiO_2 showed minor effects on the proportion of haematopoietic cells and the erythrocyte morphology in the spleen. This included small transient changes in the proportion of erythroblast and lymphocytes when compared to controls. An increase in the proportion of erythrocytes with abnormalities (swollen red blood cells and cell with membrane abnormalities) was observed in the spleen following dietary exposure even though the incident rate was only 1-2 % of the red cells (all statistically significant, ANOVA, $P < 0.05$). Waterborne exposure to TiO_2 showed a decrease in the proportion of erythroblasts and an increase in the proportion of some types of immune cells compared to controls (ANOVA, $P < 0.05$). At day 14 of waterborne exposure to TiO_2 NPs showed a few more statistically significant changes than day 4 (ANOVA, $P < 0.05$); including an increase in the proportion of erythrocyte abnormalities (swollen red blood cells, red cells with micronuclei, and cell membrane abnormalities). The incident rate was only 1-4 % of the red cells observed compared to the control (0-1%). It was concluded that dietary exposure of TiO_2 caused minor alteration in the erythropoiesis and immune system while exposure to waterborne TiO_2 caused slightly greater

alterations in the erythropoiesis and immune system. The adverse effects via the dietary route are less severe than waterborne exposure on the immune system. The levels of changes that observed are unlikely to be clinically important to fish health.

A.1 Introduction

Trace metals are known to be toxic to fish via the water (Luoma, 1983, Taylor *et al.*, 1996) and food (Handy, 1996; Clearwater *et al.*, 2002; Handy *et al.*, 2005). There are also some concerns about the immunotoxicity from chemicals in fishes (Segner *et al.*, 2012). For example, immunotoxic of chemicals shown to cause adverse health effects by suppressing or stimulating the immune capacity of exposed organisms. Recently, a new area of sciences emerged called nanomaterials which defined as materials with a primary particle size between 1-100 NM (SCENIHR, 2010). Data on the uptake mechanisms, ecotoxicity, and target organs for nanomaterials are limited (Handy *et al.*, 2008a; Klaine *et al.*, 2008; Handy *et al.*, 2011), and one of the gaps in the data is on the effects of nanomaterials on the immune system of fishes (Handy *et al.*, 2011; Jovanović and Palić, 2012). Nanoparticles are known to cause inflammation reactions and immune responses in mammals (Ding *et al.*, 2005; Tian *et al.*, 2006; Dobrovolskaia and Mc Neil, 2007; Patlolla *et al.*, 2010). *In vitro* toxicity experiments with human fibroblast cells showed inflammation, oxidative stress, cytokine production, cellular apoptosis (necrosis), changes in gene expression in response to the CNT exposure (MWCNT, Ding *et al.*, 2005; SWCNT, Tian *et al.*, 2006; MWCNT, Patlolla *et al.*, 2010). Recently, there have also been concerns that NPs could interfere with the immune system of fishes (Jovanović and Palić, 2012). The immune system of fishes shares many common features with mammals (*e.g.*, immunoglobulins, albumin and fibrinogen, Watts *et al.*; 2001; Dobrovolskaia and Mc Neil, 2007), although in fish the

immune cells are not all organised into discrete organs. One organ of great importance is the spleen, which is considered one of the main haematopoietic organs in fishes (Press and Evensen, 1999; Lange *et al.*, 2000). The information on the haematopoietic system such as spleen in fish is scarce. However, the spleen functions as a filter to remove damaged blood cell and any foreign material from the circulation, and to maintain normal haematology of the cells in the cardiovascular system. Environmental pollutants can change the proportion of red and white pulp in the spleen (*e.g.*, in sticklebacks exposed to sewage treatment effluents, Handy *et al.*, 2002c; in rainbow trout exposed to bulk and TiO_2 NPs, Boyle *et al.*, 2013). Handy *et al.* (2011) mentioned that an increase in red pulp (mostly sinusoids containing red blood cell) may indicate the spleen was working hard to remove the damaged red blood cells, whilst an increase in white pulp (mostly immune cells) may indicate an immunological stress. The haematopoietic efficiency of the spleen has been quantified in various ways including the spleen prints method (Peters and Schwarzer, 1985). These researchers observed changes in the haematopoietic tissue (spleen and head kidney) of rainbow trout under influence of stress. Recently, a study by Boyle *et al.* (2013) showed that waterborne exposure to 1 mg l^{-1} of bulk or TiO_2 NPs materials for 14 days caused a decrease in the proportion of red pulp that a concomitant with an increase in the proportion of sinusoid space in the spleen of rainbow trout. Boyle *et al.* (2013) also found an increase in the number of melanomacrophage deposits in the spleen with both bulk and TiO_2 NP treatments, with more elevation with NP treatment.

The objective of the present study was to evaluate the effects of dietary or waterborne exposure to TiO_2 NPs on the spleen contents (erythrocyte morphology and haematopoietic cells) of rainbow trout by using spleen prints. Some of the exposure data

and general toxic effects on these same fish for dietary and waterborne TiO_2 NPs are published elsewhere (Federici *et al.*, 2007; Ramsden *et al.*, 2009).

A.2 Materials and Methods

A.2.1 Experimental design

The spleens examined here for TiO_2 NPs effects were obtained from fish exposed to either dietary or waterborne TiO_2. Exposure of juvenile rainbow trout to dietary TiO_2 NPs is reported in Ramsden *et al.* (2009). Briefly, fish was exposed in triplicate (3 tanks/treatments) a control diet of no added TiO_2 and TiO_2 NP contaminated diets (10 and 100 mg kg^{-1} dry weight feed TiO_2 NPs) for 8 weeks and followed by 2 weeks recovery on normal food. The feeds for fish were prepared by spraying dispersed stock solutions of TiO_2 NPs onto the food pellets. The exposure concentration and periods when spleens were collected are summarised in Table A.1. Waterborne exposure to TiO_2 NPs is reported in Federici *et al.* (2007). Briefly, a triplicated semi-static exposure method was used with replacement of the water every 12 hours to ensure the exposure concentration of material was maintained. Sonicated stock solutions of TiO_2 NPs were used for re-dosing each tank. Fish were exposed to various concentrations of TiO_2 NPs up to 1 mg l^{-1} for up to 2 weeks (see Table A.1).

Table A.1 Summary of the exposure conditions and nominal TiO_2 concentrations used in dietary or waterborne exposure studies with juvenile rainbow trout.

Nanoparticle	Types of exposure	Nominal concentrations	Actual measured Ti concentrations in the food or water	Exposure duration	Times when spleens were collected	References
TiO_2 NPs	dietary	10 and 100 mg kg^{-1}	5.4 and 53.6 mg kg^{-1} feed	10 weeks	8 weeks exposure followed by a 2 weeks recoveries	Ramsden *et al.* (2009)
TiO_2 NPs	waterborne	0.1 and 1 mg l^{-1}	(mean ± S.E.M., n = 36/ treatments) 0.095 ± 0.006 mg l^{-1} 0.965 ± 0.021 mg l^{-1}	14 days	7 and 14 days	Federici *et al.* (2007)

A.2.2 Characterisation of TiO_2 nanoparticles

The characterisation of the TiO_2 NPs used here has been reported and both exposures used material from the same batch of powder. The TiO_2 was characterised for the water exposure in Federici *et al.* (2007) and in Ramsden *et al.* (2009) for the dietary studies. Briefly, for all TiO_2 NPs experiments, dry powder of TiO_2 NPs ("Aeroxide" P25 TiO_2, DeGussa AG, supplied via Lawrence Industries, Tamworth, UK) was used. This was made of (revised manufacture's information); crystal structure of 25 % rutile and 75 % anatase TiO_2, purity was at least 99 % TiO_2 (maximum impurity stated was 1 % Si), and with an average particle size of 21 nm with a specific surface area of 50 ± 15 $m^2\ g^{-1}$. A 10 g l^{-1} stock solution of TiO_2 NPs was made (no solvents) by dispersing the NPs in ultrapure (Millipore) water with sonication (bath type sonicator, 35 kHz frequency, fisherbrand FB 11010, Germany) for 6 h. Chemical analysis of stock solutions revealed no metal impurities and the batch was high (data not shown), with a measured mean primary particle size of 24.1 ± 2.8 nm (mean ± SEM, n = 100 electron microscope images, see Federici *et al.*, 2007). For the dietary experiment, the same 10 g l^{-1} stock solution of TiO_2 NPs was sonicated for 8 h, and then either 1 or 10 ml of the stock was added to 49 and 40 ml of ultrapure water to make a 0.2 or 2.0 g l^{-1} TiO_2 NP dilution that could be sprayed onto the food for 10 and 100 mg kg^{-1} treatments respectively. After that, the diluted TiO_2 solutions were sonicated for further 15 min just before spraying to ensure even delivery of the material through the spray nozzle. One kilogram of commercial feed was placed in a commercial food mixer (Kenwood Catering Professional food mixer XKM810) and gradually sprayed with the appropriate TiO_2 NP solution. The TiO_2 NPs immediately coated the feed, and was then sealed in by spraying the food with a 10 % bovine gelatine (BDH, Poole, UK) solution. The gelatine coat was allowed to dry, after that feed was transferred into airtight containers for

storage. The control diet was made exactly the same way, except that the TiO_2 solution was replaced by an equal volume of ultrapure water (see Ramsden *et al.*, 2009 for details).

A.2.3 Preparation of spleen prints

Spleen samples were collected from juvenile rainbow trout at the time points in Table A.1 of the exposure to dietary and waterborne of TiO_2 NPs. Spleen prints were prepared as described in Chapter 2 (see 2.10). Briefly, spleen prints was collected from the trout, then stained with May-Grünwald Giemsa stain and scored haematopoietic cells and cellular pathologies as described in Chapter 2 (see 2.10.1).

A.2.4 Statistical analysis

All data were presented as % mean ± S.E.M and analysed by StatGraphics Plus version 5.1 as described in Chapter 2 (see 2.11).

A.3 Results

A.3.1 Dietary exposure to titanium dioxide nanoparticles

Dietary exposure to TiO_2 NP showed small changes in haematopoietic cells in the spleen after 8 weeks of exposure (Fig. A.1). After eight weeks of exposure to 10 and 100 mg kg^{-1} TiO_2 NP, the spleen image showed the presence of different types of immature (haemocytoblast, erythroblast) and mature red blood cells (erythrocytes) compared to controls and recovery phase (Fig. A.1). An immune cells such as neutrophils and macrophages were also observed with both TiO_2 concentrations compared to the control and the recovery phase image (Fig. A.1). Quantitative spleen print analysis showed that exposure to both TiO_2 concentrations caused a small transient

change in the proportion of erythroblast, but still a statistically significant change (ANOVA, $P < 0.05$), although the proportion of erythrocytes remained the same after 8 weeks of exposure when compared to controls and to the recovery phase (Table A.2). The proportion of lymphocytes also showed a small but statistically significant increase (ANOVA, $P < 0.05$) after 8 weeks of exposure to 100 mg kg $^{-1}$ of the TiO_2 NP treatment compared to control groups and the recovery phase (Table A.2). After 8 weeks of exposure, the concentration effect exhibited a statistically significant decrease (ANOVA, $P < 0.05$) in the proportion of erythroblast and an increase in the proportion of lymphocytes as well as neutrophils were observed with the highest TiO_2 concentration compared to the lowest concentration of TiO_2 NPs (Table A.2).

Erythrocyte morphology in the spleen prints also showed small changes after 8 weeks of exposure to TiO_2 NPs (erythrocytes recognised as having lesions; Fig. A.1). These changes included swollen erythrocytes, red blood cells with dividing nuclei, and cells with membrane abnormality. After an additional two weeks of recovery on normal food, less changes were observed in the erythrocyte morphology in the spleen prints with both TiO_2 concentrations (Fig. A.1). A quantitative analysis of erythrocyte abnormalities confirmed that dietary exposure to 10 and 100 mg kg $^{-1}$ of the TiO_2 for 8 weeks caused a small but statistically significant increase in the proportion of swollen erythrocytes and cells with membrane abnormality compared to controls (ANOVA, $P < 0.05$; Table A.3). For most of the abnormalities, the incident rate was only 1-2 % of the red blood cells observed, regardless of the concentration. High concentration of TiO_2 treatment showed more increased in the proportion of swollen red cells and cells with membrane abnormality compared to the 10 mg kg $^{-1}$ TiO_2 treatment (ANOVA, $P < 0.05$; Table A.3). No changes in the proportion of erythrocyte abnormalities were

observed after 2 weeks of recovery (Table A.3). This level of changes is unlikely to be clinically important to fish health.

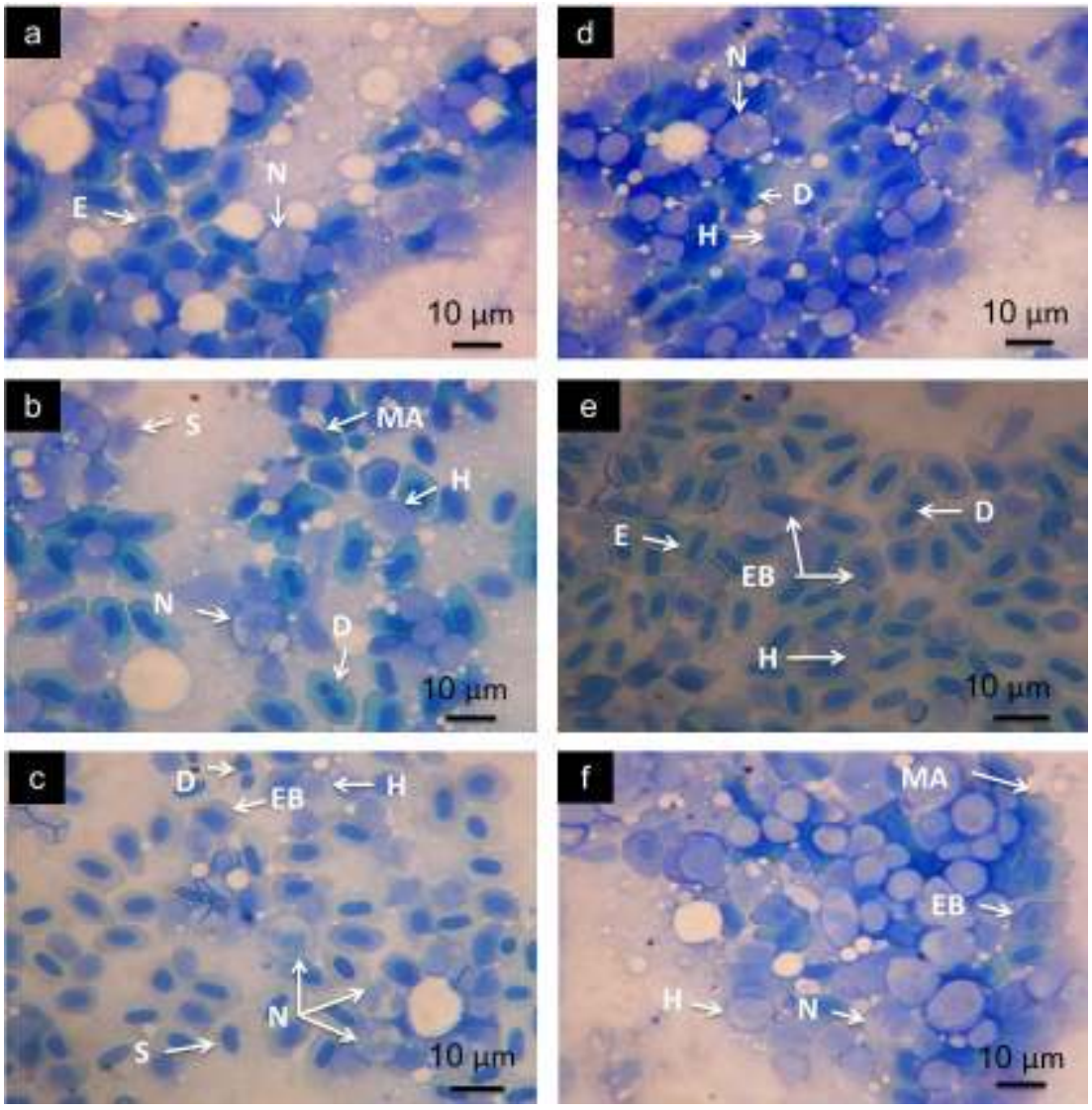

Figure A.1 Spleen prints from rainbow trout following dietary exposure to TiO_2 NPs for 8 weeks (left column) and an additional 2 weeks of recovery which fish were feeding on normal food (right column). For 8 weeks, the panels include (a) control, (b) 10 mg kg^{-1} of TiO_2, (c) 100 mg kg^{-1} of TiO_2. After an additional 2 weeks recovery, the panels include (d) control, (e) 10 mg kg^{-1} of TiO_2, (f) 100 mg kg^{-1}. Erythrocytes (E), neutrophils (N), swollen red cells (S), cells with membrane abnormality (MA), haemcytoblast (H), red cells with dividing nuclei (D), and erythroblast (EB). Spleen prints were obtained from the fish used in Ramsden *et al.* (2009). Scale bar indicates magnification, smear stained with May-Grünwald Giemsa stain.

Table A.2 The percentage of red and white blood cells in spleen prints from rainbow trout exposed to dietary TiO_2 nanoparticles for 8 weeks and followed by 2 weeks of recovery. (During recovery state fish feed on normal food)

% of haematopoietic cells	Time/ week	Treatments		
		Control	10 mg kg^{-1} TiO_2	100 mg kg^{-1} TiO_2
Haemocytoblast	8	5.4 ± 0.3 (5)	5.4 ± 0.2 (6)	5.7 ± 0.1 (6)
	2	6.6 ± 0.3 (6) $^{+}$	6.8 ± 0.1 (6) $^{+}$	6.0 ± 0.6 (5) $^{+}$
Erythroblast	8	13.6 ± 0.8 (5)	15.2 ± 0.3 (6) *	11.8 ± 0.2 (6) *$^{\#}$
	2	11.3 ± 0.4 (6) $^{+}$	11.4 ± 0.7 (6) $^{+}$	12.2 ± 0.4 (5)
Progranulocytes	8	0.3 ± 0.1 (5)	0.8 ± 0.1 (6)	0.6 ± 0.2 (6)
	2	0.5 ± 0.3 (6)	Not observed (6) $^{+}$	0.3 ± 0.1 (5)
Erythrocytes	8	77.2 ± 1.0 (5)	76.4 ± 0.2 (6)	76.3 ± 0.5 (6)
	2	77.8 ± 0.4 (6)	77.1 ± 0.6 (6)	76.1 ± 0.6 (5)
Lymphocytes	8	0.2 ± 0.1 (5)	0.2 ± 0.1 (6)	2.9 ± 0.2 (6) *$^{\#}$
	2	0.4 ± 0.2 (6)	0.8 ± 0.2 (6) $^{+}$	Not observed (5) $^{+\#}$
Neutrophils	8	3.0 ± 0.2 (5)	2.6 ± 0.3 (6)	4.1 ± 0.5 (5) $^{\#}$
	2	3.2 ± 0.4 (6)	2.8 ± 0.1 (6)	3.0 ± 0.3 (6) $^{+}$
Macrophages	8	Not observed (5)	0.2 ± 0.1 (6)	0.3 ± 0.1 (6) *
	2	Not observed (6)	Not observed (6)	Not observed (5) $^{+}$

Data are means of proportional cells ± S.E.M. (n = number of fish). (*) Statistically significant difference between control and treatment within row (ANOVA, $P < 0.05$). (#) Statistically significant difference between low and high concentration within row (ANOVA, $P < 0.05$). (+) Statistically significant difference between 8 weeks exposures and the subsequent 2 weeks recovery (time effects, ANOVA, $P < 0.05$). Not observed, the cell was absent from all the spleen prints examined from all the fish (a mean of zero).

Table A.3 The percentage of erythrocytes with abnormalities in the spleen prints from rainbow trout exposed to dietary TiO_2 nanoparticles (NPs) for 8 weeks and followed by 2 weeks of recovery. (During recovery state fish feed on normal food)

Types of erythrocyte abnormality	Time/ week	Treatments		
		Control	10 mg kg^{-1}	100 mg kg^{-1}
Swollen cells	8	0.6 ± 0.1 (6)	1.4 ± 0.1 (6) *	2.5 ± 0.2 (5)*#
	2	0.4 ± 0.2 (5)	0.7 ± 0.1 (6) +	0.8 ± 0.2 (6) +
Cells with a dividing nuclei	8	12.5 ± 1.1 (6)	12.1 ± 0.4 (6)	12.4 ± 0.6 (5)
	2	6.2 ± 0.4 (5) +	7.6 ± 0.4 (6) +	6.8 ± 0.2 (6) +
Cells with membrane abnormality	8	1.3 ± 0.3 (6)	1.9 ± 0.2 (6) *	3.6 ± 0.2 (5) *#
	2	1.2 ± 0.1 (5)	0.8 ± 0.1 (6) +	1.4 ± 0.2 (6) +

Data are means of proportional cells ± S.E.M. (n = number of fish). (*) Statistically significant difference between control and treatment within row (ANOVA, $P < 0.05$). (#) Statistically significant difference between low and high concentration within row (ANOVA, $P < 0.05$). (+) Statistically significant difference between 8 weeks exposures and the subsequent 2 weeks recovery (time effects, ANOVA, $P < 0.05$).

A.3.2 Waterborne exposure to titanium dioxide nanoparticles

Differentiation of the haematopoietic cells in spleen prints after 7 and 14 days of exposure to 0.1 and 1 mg l^{-1} of TiO_2 NP showed the presence of immature cells (erythroblast and haemocytoblast) and mature cells (erythrocytes) compared to control images (Fig. A.2). Waterborne exposure to both TiO_2 concentrations also showed the presence of different types of immune cells such as neutrophils, lymphocytes, monocytes, and macrophages compared to the control image (Fig. A.2). A quantitative analysis of spleen prints showed a statistically significant transient change (ANOVA, $P < 0.05$) in the proportion of haemocytoblast and erythroblast, although the proportion of erythrocytes remained the same at the end of exposure with both TiO_2 concentrations compared to controls (Fig. A.2; Table A.4). For the immune immature cells, the proportion of progranulocytes also showed a small increase after 7 days of exposure to

0.1 mg l^{-1} of TiO_2 NP compared to the control (ANOVA, $P < 0.05$). The transient statistically significant changes depending on the TiO_2 concentration were shown in the proportion of lymphocytes at day 14 when compared to controls (ANOVA, $P < 0.05$; Table A.4). The proportion of neutrophils and monocytes showed a small increase after 7 days of exposure to TiO_2 compared to controls (all statistically significant, ANOVA, $P < 0.05$; Table A.4). The incident rate of immature and immune cell changes was only 1- 4 % of the haematopoietic cells on the spleen. Some measurements were approaching the limits of experimental error. For example, quantitative scoring of the spleen prints at day 14 found no thrombocytes (not observed, mean score of zero, Table A.5). Earlier on in the study, however, thrombocytes were counted in the control (*e.g.*, % mean ± S.E.M = 0.9 ± 0.3 on day 7, $n = 6$). However, the proportion of haemocytoblast and lymphocytes also showed concentration (greater effects with high concentration) and time effects (greater effects at day 14 than 7) (Table A.4).

Increasing the presence of erythrocytes abnormality was observed in the spleen prints of trout following waterborne exposure to 0.1 and 1 mg l^{-1} of the TiO_2 NPs at each time points (Fig. A.2). These abnormalities included swollen erythrocytes, red cells with micronuclei, and cells with membrane abnormality. A quantitative analysis of the erythrocytes abnormality showed exposure to 0.1 mg l^{-1} TiO_2 at each time point caused a small but statistically significant increase in the proportion of swollen red cells, cells with micronuclei, and cells with membrane abnormality compared to the control (ANOVA, $P < 0.05$; Table A.5). Whereas, exposure to 1 mg l^{-1} of TiO_2 NP treatment showed a small increase in the proportion of swollen blood cells (at day 7 only) and cells with membrane abnormality (at day 14 only) compared to controls (all statistically significant, ANOVA, $P < 0.05$). The incident rate of most abnormalities was only 1-4 % of the red blood cells observed, regardless of the time point (Table A.5).

Concentrations effects within each time point showed a small but statistically significant increase in the proportion of swollen red cells and cells with dividing nuclei at 1 mg l^{-1} TiO_2 treatment when compared to the 0.1 mg l^{-1} TiO_2 NP treatment (ANOVA, $P < 0.05$; Table A.5). The proportion of swollen red blood cells and cells with membrane abnormality also showed time effects which include more elevation at day 14 than day 7.

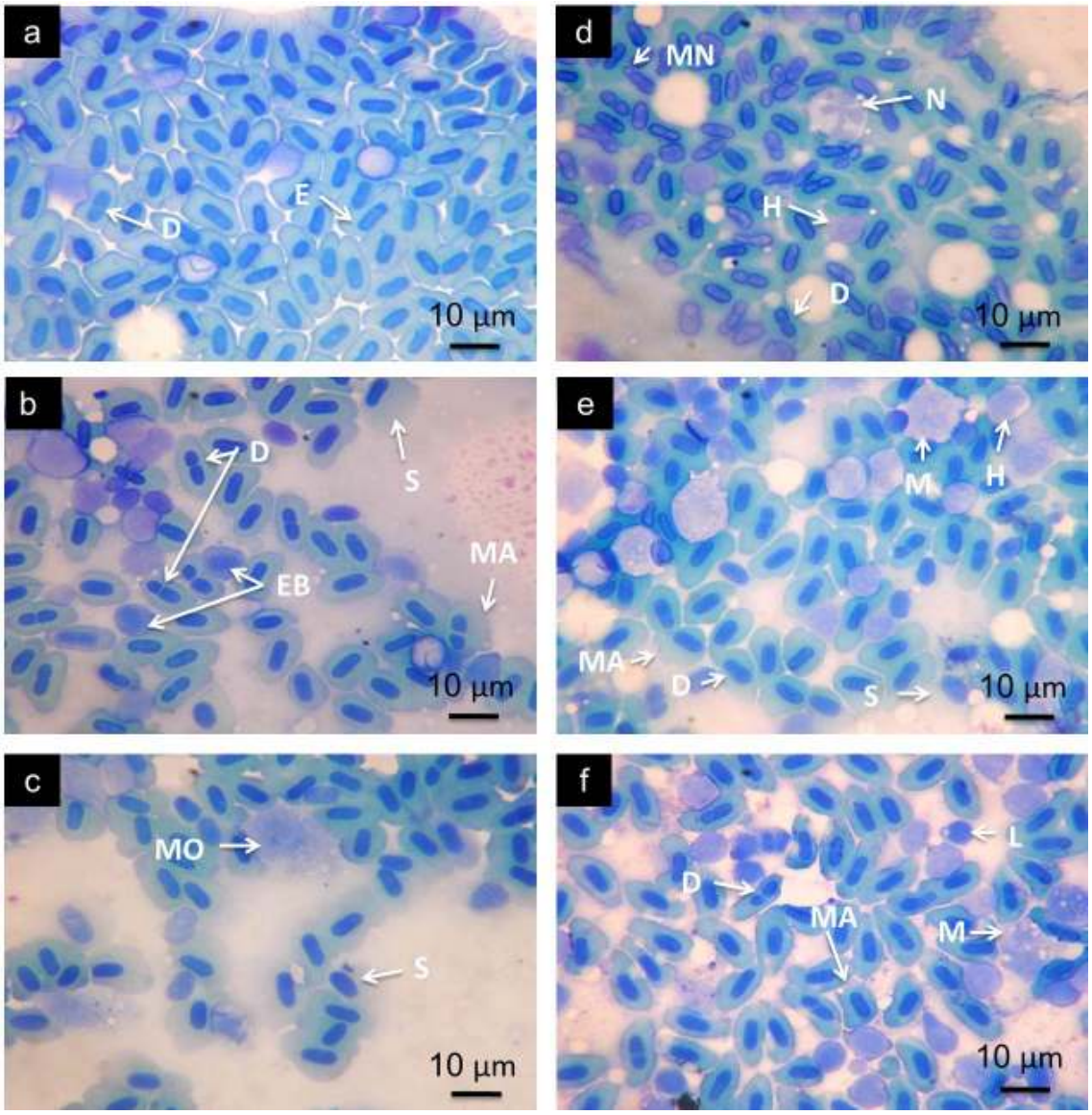

Figure A.2 Spleen prints from rainbow trout following exposure to waterborne TiO_2 NPs for 7 days (left column) and 14 days (right column). For 7 days, the panels include (a) control, (b) 0.1 mg l^{-1} of TiO_2, (c) 1 mg l^{-1} TiO_2. For 14 days, the panels include (d) control, (e) 0.1 mg l^{-1} of TiO_2 , (f) 1 mg l^{-1} of TiO_2. Swollen red cells (S), red blood cells with a dividing nucleus (D), erythroblast (EB), cells with membrane abnormality (MA), monocytes (MO), cells with micronuclei (MN), neutrophils (N), haemocytoblast (H), macrophages (M), lymphocytes (L). Spleen prints were obtained from the fish used in Federici *et al*. (2007). Scale bar indicates magnification, smear stained with May-Grünwald Giemsa stain

Table A.4 The percentage of red and white blood cells in spleen prints from rainbow trout exposed to waterborne TiO_2 nanoparticles for 7 and 14 days.

% of the haematopoietic cells	Time/ days	Treatments		
		Control	0.1 mg l^{-1} TiO_2	1 mg l^{-1} TiO_2
Haemocytoblast	7	Not observed (6)	0.8 ± 0.4 (6)	2.5 ± 0.4 (6) *#
	14	3.1 ± 0.3 (6) +	2.3 ± 0.2 (5) *+	2.3 ± 0.1 (5)
Erythroblast	7	12.4 ± 1.0 (6)	7.7 ± 0.5 (6) *	8.1 ± 0.8 (6) *
	14	7.5 ± 0.7 (6)	7.0 ± 0.2 (5)	4.9 ± 0.1 (6) *+
Progranulocytes	7	0.3 ± 0.2 (6)	1.0 ± 0.1 (6) *	0.6 ± 0.2 (6) #
	14	0.6 ± 0.1 (6)	0.8 ± 0.1 (5)	0.4 ± 0.1 (5)
Erythrocytes	7	86.0 ± 0.7 (6)	87.7 ± 1.3 (6)	86.1 ± 1.0 (6)
	14	85.0 ± 0.4 (6)	86.5 ± 0.9 (5)	86.7 ± 0.5 (5)
Lymphocytes	7	1.0 ± 0.4 (6)	1.5 ± 0.2 (6)	1.1 ± 0.1 (6)
	14	1.9 ± 0.2 (6) +	0.9 ± 0.2 (5) *+	3.1 ± 0.3 (5) *#+
Neutrophils	7	Not observed (6)	0.9 ± 0.4 (6) *	1.0 ± 0.2 (6) *
	14	1.5 ± 0.3 (6) +	1.6 ± 0.2 (5)	1.9 ± 0.1 (5) +
Macrophages	7	Not observed (6)	0.3 ± 0.1 (6)	0.6 ± 0.2 (6) *
	14	0.3 ± 0.1 (6)	0.7 ± 0.2 (5) *	0.6 ± 0.1 (5)
Monocytes	7	Not observed (6)	0.3 ± 0.1 (6) *	Not observed (6)
	14	0.1 ± 0.1 (6)	0.3 ± 0.1 (5) *	Not observed (5)
Thrombocytes	7	0.9 ± 0.3 (6)	0.1 ± 0.1 (6) *	Not observed (6) *
	14	Not observed (6) +	Not observed (5)	Not observed (5)

Data are means of proportional cells ± S.E.M. (n = number of fish). (*), statistically significant difference between control and treatment within row (ANOVA, $P < 0.05$). (#), statistically significant difference between low and high concentration within row (ANOVA, $P < 0.05$). (+), statistically significant difference between 7 and 14 days (time effects, ANOVA, $P < 0.05$). Not observed, the cell was absent from all the spleen prints examined from all the fish (a mean of zero).

Table A.5 The percentage of erythrocytes with abnormalities in spleen prints from rainbow trout exposed to waterborne TiO_2 nanoparticles for 7 and 14 days.

% of erythrocytes abnormalities	Time/ days	Treatments		
		Control	0.1 mg l^{-1} TiO_2	1 mg l^{-1} TiO_2
Swollen cells	7	Not observed (6)	0.2 ± 0.1 (6)	0.9 ± 0.1 (6) *#
	14	0.2 ± 0.1 (6)	1.4 ± 0.2 (5) *+	Not observed (5) #+
Cells with micronuclei	7	0.2 ± 0.1 (6)	2.1± 1.0 (6) *	Not observed (6) #
	14	Not observed (6)	0.2 ± 0.1 (5) +	0.2 ± 0.1 (5)
Cells with a dividing nuclei	7	5.7 ± 0.8 (6)	8.2 ± 1.5 (6) *	5.5 ± 0.9 (6)
	14	18.4 ± 3.1 (6) +	4.1 ± 0.3 (5) *	12.9 ± 1.7 (5) *+#
Cells with membrane abnormalities	7	1.8 ± 0.4 (6)	2.9 ± 0.6 (6) *	1.7 ± 0.4 (6) #
	14	0.2 ± 0.1 (6) +	4.3 ± 0.4 (5) *	4.0 ± 0.3 (5) *+

Data are means of proportional cells ± S.E.M. (n = number of fish). (*), statistically significant difference between control and treatment within row (ANOVA, $P < 0.05$). (#), statistically significant difference between low and high concentration within row (ANOVA, $P < 0.05$). (+), statistically significant difference between 7 and 14 days (time effects, ANOVA, $P < 0.05$). Not observed, the cell was absent from all the spleen prints examined from al/l the fish (a mean of zero).

A.4 Discussion

Information on the effects of metals and metal oxide nanoparticles on the spleen or blood component in the haematopoietic tissues of fish are scarce. In this study, the results show that dietary exposure to TiO_2 NPs caused less effect on the proportion of haematopoietic cells and the erythrocyte morphology compared to controls. However, responses were very minor compared to controls of the different routes of exposure. Waterborne exposure to the same materials (TiO_2 NPs) showed greater effects on the spleen contents of rainbow trout.

A.4.1 Effects of dietary TiO_2 NPs exposure on the spleen

Dietary exposure to TiO_2 NPs caused small changes in the haematopoietic cells in the spleen which included an increase in the proportion of erythroblasts, but the number of erythrocytes remained the same. The proportion of erythrocytes with abnormalities also showed small changes. This could indicate the spleen function is not affected by TiO_2, or the amount of TiO_2 that reached the spleen is not adequate to induce greater lesions in the spleen contents. Ramsden *et al.* (2009) found that dietary exposure to sub-lethal of TiO_2 NPs gave normal growth level, no changes in the haematology and no evidence of osmotic stress, although Ti was accumulated in the spleen and other organs (gill, gut, liver and brain) with minor organ pathology. The presence of measurable levels of Ti in the spleens indicated that organ as an early target for dietary Ti exposure (Ramsden *et al.*, 2009). The level of Ti accumulation was decreased in the spleen after six and eight weeks of dietary exposure and Ramsden *et al.* (2009) suggested that the spleen is able to regulate the excess TiO_2 during the exposure. Ramsden *et al.* (2009) also found an increase in the proportion of red pulp after 8 weeks of exposure to 100 mg kg^{-1} of TiO_2 treatment. Therefore, the spleen prints results could reflect that the spleen was working to supply red blood cells into the circulatory system after removal of damaged cells from the circulation. After two weeks of recovery, a decrease in the proportion of erythrocytes with abnormalities (present study) and the proportion of red pulp (Ramsden *et al.*, 2009) was observed in the spleen of trout. This suggests that the spleen was working to filter damaged red blood cells to return blood (and spleen function) to normal levels. For the immune response, a small increase in the proportion of immune cells that observed here could suggest the spleen responded by an induced immune cells to counteract these materials. The presence of granular deposits associated with the activity of fixed macrophages in the same samples of the spleen from all TiO_2 treatments that observed in Ramsden *et al.* (2009) could suggests normal

phagocytic functions. In fishes, the primary target of nanoparticle immunotoxicity is the cell mediated immunity and the phagocytic cells (Jovanović and Palic 2012). The increasing immune cells and removing foreign materials by ingesting them through a phagocytosis process has been reported in Dobrovolskaia *et al.* (2007).

A.4.2 Effects of waterborne TiO_2 NPs exposure on the spleen

Waterborne exposure to TiO_2 NPs caused a small decrease in the proportion of immature red blood cells, but the proportion of mature erythrocytes remained the same after 14 days of exposure. These results suggest that fish suffered from hypoxia (see gill injury; Federici *et al.*, 2007). A study by Federici *et al.* (2007) found that waterborne exposure to sub-lethal concentration of TiO_2 NPs caused organ pathologies (gill, liver, and brain), biochemical disturbances, oxidative stress and no major haematological disturbance. The finding of Federici *et al.* (2007) may be explained by the results found in the present study on the proportion of immature and mature erythrocytes in the spleen prints that collected from the same experiment. Usually, a confounding factor for any study of the spleen in fishes exposed via the water column is the indirect effects of gill injury and systemic hypoxia on the spleen function. Gill injury could stimulate the conscription of blood cells from the spleen into the circulation. Recent study by Bilberg *et al.* (2010) showed that exposure to silver NPs can reduce blood pO_2 in fish.

The transient increased in the proportion of immune cells in the spleen prints of trout following exposure to TiO_2 that observed here could indicate the spleen responding to the NPs by producing immune cells (lymphocytes, neutrophils, and macrophages) as an immune response. A small proportion of thrombocytes that observed here could attribute to the experimental error instead of biological impact.

The current study also found a small increase in the proportion of erythrocytes with abnormalities compared to the control. Observation cells with dividing nuclei in the spleen prints are not considered as a type of erythrocyte abnormalities, but are evidence of stimulation of reticulocytes for dividing to produce new cells. These new cells compensated to the deficiencies of the blood cells in the circulatory system. Whereas, the swollen red cells and cells with membrane abnormality that observed in this study could result due to the osmotic influx of water that lead in the worst case to haemolysis.

Differences between dietary and waterborne exposure

The current study provides details about the effects of dietary and waterborne exposure to TiO_2 NPs on the spleen contents of rainbow trout by using spleen prints method. Over all, the dietary exposure to TiO_2 NPs had minor effects on the spleen contents of trout compared to waterborne exposure (morphology of erythrocytes and haematopoiesis cells). This suggests that dietary exposure to TiO_2 NPs had a lower bioavailability than waterborne NPs. Low oral bioavailability of NPs was found during dietary exposure in Ramsden *et al.* (2009) study, because this route of exposure caused minor effects on the haematology parameters (red or white blood cell counts, haematocrits, whole blood haemoglobin) with no impact on growth or nutritional performance. Ramsden *et al.* (2009) also found more elevation of Ti levels in the spleen after 2 weeks of exposure than 4 or 6 weeks, and these levels were sharply decreased at week 8 of exposure. These authors suggest that fishes were able to regulate excess TiO_2 in the spleen. Therefore, the minor changes that observed here in the spleen prints contents may be due to the Ramsden *et al.* (2009) observation, but the function of

spleen did not affect by this route of exposure to TiO_2 NPs. However, the dietary exposure is less dangerous to the immune system of fishes.

Waterborne exposure to TiO_2 NPs induces lesions in the gills and other internal organs, with no major haematological or blood disturbances (Federici *et al.*, 2007), as well as small changes in the spleen contents (in the current study). Changes in the spleen prints contents are therefore not necessarily indicative of NM uptake, or direct NM toxicity on the spleen could be occurred due to hypoxia. Therefore, the waterborne exposure is more dangerous to the respiratory health of fishes than dietary exposure. Additionally, the waterborne exposure to TiO_2 showed greater effects on the immune system of fish by producing different types of immune cells to phagocytic the effects of NPs. Overall, the levels of changes observed with dietary and waterborne exposure to TiO_2 are unlikely to be clinically important to fish health.

Appendix B: Poster presentation

THE EFFECTS OF NANOCOPPER COMPARED TO AQUEOUS COPPER SULPHATE ON RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Introduction

Emerging data has indicated that some types of engineered nanoparticles (NPs) are toxic to fish[1], and given the well known toxicity of dissolved metals, there are also concerns about metal NPs. The principle target organs for dissolved Cu are the gills, liver and intestine with effects including tissue injury and diminished physiological function including oxidative and osmoregulatory stress.
The present study aimed to assess whether copper nanoparticles(Cu-NPs) exhibited a different mode of toxicity compared to an equal mass of dissolved copper in rainbow trout .

Materials & Methods

Experimental Design

- Fish were exposed to 0, 20, or 100 µg l^{-1} of Cu as $CuSO_4.5H_2O$ or Cu-NPs for up to 10 days.
- A semi-static exposure regime was used with no feeding during the experiment.
- Water quality parameters were measured daily (Table 1).
- Sampling was carried out on days 0, 4, and 10 for haematology, trace metal analysis (ICP-OES), immune response (spleen prints), biochemistry (not presented) and histology.

Table 1. Water quality parameters measured throughout the 10 day exposure (as there were no tank effects data from all tanks were pooled and are presented as mean ± SD, n = 150-300).

Parameter	Temperature (°C)	saturated O_2 (%)	pH	Ammonium + water change (mg l^{-1})	Ammonium + water change (mg l^{-1})	Total hardness (mg l^{-1} $CaCO_3$)	Background Cu levels (µg l^{-1})
Data	16.0 ± 0.12	82.5 ± 3.66	8.06 ± 0.06	0.40 ± 0.62	0.29 ± 0.1	72.02 ± 1.2	0.04 ± 0.07

Copper Nanoparticles

- Sigma-Aldrich, >99.9% purity, nominal particle size <50 nm (from manufacturers information).
- Measured primary particle size ; 87 ± 27 nm (TEM, n = 50, Fig.1).
- Particle size distribution in a 1 g l^{-1} stock showed a mean aggregate size of 216 ± 122 nm, mode = 48 nm (NanoSight).

Fig. 1. Cu-NP primary particle size and particle size assessed by transmission electron microscopy .

Results

Mortality

Fish from all treatments experienced mortality over 10 days of exposure (Fig. 2). However, by day 4 >66 % of fish from the 100 µg l^{-1} $CuSO_4$ treatment died and this treatment was therefore terminated.

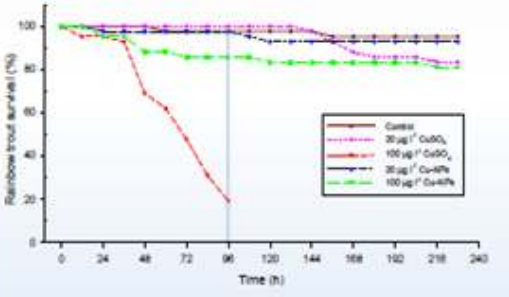

Fig. 2. Survival of rainbow trout exposed to 20 or 100 µg l^{-1} Cu-NPs or $CuSO_4$ for up to 10 days. Blue dashed line indicates end of 96 h exposure and termination of 100 µg l^{-1} $CuSO_4$ treatment only.

Copper Accumulation and Branchial Electrolytes

- Increased Cu accumulation in the gills of fish exposed to $CuSO_4$ and Cu-NPs compared to control fish was observed (Fig. 3a).
- There was no statistically significant accumulation of Cu in the liver, intestine, brain, muscle, or spleen of fish from any treatment (ANOVA or Kruskal–Wallis, $P > 0.05$); although a non-significant trend of >Cu in the spleen of Cu-NP exposed fish was observed; values of (in µmol g^{-1}, mean ± SEM) 0.05 ± 0.008, 0.11 ± 0.04, 0.09 ± 0.06; and 0.06 ± 0.01, 0.13 ± 0.06, 0.06 ± 0.01 for control, 20, and 100 µg l^{-1} Cu-NP exposed fish at days 4 and 10 respectively.
- A (not statistically significant) trend of reduced gill K in fish from all Cu treatments (data not shown).
- A significant depletion in branchial Na levels of both Cu treatments was observed after 4 days of exposure and was still evident at day 10 (Fig. 3b).

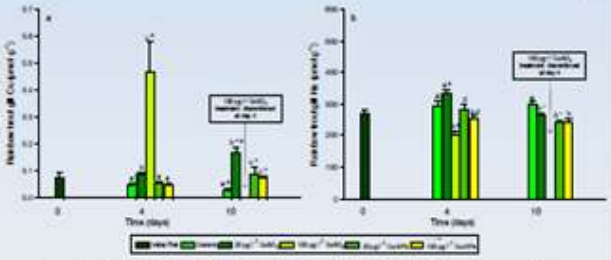

Fig. 3a. Cu accumulation and Fig. 3b. Na levels, in the gills of rainbow trout. Different letter denotes statistically a significant difference between treatments at each time point (ANOVA or Kruskal–Wallis, $P < 0.05$); * indicates significantly different than initial fish (Host, $P < 0.05$); ^ indicates significantly different from same treatment at the previous time point (Host, $P < 0.05$).

Haematology & Spleen Prints

- No differences in haemoglobin, red blood cell counts, haematocrit, or plasma Na^+, K^+, Cl^-, or glucose levels of any treatment (ANOVA or Kruskal–Wallis, $P > 0.05$).
- Changes to erythrocytes seen in spleen prints from both Cu treatments (more pronounced in $CuSO_4$ exposed fish; Fig. 4).

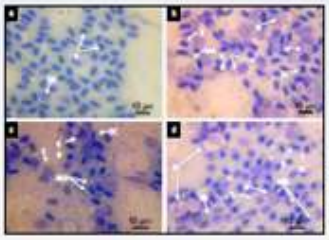

Fig. 4. Spleen prints in rainbow trout following exposure to (a) control, (b) 20 µg l^{-1} $CuSO_4$, (c) 20 µg l^{-1} Cu-NPs, or (d) 100 µg l^{-1} Cu-NPs for 10 d. (EE) erythroblast, (E) erythrocyte, (N) neutrophil, (L) swollen cell, (SC) small cell, (M) macrophage, (H) haemoblast, (MA) membrane abnormalities, (D) dividing nucleus . Stained with Giemsa.

Histology

- Fish from both Cu treatments exhibited histological pathologies at days 4 and 10 (Figs. 5, 6, and 7 for day 10) which were largely absent in the control fish.
- The more severe and frequent lesions were seen in the $CuSO_4$ treatments.
- Note: No 100 µg l^{-1} $CuSO_4.5H_2O$ treatment at day 10.

(Electron were 7 µm and stained with Mallory's trichrome (gills) or H&E (liver, spleen), x 400).

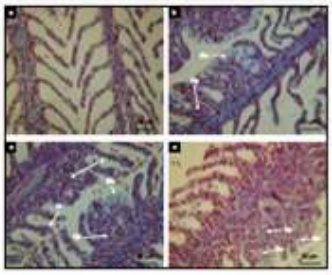

Fig. 5. Gill morphology in rainbow trout following exposure to (a) control, (b) 20 µg l^{-1} $CuSO_4.2H_2O$ and (c) 20 µg l^{-1} Cu-NPs, or (d) 100 µg l^{-1} Cu-NPs for 10 d. (Ma) swollen mucocytes, (Hp) hyperplasia (Hp), (F) fusion, (De) oedema, and clubbed tips.

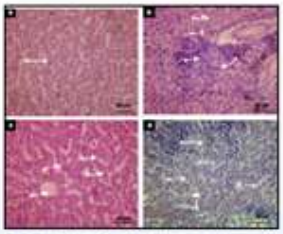

Fig. 6. Liver morphology in trout following exposure to (a) control, (b) 20 µg l^{-1} $CuSO_4.2H_2O$ and (c) 20 µg l^{-1} Cu-NPs, or (d) 100 µg l^{-1} Cu-NPs for 10 d. (Ag) aggregation of vacuoles, (IH) inflammatory hepatitis, (L) lipidosis, (M) foci of melanomacrophage, (N) necrosis, (PN) pyknotic nuclei, (S) normal sinusoid space.

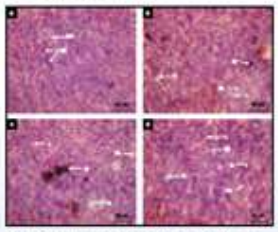

Fig. 7. Spleen morphology in trout following exposure to (a) control, (b) 20 µg l^{-1} $CuSO_4.2H_2O$ and (c) 20 µg l^{-1} Cu-NPs, or (d) 100 µg l^{-1} Cu-NPs for 10 d. Spleen from control fish showed normal histology, with defined red (RP) and white (WP) pulp. (DL) depletion of lymphoid tissues, (L) lipidosis, (M) foci of melanomacrophage, (N) necrosis, (SE) swollen erythrocyte.

Conclusions

- Both Cu treatments were toxic to rainbow trout with dissolved Cu more toxic than Cu-NPs.
- Cu uptake in the gills of fish from both treatments; more pronounced in dissolved Cu exposed fish.
- There were no major affects on haematology from either treatment.
- Dissolved Cu and Cu-NPs both caused pathologies in gills, liver and spleen. However, this was largely without appreciable Cu accumulation in Cu-NP exposed fish raising the possibility of these NPs acting in a similar manner to surface acting metals (e.g., aluminium[2]).
- There were possible effects on spleen function which requires further investigation.
- Cu-NPs potentially affects gill Na, though plasma Na^+, along with K^+, and Cl^-, remained similar to control levels.
- Ongoing work includes the assessment of affects on osmoregulation (e.g., Na^+/K^+-ATPase), indications of oxidative stress, brain & intestine histology, and quantification of the rate of dissolution of Cu from Cu-NPs.

Acknowledgments: Assistance from Dr Andy Fisher (ICP-OES), Mike Hoskings (histology) and Andy Arkell (sampling and haematology) is greatly appreciated.

1 Handy et al., 2008. Ecotoxicology 17, 396–409.
2 Handy & Eddy, 1989. J. Fish Biol. 34, 865–874.

Effects Of Dissolved Copper and Nanocopper on Histopathology and Haematopoietic Organs of Rainbow Trout (*Oncorhynchus mykiss*)

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Introduction

- Increasing use of copper and copper nanoparticles (NPs) is likely to results in release of these pollutants into aqueous environment; however, it is unclear what types of lesions these materials cause in fish.
- Ecotoxicological Studies revealed that some types of engineered nanoparticles are toxic to fish (1).
- Gill, liver, spleen, and intestine are considered target organs for dissolved Cu with some toxic effects; including oxidative stress, osmoregulatory disturbances and tissue injury (2).
- The present study aimed to determine the effects of dissolved Cu and Cu-NPs on organ integrity in trout; and to compare the effects of Cu metal with Cu-nanoparticles to identify any nano-specific pathologies.

Methodology

- Fish were exposed to 0, 20, or 100 µg/L of Cu as $CuSO_4.5H_2O$ and Cu-NPs for up to 10 days using a semi-static exposure regime with no feeding during the experiment.
- Water quality parameter were measured daily.
- Sample of tissues were carried out on day 0, 4, and 10 for histology and spleen prints.

Results

Mortality

Fish in all treatments experienced mortality over 10 days of exposure.

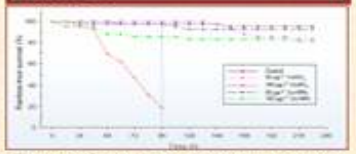

Figure 1. Survivals of rainbow trout exposed to 20 or 100 µg/L of $CuSO_4$ or Cu-NPs for up to 10 days. The middle dash line indicates end of 96 h exposure and termination of 100 µg/L $CuSO_4$ treatment only.

Histology

- Fish from both Cu treatments showed histological pathologies at days 4 and 10 (Figures 2, 3, 4, 5, 6 and 7 for day 10) which were largely absent in control fish.
- The injuries observed were greater with Cu than Cu-NPs.
- **Note:** No 100 µg/L $CuSO_4.5H_2O$ treatment at day 10.
- Sections were 7 µm and stained with Mallory's trichrome (gill) or H&E (Liver, spleen and gut), x400

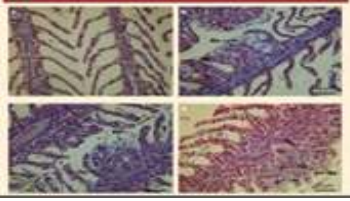

Figure 2. Gill morphology in rainbow trout following exposure to (a) control, (b) 20 µg/L $CuSO_4$, (c) 20 µg/L Cu-NPs and (d) 100 µg/L Cu-NPs for 10 days. (Oe) oedema, (Ct) club tips, (An) lamellar aneurism, (Ms) swollen mucocyte, (Hp) hyperplasia and (F) Fusion.

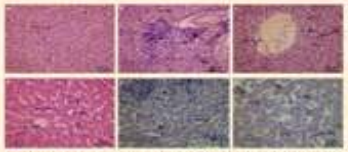

Figure 3. Liver morphology in trout following exposure to (a) control, (b and c) 20 µg/L $CuSO_4$,(d) 20 µg/L Cu-NPs and (e and f) 100 µg/L Cu-NPs for 10 days. (S) sinusoid space, (L) lipidosis, (Pn) pyknotic nuclei, (M) foci of melanomagrophage, (Av) aggregation of vacuoles, (Ab) aggregation of blood cell in sinusoid space, (IH) inflammatory hepatitis, (N) necrosis, (Le) lifting endothelium of blood vessel and (Oe) oedema.

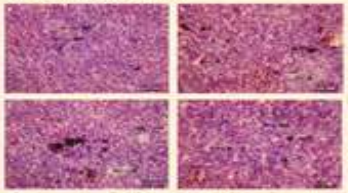

Figure 4. Spleen morphology in trout following exposure to (a) control, (b) 20 µg/L $CuSO_4$, (c) 20 µg/L Cu-NPs and (d) 100 µg/L Cu-NPs for 10 days. (RP) red and (WP) white pulp, (SE), swollen erythrocyte, (N) necrosis, (L) lipidosis, (M) foci of melanomacrophage and (DL) depletion of lymphoid tissues.

References

(1) Handy *et al.* (2008). Ecotoxicology, 17, 390- 409.

(2) Osman *et al.* (2009). Research Journal of Environmental Toxicology, 3 (1), 9-23.

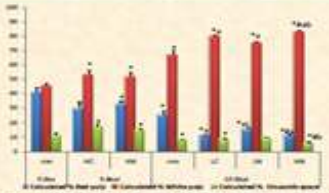

Figure 5. Changes in the proportions of red and white pulp in the spleens of trout following exposure to dissolved Cu (20, 100 µg/L) and Cu-NPs (20, 100 µg/L) for 0, 4 and 10 days. (*), significant difference from initial fish (stock fish at time zero, (ANOVA, $P < 0.05$). (a), significant difference from control within treatment in 10 days (ANOVA, $P < 0.05$). (b), significant difference from the previous Cu-NPs concentration (dose effect within time point, *t*test, $P < 0.05$). (c), significant difference between low concentration of Cu and Cu-NPs (nano-effects, *t*test, $P < 0.05$). (#), significant difference between day 4 and 10 within treatment of high Cu-NPs (time effects, *t*test, $P < 0.05$).

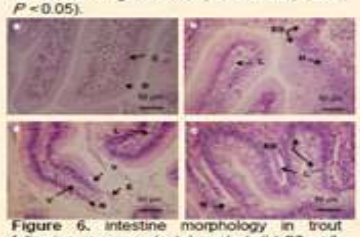

Figure 6. Intestine morphology in trout following exposure to (a) control, (b) 20 µg/L $CuSO_4$, (c) 20 µg/L Cu-NPs and (d) 100 µg/L Cu-NPs for 10 days. (C) columnar cell, (G) goblet cell, (L) lifting epithelium, (SG) swelling of goblet cells, (H) hyperplasia, (V) vacuoles in the lamina propria and lining epithelium, (N) necrosis in the lining epithelium layer and (E) erosion of villi.

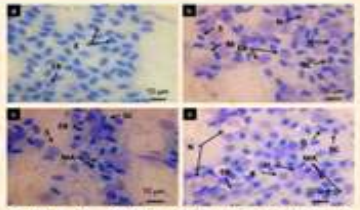

Figure 7. Spleen print in rainbow trout following exposure to (a) control, (b) 20 µg/L of $CuSO_4$, (c) 20 µg/L of Cu-NPs and (d) 100 µg/L of Cu-NPs for 10 days. (EB) erythroblast, (E) erythrocyte, (N) neutrophil, (S) swollen cell, (SC) small cell, (M) macrophage, (H) haemoblast, (MA) membrane abnormalities, (D) dividing nucleus.

Conclusions

- Both Cu treatments were toxic to rainbow trout with dissolved Cu more toxic than Cu-NPs.
- Cu and Cu-NPs caused similar types of lesion to the gill, liver, intestine and spleen tissues but Cu was more severity damage than Cu-NPs in those organs.
- Gill, liver, intestine and spleen are considered target organs for dissolved Cu and Cu-NPs.
- Dissolved Cu and Cu-NPs caused similar alteration in the component cells of the immune system but the level of alteration was more with Cu than Cu-NPs.

Acknowledgments

Dr. Ted Henry, Mike Hockings (histology) and Andy Atfield (spleen print staining) is greatly appreciated.

Histopathological Study of the Effect of Dissolved Copper and Copper Nanoparticles on Some organs of Rainbow Trout (*Oncorhynchus mykiss*)

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Background

- Increasing use of copper and copper nanoparticles (NPs) is likely to results in release of these pollutants into aqueous environment; however, it is unclear what types of lesions these materials cause in fish.
- Ecotoxicological Studies revealed that some types of engineered nanoparticles are toxic to fish (1).
- Gill, liver, spleen, and intestine are considered target organs for dissolved Cu with some toxic effects; including oxidative stress, osmoregulatory disturbances and tissue injury (2).

Objectives

The aim of this study was to determine the effects of dissolved Cu and Cu-NPs on organ integrity in trout; and to compare the effects of Cu metal with Cu-nanoparticles to identify any nano-specific pathologies.

Methods

- Fish were exposed to 0, 20, or 100 µg/L of Cu as $CuSO_4.5H_2O$ and Cu-NPs for up to 10 days using a semi-static exposure regime with no feeding during the experiment.
- Water quality parameter were measured daily.
- Sample of tissues were carried out on day 0, 4, and 10 for histology process.

Results

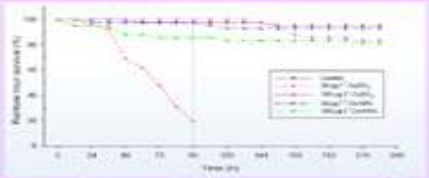

Figure 1. Survival of rainbow trout exposed to 20 or 100 µg l^{-1} Cu NPs or $CuSO_4$ for up to 10 days. Blue dashed line indicates end of 96 h exposure and termination of 100 µg l^{-1} $CuSO_4$ treatment only.

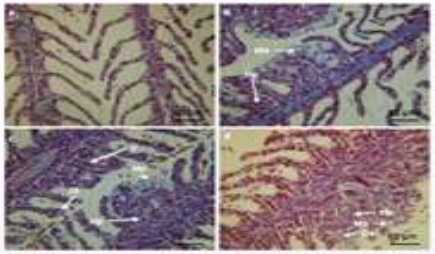

Figure 2. Gill morphology in trout following exposure to (a) control, (b) 20 µg/L $CuSO_4$, (c) 20 µg/L Cu-NPs and (d) 100 µg/L Cu-NPs for 10 days. (Oe) oedema, (Ct) club tips, (An) lamellar aneurism, (Ms) swollen mucocyte, (Hp) hyperplasia and (F) Fusion.

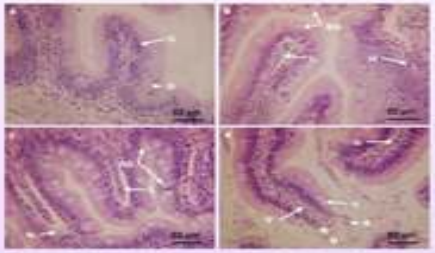

Figure 3. Intestine morphology in trout following exposure to (a) control, (b) 20 µg/L $CuSO_4$, (c) 20 µg/L Cu-NPs and (d) 100 µg/L Cu-NPs for 10 days. (C) columnar cell, (G) goblet cell, (L) lifting epithelium, (SG) swelling of goblet cells, (H) hyperplasia, (V) vacuoles in the lamina propria and lining epithelium, (N) necrosis in the lining epithelium layer and (E) erosion of villi.

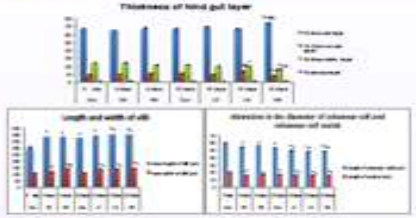

Figure 4. Alteration that observed in gut region following exposure to Cu and Cu NPs for 4 and 10 days. Data are shown mean ± S.E.

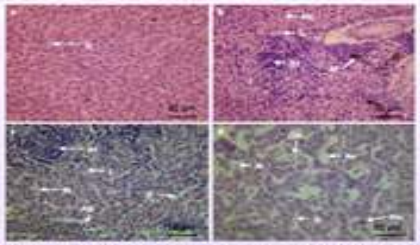

Figure 5. Liver morphology in trout following exposure to (a) control, (b) 20 µg/L $CuSO_4$, (c) 20 µg/L Cu-NPs and (d) 100 µg/L Cu-NPs for 10 days. (S) sinusoid space, (L) lipidosis, (Pn) pyknotic nuclei, (M) foci of melanomagrophage, (Av) aggregation of vacuoles, (Ab) aggregation of blood cell in sinusoid space, (IH) inflammatory hepatitis, (N) necrosis, (Le) lifting endothelium of blood vessel and (Oe) oedema

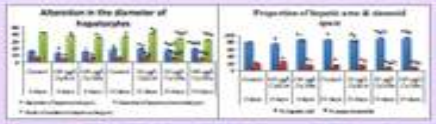

Figure 6. Alteration that observed in the liver following exposure to Cu and Cu NPs for 4 and 10 days. Data are shown mean ± S.E.

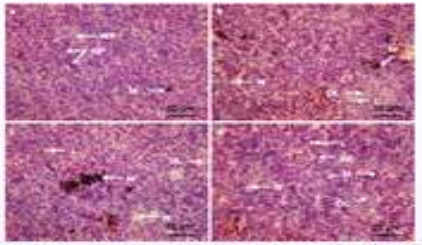

Figure 7. Spleen morphology in trout following exposure to (a) control, (b) 20 µg/L $CuSO_4$, (c) 20 µg/L Cu-NPs and (d) 100 µg/L Cu-NPs for 10 days. (RP) red and (WP) white pulp, (SE), swollen erythrocyte, (N) necrosis, (L) lipidosis, (M) foci of melanomacrophage and (DL) depletion of lymphoid tissues.

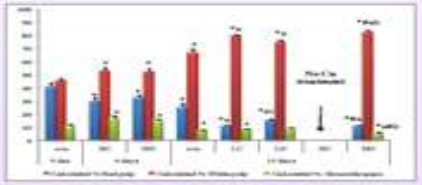

Figure 8. Changes in the proportions of red and white pulp in the spleens of trout following exposure to dissolved Cu (20, 100 µg/L) and Cu-NPs (20, 100 µg/L) for 0, 4 and 10 days.

Conclusions

- Both Cu treatments were toxic to rainbow trout with dissolved Cu more toxic than Cu-NPs.
- Cu and Cu-NPs caused similar types of lesion to the gill, liver, intestine and spleen tissues but Cu was more severity damage than Cu-NPs in those organs.
- Gill, liver, intestine and spleen are considered target organs for dissolved Cu and Cu-NPs.

References

(1) Handy *et al.* (2008). Ecotoxicology, 17, 390- 409.
(2) Osman *et al.* (2009). Research Journal of Environmental Toxicology, 3 (1), 9-23.

This work was supported by the Iraqi government

Histopathological Study of the Effect of Bulk or Titanium Dioxide Nanoparticles on Some Organs of Rainbow Trout (*Oncorhynchus mykiss*)

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Background

❖ Engineered nanoparticles (NPs) are used widely in consumer products and exposure modelling predicts releases of ng to low µg l^{-1} level of NPs into surface waters. Aquatic organisms (fish) may be affected by these particles.

❖ Ecotoxicological studies have demonstrated biochemical and ionoregulatory disturbance, as well as organ pathologies during aqueous exposure to TiO_2 NPs (1 & 2), but there is a gap in the information about the pathologies from ordinary bulk powder forms (micron scale) of the same materials.

Objectives

The aim of this study was to describe the organ pathologies in trout; and to compare the effects of bulk TiO_2 with TiO_2 NPs to identify any nano-specific pathologies.

Methods

❖ Juvenile rainbow trout were exposed to 1 mg l^{-1} of either bulk or TiO_2 NPs for up to 14 days using a semi-static exposure test with no feeding during the experiment.

❖ Water quality parameter were measured daily.

❖ Sample of tissues were carried out on day 0 and 14 for histology process.

Results

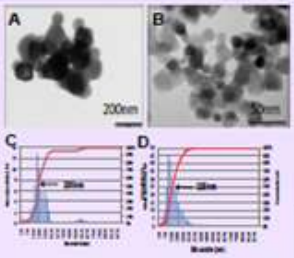

Figure 1. Electron microscope (EM) images and Nanosight graphs showing (A & B) EM images of bulk TiO_2 and TiO_2 NPs, respectively, scale bar = 200 and 50 nm in 1 g l^{-1} stock solution of bulk and TiO_2 NPs, respectively. (C & D) Nanosight graphs showing the distribution of bulk TiO_2 and TiO_2 NP in 1 mg l^{-1} stock solution respectively.

Figure 2. Gill morphology in trout after 14 days of a waterborne exposure to (a) control, (b) 1 mg l^{-1} bulk TiO_2 and (c) 1 mg l^{-1} TiO_2 NPs. Both treatments showed hyperplasia (Hp), aneurisms (An), clubbed tips (Ct), fusion (F), swollen mucocytes (SM) , and oedema (Oe).

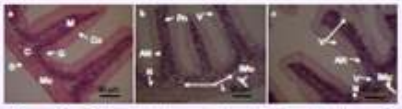

Figure 3. Gut morphology in trout after 14 days of exposure to (a) control, (b) 1 mg l^{-1} bulk TiO_2, (c) 1 mg l^{-1} TiO_2 NPs. Both treatments showed necrosis in lining epithelium layer (N), lifting epithelium (L), vacuolation in lining epithelium (V), pyknotic nuclei (Pn), increased thickness of muscular layer (IMu) and alteration in the nuclear shape (AN) compared to control columnar cell (Co) and goblet cell (G) in mucosa layer (M), compactum layer (C), muscular layer (Mu) and serosa layer (S) .

Figure 4. Liver morphology of trout following waterborne exposure to (a) control, (b) 1 mg l^{-1} of bulk and (c) 1 mg l^{-1} of TiO_2 NPs for up 14 days. The liver of control showed normal histology with normal sinusoid space (S). Both treatments showed injuries that include vacuolation (V), pyknotic nuclei (Pn), hepatitis (H) , foci of melanomacrophage deposition (M), and degeneration of hepatocytes (Dg) .

Figure 5. Spleen morphology in trout following exposure to (a) control, (b) 1 mg l^{-1} of bulk and (c) 1 mg l^{-1} of TiO_2 NPs for 14 days. The spleen of control fish showed normal histology, with defined red and white pulp as well as melanomacrophage deposits. Both treatments showed injuries that include decreased the proportion of red pulp (DRP), necrosis (N), depletion of lymphocyte (DL), vacuolation (V) and increased the number of melanomacrophage deposition (IM). The proportion of white pulp (IWP) was only increased with bulk. These injuries were greater with bulk than TiO_2 NPs.

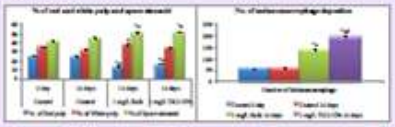

Figure 6. Alteration that observed in the proportion of red, white pulp and sinusoid space and the number of melanomacrophage deposition in the spleen. Data are mean ± S.E.M., n = 8. (*) Significant difference from initial fish with treatments (stock fish at time zero, ANOVA, $P < 0.05$). (a) Significant difference from control for day 14 with treatment (ANOVA or Kruskal-Wallis, $P < 0.05$). (#) Significant difference between bulk and TiO_2 NPs (*t* Test, $P < 0.05$).

Figure 7. Telencephalon brain morphology in trout following exposure to (a) control (b) 1 mg l^{-1} bulk, (c) 1 mg l^{-1} TiO_2 NPs for 14 days. All control groups showed normal structure of neuron (N) in telencephalon. Both treatments showed injuries that include pyknotic nuclei (P), swollen neuron (S), vacuolisation (V), appeared a lot of blood cell (B) and necrosis (NE). These injuries were more severe with bulk than TiO_2 NPs.

Figure 8. Cerebellum brain morphology in trout following exposure to (a) control (b) 1 mg l^{-1} bulk, (c) 1 mg l^{-1} TiO_2 NPs for 14 days. Control groups showed normal structure that include capsule (C), molecular layer (M) , purkinje layer (P), and granular layer (G). Both treatments showed injuries that include blood vessel abnormalities on the ventral surface (AB). These injuries were slightly more severe with bulk than TiO_2 NPs.

Conclusions

❖ Both bulk TiO_2 and TiO_2 NPs caused organs pathologies.

❖ Both treatments showed similar types of injuries in gill, liver, gut, spleen and brain tissues but in some organs, the bulk material produced slightly greater injuries than TiO_2 NPs.

❖ Gill, liver, gut, spleen and brain are considered target organs for bulk and TiO_2 NPs.

References

(1) Federici, G., Shaw, B. J. and Handy, R. D. 2007. Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): Gill injury, oxidative stress, and other physiological effects. *Aquatic Toxicology*, **84**, (4) 415-430.

(2) Hao, L., Wang, Z. and Xing, B. 2009. Effect of sub-acute exposure to TiO_2 nanoparticles on oxidative stress and histopathological changes in juvenile carp (*Cyprinus carpio*). *Journal of Environmental Sciences*, **21**, 1459-1466.

This work was supported by the Iraqi government

The Effect of pH on Copper accumulation from Copper Nanoparticles and Copper Sulphate in Rainbow Trout (*Oncorhynchus mykiss*)

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Introduction

- In aquatic organism, water pH is an important factor in copper (Cu) toxicity and accumulation. Hydrogen ions may compete for uptake with Cu ions, but pH also alters Cu speciation of the dissolved metal [1].
- Low pH in combination with high concentration of copper have been shown to cause mortality of fishes [2]. Acidic water pH is also associated with excessive mucus secretion and metal precipitation on the gills, as well as gill pathology in trout [3 & 4].
- Only a few recent studies have assessed the effects of copper nanoparticles (Cu-NPs) on fish, and details of the mechanisms of toxicity at neutral and acidic pH are currently unclear.

Aims

This study aimed to describe whether the toxicity of $CuSO_4$ is similar or less than Cu-NPs at neutral and acidic pH to the main body systems of rainbow trout.

Methods

- Juvenile rainbow trout were exposed in triplicate to either a control or 20 µg l^{-1} of either Cu as $CuSO_4$ or Cu-NPs at pH 7 and 5 (mean primary particle size at pH 7, 60 ± 2 and pH 5, 54 ± 1 nm) in a semi-static aqueous exposure regime for up to 7 days.
- Water quality parameters were measured daily.
- The lower pH was adjusted every 4 hours during 24 hours exposure by adding H_2SO_4.
- Sample of tissues were collected on day 0, 4 and 7 for metal analysis and histology.
- Dialysis experiment was performed separately to measure the dissolution rate of Cu from the Cu-NPs and $CuSO_4$ in Plymouth tap water at pH 7 and 5.

Results

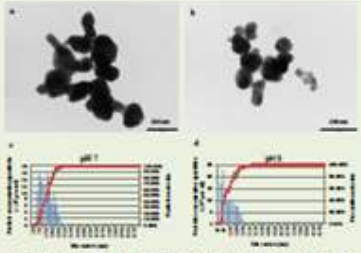

Figure 1. Particle size and particle size distribution and aggregation of 0.1 g l^{-1} Cu-NPs stock. Panels a and b are electron micrograph showing aggregated Cu-NPs with a mean primary particle size of 60 ± 2 at pH 7 and 54 ± 1 nm at pH 5 (mean ± S.E.M, n = 68 to123). Panels c and d show particle size distributions (NanoSight LM_{10}) with the concentration of particles at each size (vertical bars) and the percent of total particles at each size (line). A mean aggregate size of NPs at pH 7 was 143 ± 3 nm with a mode of 136 nm, and at pH 5 was 114 ± 11 nm with a mode of 97 nm.

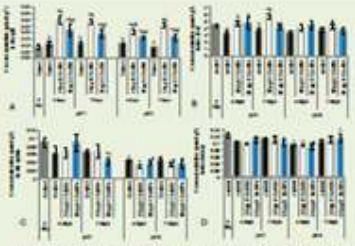

Figure 2. Cu concentrations in (a) gills, (b) liver, (c) spleen and (d) kidney of trout after 4 and 7 days of exposure at pH7 and 5. Data are mean ± S.E.M., dry weight tissue, n = 6 fish/treatment.*, indicates a statistically significant different from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). Different letter (a & b) denotes a statistically significant difference between treatment at the same time point and pH levels (ANOVA, $P < 0.05$). Σ, indicates a statistically significant different for the same treatment and pH level at the previous time point (time effects, ANOVA, $P < 0.05$). ▲, indicates a statistically significant different for the same treatment and time point at previous pH level (pH effects, ANOVA, $P < 0.05$). d, indicates a statistically significant different between $CuSO_4$ and Cu-NPs at the same pH level and time point (ANOVA, $P < 0.05$).

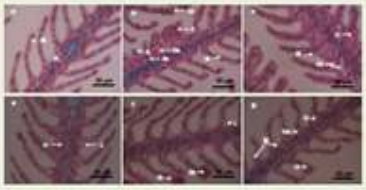

Figure 3. Gill morphology following exposure to (a) control (b) $CuSO_4$, (c) Cu-NPs at pH 7 and (e) control (f) $CuSO_4$ (g) Cu-NPs at pH 5 for 7 days. The gill of control groups showed a normal structure of primary (PL) and secondary lamellae (SL), with a minor lesion at pH 5. Exposure to $CuSO_4$ or Cu-NPs at pH 7 and 5 caused similar types of injuries that include clubbed tips (Ct), fusion (F), oedema (Oe), atrophy (A), aneurism (An), mucocytes (M), and hyperplasia (H). Scale bar indicates magnification (x400 µm), sections were 7 µm thick and stained with Mallory's trichrome.

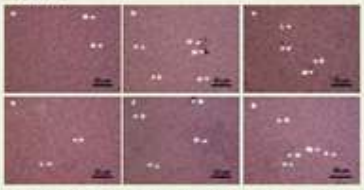

Figure 4. Liver morphology following exposure to (a) control (b) $CuSO_4$, (c) Cu-NPs at pH 7 and (e) control (f) $CuSO_4$ (g) Cu-NPs at pH 5 for 7 days. The liver of control groups showed a normal structure with sinusoid space (S) and small deposit of melanomacrophage (M). Both Cu treatments at pH 7 and 5 showed similar changes that include, lipidosis (L), occasional inflammatory cells like hepatitis (IF), cell with pyknotic nuclei (P), degeneration of hepatic cell (Dg). Scale bar indicates magnification (x400 µm), section were 7 µm thick and stained with H&E.

This work was supported by the Iraqi government

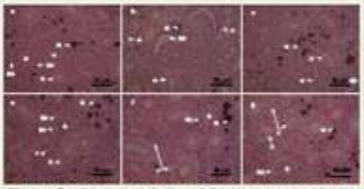

Figure 5. Kidney morphology following exposure to (a) control (b) $CuSO_4$, (c) Cu-NPs at pH 7 and (e) control (f) $CuSO_4$ (g) Cu-NPs at pH 5 for 7 days. Kidney of control fish showed a normal histology with parietal epithelium of Bowman's capsule (BC), glomerulus (G), Bowman's space (BS), renal tubules (R), haematopoietic tissues (H) and melanomacrophages deposits (M). Similar pathologies were observed with both Cu treatments at pH 7 and 5 that include necrosis (N), vacuolisation (V), and degeneration of renal tubule (D). Scale bar indicates magnification (x400 µm), section were 7 µm thick and stained with H&E.

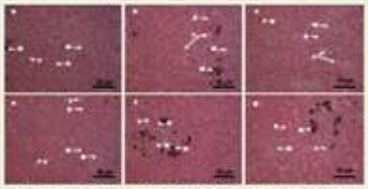

Figure 6. Spleen morphology following exposure to (a) control (b) $CuSO_4$, (c) Cu-NPs at pH 7 and (e) control (f) $CuSO_4$ (g) Cu-NPs at pH 5 for 7 days. The spleen of control groups showed a normal structure of white (W) and red (R) pulp, and melanomacrophage deposits (M), with a minor changes at pH 5. Both Cu treatments ($CuSO_4$ and Cu-NPs) at pH 7 and 5 showed changes that include vacuole formation (V) and increased the number of melanomacrophage deposits). Scale bar indicates magnification (x400 µm), section were 7 µm thick and stained with H&E.

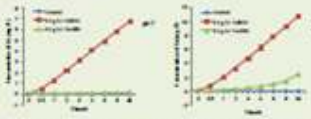

Figure 7. Measurement of dissolved Cu in the external medium of beakers at pH 7 and 5 during dialysis experiments. The result (mean of triplicates) show an increase in dissolution of Cu from Cu-NPs at pH 5 compared to pH 7.

Conclusions

- Cu accumulation was seen in the gills and liver with either $CuSO_4$ or Cu-NPs treatments at pH 7 and 5.
- Both $CuSO_4$ and Cu-NPs treatments at pH 7 and 5 showed organs pathologies.
- Both Cu treatments at pH 7 and 5 showed similar types of injuries in gill, liver, kidney and spleen, but these injuries were more severe at pH 5 than pH 7.
- Dialysis experiment showed increased release of dissolved Cu from both $CuSO_4$ and Cu-NPs at pH 5 compared to pH7.

References

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2. Çogun and Kargin (2004). *Chemosphere*, 55, 277-282.
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4. Chevalier, et al. (1966). *Canadian Journal of Zoology*, 63, 2062-2070.

The Effects of Waterborne Copper Nanoparticles and Copper Sulphate on rainbow trout (*Oncorhynchus mykiss*).

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Background & Aims

❖ Engineered nanoparticles (NPs) are used widely in consumer products and exposure modelling predicts releases of ng to low µg l^{-1} level of NPs into surface waters. Aquatic organisms (fish) may be affected by these particles.

❖ The toxicity of $CuSO_4$ to fishes is well known with injury to the gills, ionoregulatory disturbances, and other physiological effects during aqueous exposure. However, in comparison relatively little is known about the effects of nano Cu on fishes.

❖ The **aim** of this study was to compare the effects of Cu-NPs to equal concentrations of soluble Cu (as $CuSO_4$) during waterborne exposure to juvenile rainbow trout.

Methods

❖ Trout were exposed in triplicate to 0, 20 or 100 µg l^{-1} of either Cu as $CuSO_4$ or Cu-NPs (mean primary particle size, 87 ± 27 nm) in a semi-static aqueous exposure regime.

❖ Water quality was checked before and after daily water changes. Fish were sampled at days 0, 4, and 10 for tissue trace elements, haematology, biochemistry, and histology.

Results

Figure 1. Particle size distribution in 1 g l^{-1} Cu-NP stock. (a) Electron micrograph showing aggregated Cu-NPs with a mean particle size of 87 ± 27 nm (mean ± S.D., n = 50), and (b) Particle size distributions (NanoSight LM10) with a calculated mean aggregate size of 216 ± 122 nm and a mode of 48 nm (n = 6).

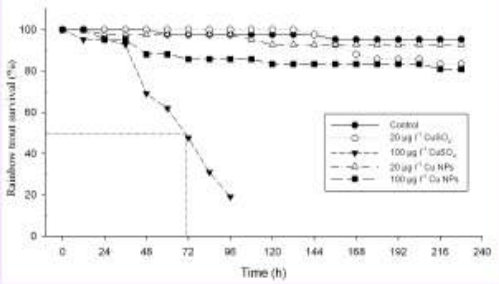

Figure 2. The effects of $CuSO_4$ and Cu-NPs on survival in rainbow trout. Data are mean of triplicate treatments (errors bars are not shown for clarity). Note the termination of the 100 µg l^{-1} $CuSO_4$ treatment after 4 days due to high mortality.

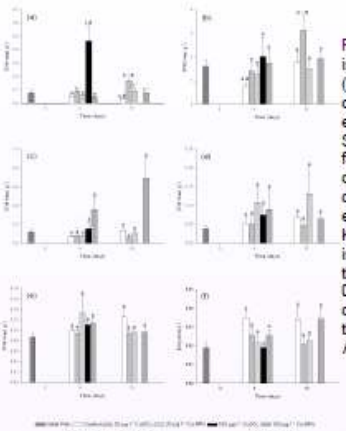

Figure 3. Copper concentrations in (a) gills; (b) liver; (c) intestine; (d) spleen; (e) brain; (f) muscle of trout after 4 and 10 days of exposure. Data are mean ± S.E.M., dry weight tissue, n = 6 fish/treatment. Different letter denotes a statistically significant difference between treatments at each time point (ANOVA or Kruskal–Wallis, $P < 0.05$); # indicates significantly different than initial fish (ANOVA, $P < 0.05$), * indicates significantly different from same treatment at the previous time point (ANOVA, $P < 0.05$).

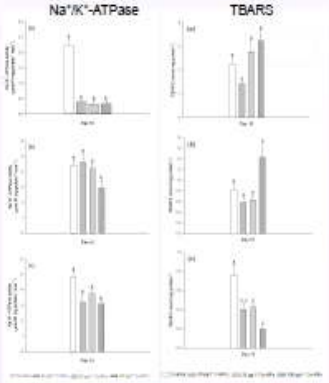

Figure 4. Na^+/K^+-ATPase activity (left panels) and TBARS (right panels) in crude homogenates from (a) gills; (b) intestine; (c) brain of trout after 10 days of exposure to 0 (clear bar), 20 µg l^{-1} $CuSO_4$ (grey bar), 20 µg l^{-1} Cu-NPs (white bar with diagonal lines), or 100 µg l^{-1} Cu-NPs (grey bar with diagonal lines). Data are mean ± S.E.M., n = 6 fish/treatment. Different letters indicate significant differences between treatments within tissues (ANOVA or Kruskal-Wallis, $P < 0.05$).

Figure 5. Gill morphology in the gill of trout following waterborne exposure to (a) control, (b) 20 µg l^{-1} Cu as $CuSO_4$, (c) 20 µg l^{-1} Cu as Cu-NPs and (d) 100 µg l^{-1} Cu as Cu-NPs at day 10. The gills of control fish showed normal histology, whilst all treatments showed injuries that include oedema (Oe), clubbed tips (Ct), mucocytes swollen (Ms), hyperplasia (Hp) and fusion (F). These injuries were greater with Cu-NPs than $CuSO_4$ at day 10 with some types of lesion. Scale bar indicates magnification; sections were 7 µm thick and stained with Mallory's trichrome.

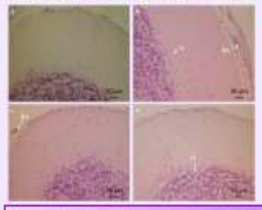

Figure 6. Cerebellum morphology in the brain of rainbow trout following waterborne exposure to (a) control, (b) 20 µg l^{-1} of Cu as $CuSO_4$, (c) 20 µg l^{-1} of Cu as Cu-NPs, or (d) 100 µg l^{-1} of Cu as Cu-NPs for 10 days. The cerebellum of control fish showed normal histology. All Cu-treatments showed similar types of injuries, but the severity was worse for the equivalent Cu-NP exposure by day 10. Injuries include blood vessel abnormality on the ventral surface (BA), necrosis between the molecular and granular layers (N). Section were 7 µm, stained with H&E.

Conclusion

Overall, Cu-NPs have similar types of toxic effects to $CuSO_4$, which can occur at lower tissue Cu concentrations than expected for the dissolved metal.

Reference: Shaw et al. (2012) Aquatic Toxicology, 116-117, 90-101.

G. Al-Bairuty was funded by a PhD studentship from the Iraqi Government, and the experiments were supported by a NERC grant to R. Handy (NE/G001812/1).

Effects of Waterborne Exposure to Dissolved Copper or Copper Nanoparticles on the Spleen of Juvenile Rainbow Trout (*Oncorhynchus mykiss*)

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Introduction

- Data on the immunotoxicity of metal-containing nanoparticles (Cu-NPs) compared to ordinary forms of dissolved copper ($CuSO_4$) is scarce, and the spleen is implicated as an important target organ.
- The spleen is considered one of the main haematopoietic organs in fishes [1] and has a role in removing foreign material and damaged blood cells from the circulation.
- The proportions of red and white pulp in the spleen are known to change following exposure to environmental pollutants and NPs [2,3].
- However, changes in splenic red pulp in trout following waterborne TiO_2 exposure have been suggested to arise from a haematological response to systemic hypoxia [3], highlighting some non-immune functions of the spleen and the potential for secondary toxicity not associated with metal accumulation in the spleen.

Aims

This study aimed to investigate the toxicity of dissolved Cu (as $CuSO_4$) compared to Cu-NPs on the spleen of rainbow trout as well as the immunological response by using spleen prints.

Methods

- Juvenile rainbow trout were exposed to control (no added Cu), 20 or 100 µg/L of either Cu as $CuSO_4.5H_2O$ or Cu-NPs for 0, 4, and 10 days using a semi-static exposure regime with no feeding during the experiment.
- Water quality parameter were measured daily.
- Sample of tissues were carried out on day 0, 4, and 10 for histology and day 4 and 10 for spleen prints.

Results

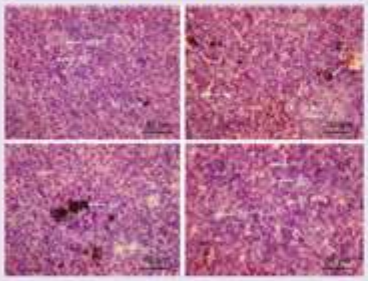

Figure 1. Spleen morphology in rainbow trout following exposure to (a) control, (b) 20 µg l^{-1} of Cu as $CuSO_4$, (c) 20 µg l^{-1} of Cu as Cu-NPs and (d) 100 µg $l^{-1}$of Cu as Cu-NPs for 10 days. Spleen from control fish showed normal histology, with defined red (RP) and white (WP) pulp. At day 10, all treatments showed similar types of injuries. These injuries include occasional necrotic cell (N), depletion of lymphoid tissues (DL), foci of melanomacrophage (M), vacuole formation (V), and swollen erythrocyte (SE). Scale bar indicates x 400 magnification; sections were 7 µm thick and stained with H & E.

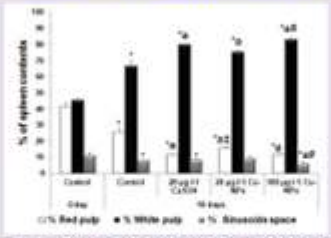

Figure 2. Changes in the proportions of red & white pulp and sinusoids space in the spleen of rainbow trout exposed to $CuSO_4$ (20 µg l^{-1}) and Cu-NPs (20 or 100 µg l^{-1}) for 0 and 10 days. Data are proportion means ± S.E.M., n = 6 fish/treatment. *, significant difference from initial fish (stock fish at time zero, ANOVA, $P < 0.05$). a, significant difference from control within treatment (ANOVA, $P < 0.05$). #, significant difference from the previous Cu-NPs concentration (dose effect, ANOVA, $P < 0.05$). ‡, significant difference between the 20 µg l^{-1} of $CuSO_4$ and Cu-NPs (material-type effects, Hest, $P < 0.05$). Note the termination of the 100 µg l^{-1} $CuSO_4$ treatment after 4 days for ethical reasons.

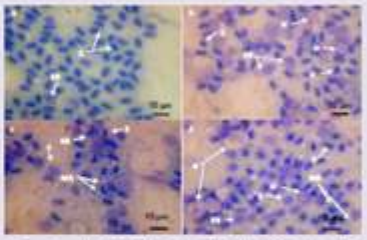

Figure 3. Spleen prints of rainbow trout following exposure to dissolved Cu as $CuSO_4$ and Cu-NPs for 10 days. (a) control, (b) 20 µg l^{-1} of $CuSO_4$, (c) 20 µg l^{-1} of Cu-NPs (d) 100 µg l^{-1} of Cu-NPs. Both Cu treatments showed similar changes in the haematopoietic cells in the spleen. Erythroblast (EB), erythrocyte (E), neutrophil (N), swollen cell (S), haemocytoblast (H), cell with membrane abnormality (MA), cells with a dividing nuclei (D). Scale bar indicate magnification. Smear stained with May-Grunwald Giemsa.

Table 1 Quantitative analysis of the proportion of haematopoietic cells in the spleen prints of rainbow trout that exposed to 20 and 100 µg l^{-1} of either dissolved Cu as $CuSO_4$ and Cu-NPs for 4 and 10 days.

% of haematopoietic cells	Time /day	Treatments				
		Control	20 µg l^{-1} $CuSO_4$	20 µg l^{-1} Cu-NPs	100 µg l^{-1} $CuSO_4$	100 µg l^{-1} Cu-NPs
Haemocytoblast	4	4.4 ± 0.4 (6)	2.8 ± 0.4 (6) *	4.3 ± 0.2 (6) ‡	5.0 ± 1.2 (3) #	4.3 ± 0.4 (6)
	10	5.5 ± 0.3 (6)	5.6 ± 0.4 (6) x	6.3 ± 0.4 (6) x	-	5.0 ± 0.2 (6) #
Erythroblast	4	7.8 ± 0.4 (6)	6.8 ± 0.4 (6)	7.9 ± 0.5 (6)	20.2 ± 3.4 (3) *#	12.8 ± 0.7 (6) *+#
	10	13.5 ± 1.0 (6) x	11.3 ± 0.4 (6) x	12.9 ± 0.5 (6) x		12.9 ± 0.2 (6)
Progranulocyte	4	2.4 ± 0.1 (6)	0.9 ± 0.1 (6) *	1.3 ± 0.4 (6) *	0.5 ± 0.3 (3) *	1.3 ± 0.2 (6) *+
	10	1.3 ± 0.2 (6) x	1.4 ± 0.2 (6)	1.3 ± 0.1 (6)	-	1.5 ± 0.2 (6)
Erythrocyte	4	79.8 ± 0.7 (6)	82.3 ± 0.8 (6)	82.0 ± 0.4 (6)	70.7 ± 3.4 (3) *#	76.1 ± 0.9 (6) *+#
	10	77.5 ± 1.3 (6)	73.8 ± 0.9 (6) *x	74.8 ± 0.6 (6) x	-	75.2 ± 0.6 (6)
Lymphocyte	4	Not observed (6)	5.0 ± 0.7 (6) *	1.9 ± 0.5 (6) *‡	1.8 ± 0.4 (3) #	1.8 ± 0.4 (6) *
	10	Not observed (6)	3.3 ± 0.6 (6) *x	0.8 ± 0.4 (6) ‡		0.7 ± 0.2 (6)
Neutrophil	4	5.1 ± 0.3 (6)	1.8 ± 0.5 (6) *	2.7 ± 0.3 (6) *	1.5 ± 0.5 (3) *	3.4 ± 0.2 (6) *+
	10	2.2 ± 0.3 (6) x	3.5 ± 0.2 (6) *x	3.2 ± 0.7 (6)		3.9 ± 0.2 (6) *
Macrophage	4	0.3 ± 0.2 (6)	0.4 ± 0.2 (6)	0.5 ± 0.2 (6)	Not observed (3)	Not observed (6)
	10	Not observed (6)	0.4 ± 0.2 (6)	0.3 ± 0.1 (6)		0.4 ± 0.2 (6)

Data are mean proportion ± S.E.M. *, significant difference between control and treatments within a time point (ANOVA, $P < 0.05$). #, significantly difference between low and high concentration of either $CuSO_4$ or Cu-NPs at the same time point (concentration effects, ANOVA, $P < 0.05$). ‡, significantly difference between low concentration of $CuSO_4$ and Cu-NPs at the same time (material-type effects, ANOVA, $P < 0.05$). +, significantly difference between high concentration of $CuSO_4$ and Cu-NPs at the same time (material-type effects, ANOVA, $P < 0.05$). x, significantly difference between day 4 and 10 within treatments (time effects, ANOVA, $P < 0.05$). Note that there are no data for 100 µg l^{-1} $CuSO_4$ treatment at day 10 due to the stop of this treatment at day 4 following high mortality.

Table 2 Quantitative analysis of abnormal erythrocytes in the spleen prints of rainbow trout that exposed to 20 and 100 µg l^{-1} of either dissolved Cu as $CuSO_4$ or Cu-NPs for 4 and 10 days.

% of erythrocyte abnormalities	Time /day	Treatments				
		Control	20 µg l^{-1} $CuSO_4$	20 µg l^{-1} Cu-NPs	100 µg l^{-1} $CuSO_4$	100 µg l^{-1} Cu-NPs
Swollen blood cells	4	1.1 ± 0.3 (6)	4.8 ± 0.3 (6) *	3.9 ± 0.4 (6) *	3.7 ± 0.4 (3) #*	2.8 ± 0.5 (6) *#
	10	1.0 ± 0.3 (6)	4.3 ± 0.2 (6) *	3.8 ± 0.6 (6) *	-	2.3 ± 0.4 (6) #
Small cell size	4	Not observed (6)	0.8 ± 0.5 (6)	1.7 ± 0.6 (6) *	1.5 ± 0.3 (3) *	1.4 ± 0.4 (6) *
	10	Not observed (6)	1.7 ± 0.2 (6) *	2.1 ± 0.2 (6) *		1.6 ± 0.2 (6) *
Cells with a dividing nucleus	4	9.8 ± 1.0 (6)	8.1 ± 0.5 (6)	8.5 ± 0.3 (6)	10.3 ± 2.2 (3)	7.3 ± 0.7 (6) *+
	10	7.2 ± 0.2 (6) x	10.8 ± 1.8 (5) *x	9.5 ± 0.7 (6)	-	7.8 ± 0.3 (6)
Cells with membrane abnormalities	4	1.8 ± 0.2 (6)	2.5 ± 0.3 (6)	3.7 ± 0.3 (6) *‡	6.7 ± 0.6 (3) *#	4.9 ± 0.3 (6) *#+
	10	2.8 ± 0.2 (6)	7.4 ± 0.4 (6) *x	7.0 ± 0.5 (6) *x	-	5.3 ± 0.5 (6) *#x

The same label as explained in Table 1.

Conclusions

- Dissolved Cu and Cu-NPs caused similar types of pathologies and alteration in the proportion of erythropoiesis as well as immune response in the spleen.
- Dissolved Cu has slightly more effect on the spleen than Cu-NPs.
- Both dissolved Cu and Cu-NPs may present an immunotoxic hazard to trout.

References

- (1) Handy et al. (2011). Journal of Fish Biology 79, 821-853.
- (2) Handy et al. (2002). Ecotoxicology 11, 467-479.
- (3) Boyle et al. (2013). Aquatic Toxicology 126, 116-127.

This work was supported by the Iraqi government

Appendix C: Published papers

Journal of Fish Biology (2011) **79**, 821–853
doi:10.1111/j.1095-8649.2011.03080.x, available online at wileyonlinelibrary.com

REVIEW PAPER

Effects of manufactured nanomaterials on fishes: a target organ and body systems physiology approach

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(Received 11 October 2010, Accepted 6 July 2011)

Manufactured nanomaterials (NM) are already used in consumer products and exposure modelling predicts releases of ng to low μg l^{-1} levels of NMs into surface waters. The exposure of aquatic ecosystems, and therefore fishes, to manufactured NMs is inevitable. This review uses a physiological approach to describe the known effects of NMs on the body systems of fishes and to identify the internal target organs, as well as outline aspects of colloid chemistry relevant to fish biology. The acute toxicity data, suggest that the lethal concentration for many NMs is in the mg l^{-1} range, and a number of sublethal effects have been reported at concentrations from *c.* 100 μg to 1 mg l^{-1}. Exposure to NMs in the water column can cause respiratory toxicity involving altered ventilation, mucus secretion and gill pathology. This may not lead, however, to overt haematological disturbances in the short term. The internal target organs include the liver, spleen and haematopoietic system, kidney, gut and brain; with toxic effects involving oxidative stress, ionoregulatory disturbances and organ pathologies. Some pathology appears to be novel for NMs, such as vascular injury in the brain of rainbow trout *Oncorhynchus mykiss* with carbon nanotubes. A lack of analytical methods, however, has prevented the reporting of NM concentrations in fish tissues, and the precise uptake mechanisms across the gill or gut are yet to be elucidated. The few dietary exposure studies conducted show no effects on growth or food intake at 10–100 mg kg^{-1} inclusions of NMs in the diet of *O. mykiss*, but there are biochemical disturbances. Early life stages are sensitive to NMs with reports of lethal toxicity and developmental defects. There are many data gaps, however, including how water quality alters physiological responses, effects on immunity and chronic exposure data at environmentally relevant concentrations. Overall, the data so far suggest that the manufactured NMs are not as toxic as some traditional chemicals (*e.g.* some dissolved metals) and the innovative, responsible, development of nanotechnology should continue, with potential benefits for aquaculture, fisheries and fish health diagnostics.

Key words: behaviour; blood cells; cardiovascular system; gastrointestinal tract; nanoparticles; nervous system.

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Aquatic Toxicology 116–117 (2012) 90–101

Contents lists available at SciVerse ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Effects of waterborne copper nanoparticles and copper sulphate on rainbow trout, (*Oncorhynchus mykiss*): Physiology and accumulation

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ARTICLE INFO

Article history:
Received 20 December 2011
Received in revised form 22 February 2012
Accepted 24 February 2012

Keywords:
Fish
Nanoparticles
Copper toxicity
Copper accumulation
Ionic regulation
TBARS
Sodium

ABSTRACT

Emerging data suggests that some types of nanoparticles (NPs) are toxic to fish, and given the well-known toxicity of dissolved metals, there are also concerns about whether metal-containing NPs present a similar or different hazard to metal salts. In this study, juvenile rainbow trout were exposed in triplicate to either a control, 20 or 100 $\mu g l^{-1}$ of either Cu as $CuSO_4$ or Cu-NPs (mean primary particle size, 87 ± 27 nm) in a semi-static aqueous exposure regime. Fish were sampled at days 0, 4, and 10 for tissue trace elements, haematology, and biochemistry. By day 4, fish from the 100 $\mu g l^{-1}$ Cu as $CuSO_4$ treatment showed 85% mortality (treatment subsequently terminated) compared to 14% in the 100 $\mu g l^{-1}$ Cu-NP exposed fish. Mortality at day 10 was 4, 17, 10, and 19% in the control, 20 $\mu g l^{-1}$ Cu as $CuSO_4$, 20 and 100 $\mu g l^{-1}$ Cu-NP treatments, respectively. Copper accumulation was seen in the gills of fish from all Cu treatments, and was statistically significant in both $CuSO_4$ treatments at day 4 and all Cu treatments at day 10 compared to controls. No statistically significant Cu accumulation was seen in the spleen, brain or muscle of fish from any treatment, although an elevation in intestinal Cu was seen in the high Cu-NP treatment throughout. There were some transient changes in haematology and depletion of plasma Na^+ that was treatment-related, with some differences between the nano form and metal salt, but Cu-NPs were not overtly haemolytic. A 6-fold decrease in branchial Na^+/K^+-ATPase activity in all Cu treatments (compared to controls), depletion of plasma and carcass ion concentrations suggest that Cu-NPs are an ionoregulatory toxicant to rainbow trout. Statistically significant decreases in Na^+/K^+-ATPase activity were also seen in the brains and intestine, and whilst there was no material-type effect in the former, this was only observed in the gut of fish exposed to 100 $\mu g l^{-1}$ Cu-NPs. There were material-dependent changes in tissue thiobarbituric acid reactive substances (TBARS), and in the gill the Cu-NPs caused a larger (though non-significant compared to control) increase in TBARS than the equivalent metal salt treatment (the latter actually being significantly reduced compared to all other treatments). Overall, these data show that Cu-NPs have similar types of toxic effects to $CuSO_4$, which can occur at lower tissue Cu concentrations than expected for the dissolved metal.

1. Introduction

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

Following waterborne exposure, dissolved copper from metal salts tends to accumulate initially in the gills, reflecting the route of exposure, and then in the internal organs (e.g., Stagg and Shuttleworth, 1982; Grosell et al., 1997; McGeer et al., 2000). The

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0166-445X/$ – see front matter © 2012 Published by Elsevier B.V.
doi:10.1016/j.aquatox.2012.02.032

Aquatic Toxicology 126 (2013) 104–115

Contents lists available at SciVerse ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Histopathological effects of waterborne copper nanoparticles and copper sulphate on the organs of rainbow trout (*Oncorhynchus mykiss*)

Genan A. Al-Bairuty[a], Benjamin J. Shaw[a], Richard D. Handy[a,*], Theodore B. Henry[a,b]

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ARTICLE INFO

Article history:
Received 31 July 2012
Received in revised form 3 October 2012
Accepted 7 October 2012

Keywords:
Copper nanoparticles
Gill pathology
Renal pathology
Brain injury
Hypoxia
Copper toxicity
Fish

ABSTRACT

It is unclear whether copper nanoparticles are more toxic than traditional forms of dissolved copper. This study aimed to describe the pathologies in gill, gut, liver, kidney, brain and muscle of juvenile rainbow trout, *Oncorhynchus mykiss*, exposed in triplicate to either a control (no added Cu), 20 or 100 $\mu g l^{-1}$ of either dissolved Cu (as $CuSO_4$) or Cu-NPs (mean primary particle size of 87 ± 27 nm) in a semi-static waterborne exposure regime. Fish were sampled at days 0, 4, and 10 for histology. All treatments caused organ injuries, and the kinds of pathologies observed with Cu-NPs were broadly of the same type as $CuSO_4$ including: hyperplasia, aneurisms, and necrosis in the secondary lamellae of the gills; swelling of goblet cells, necrosis in the mucosa layer and vacuole formation in the gut; hepatitis-like injury and cells with pyknotic nuclei in the liver; damage to the epithelium of some renal tubules and increased Bowman's space in the kidney. In the brain, some mild changes were observed in the nerve cell bodies in the telencephalon, alteration in the thickness of the mesencephalon layers, and enlargement of blood vessel on the ventral surface of the cerebellum. Changes in the proportional area of muscle fibres were observed in skeletal muscle. Overall the data showed that pathology from $CuSO_4$ and Cu-NPs were of similar types, but there were some material-type effects in the severity or incidence of injuries with Cu-NPs causing more injury in the intestine, liver and brain than the equivalent concentration of $CuSO_4$ by the end of the experiment, but in the gill and muscle $CuSO_4$ caused more pathology.

1. Introduction

The pathological effects of waterborne copper, and target organs for Cu toxicity are reasonably well known in fish (Wilson and Taylor, 1993; Handy, 2003; Grosell et al., 2007; Mustafa et al., 2012). The gill is considered the main route for waterborne Cu uptake, and injuries include oedema, epithelial lifting and fusion of the lamellae during acute toxicity (Sola et al., 1995), and changes in the proportions of epithelial cells and mucocytes are noted in chronic exposure (Dang et al., 1999). For dietary Cu exposure, the gill does not show acute pathology, but instead, changes can occur in the intestinal mucosa including intestinal cell proliferation and apoptosis (Berntssen et al., 1999). The liver is the central compartment for handling Cu in teleost fish (Grosell et al., 1996), and Cu is excreted in the bile. However, waterborne (Baker, 1969; Figueiredo-Fernandes et al., 2007) or dietary (Handy et al., 1999) exposure to Cu can produce liver pathology in fishes. There are concerns that Cu may be immunotoxic, although studies on the haematopoietic system of trout with Cu are limited, melanomacrophage aggregates are noted in trout kidney during Cu exposure (Handy, 2003).

Copper is also known to effect excitable tissue such as nerve and muscle. Additions of Cu alter the electrical properties of epithelia (e.g., Na channels in frog skin, Flonta et al., 1998), and while excess Cu in the CNS results in brain pathology in mammals (e.g., Wilsons disease, Menkes, 1999), and vacuole formation in the brain of trout has been observed from dietary Cu exposure (Handy, 2003). Fish do show altered behaviour and locomotor activity during Cu exposure (Handy et al., 1999; Campbell et al., 2002), but the relative contributions of CNS and skeletal muscle pathology to such events is less clear.

Relatively recently, a new form of Cu metal has been engineered comprising of Cu nanoparticles (Cu-NPs), which are one type of metal-containing nanomaterials (review on "nano metals", Shaw and Handy, 2011). However, despite an emerging body of literature on the ecotoxicity of nanomaterials (Moore, 2006; Handy et al., 2008; Klaine et al., 2008; Kahru and Savolainen, 2010; Handy et al., 2011), less attention has been given to organ pathologies in aquatic species. Some evidence is emerging on Cu-NPs in fish and waterborne exposure to high concentrations (1.5 $mg l^{-1}$ Cu-NPs for 48 h) caused oedema in the gills of zebrafish (Griffitt et al., 2007). Other nanomaterials also cause gill pathology (TiO_2 NPs, Federici et al.,

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http://dx.doi.org/10.1016/j.aquatox.2012.10.005

Aquatic Toxicology 126 (2013) 116–127

Contents lists available at SciVerse ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Subtle alterations in swimming speed distributions of rainbow trout exposed to titanium dioxide nanoparticles are associated with gill rather than brain injury

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ARTICLE INFO

Article history:
Received 26 July 2012
Received in revised form 5 October 2012
Accepted 9 October 2012

Keywords:
Locomotion
Oncorhynchus mykiss
Hypoxia
Bioenergetics
Nanomaterials
Neurotoxicity

ABSTRACT

The effects of engineered nanomaterials on fish behaviours are poorly understood. The present study aimed to determine the locomotor behaviours of trout during waterborne exposure to titanium dioxide nanoparticles (TiO_2 NPs) as well as inform on the underlying physiological mechanisms involved. Trout were exposed to either control (without TiO_2), 1 mg l^{-1} TiO_2 NPs or 1 mg l^{-1} bulk TiO_2 for 14 days. Titanium dioxide exposure resulted in 31 (bulk) and 22 fold (nano) increases in the Ti concentrations of gill tissue compared to controls, but there were no measurable increases of Ti in the internal organs including the brain. Gill pathologies were observed in both TiO_2 treatments. Locomotor behaviours were quantified using video tracking software and the proportion of time spent swimming at high speed (>20 cm s^{-1}) was significantly decreased in fish exposed to TiO_2 NPs, compared to controls, but not fish exposed to bulk TiO_2. The shift in swimming speed distribution in the TiO_2 NP-exposed fish was associated with decreased area of red pulp in the spleen, increases in haematocrit and whole blood haemoglobin, all consistent with a compensation for respiratory hypoxia without the accumulation of plasma lactate. Fish exposed to TiO_2 NPs also retained competitive abilities when paired with controls in aggressive social encounters. The duration of competitive contests, the level of aggression and contest outcome were not affected by NP exposure. Neurological injury did not explain the changes in locomotor behaviour, although there was some apparent enlargement of the blood vessels on the brain. Whole brain homogenates showed a statistically significant increase in oxidative stress defences such as the total glutathione pool, but without loss of Na^+K^+-ATPase or acetylcholinesterase activities.

1. Introduction

The ecotoxicity of engineered nanomaterials (ENMs) has been the subject of intense research in the last five years (reviews, Moore, 2006; Handy et al., 2008; Klaine et al., 2008; Kahru and Dubourguier, 2010; Klaine et al., 2012). Concerns have been raised about the effects of nano metals on fishes (Shaw and Handy, 2011), and also on the sublethal effects of ENMs on the different body systems of fish (Handy et al., 2011). In general, sparingly soluble metal or metal oxide ENMs are acutely toxic to fishes at high mg l^{-1} concentrations, with sublethal effects on body systems occurring at concentrations around 1 mg l^{-1} or less (Handy et al., 2011).

Sublethal effects associated with exposure to TiO_2 nanoparticles (TiO_2 NPs) in the laboratory are documented for some body systems in fishes. These include damage to the gill epithelium and some loss of sinusoid space with foci of lipidosis in the liver of rainbow trout (*Oncorhynchus mykiss*) during 14 day exposures to 1 mg l^{-1} TiO_2 NPs (Federici et al., 2007). Alterations of the proportions of red and white pulp in the spleen have also been observed in trout, with concerns for the function of the haematopoietic system (Handy et al., 2011). Renal function appears to be largely unaffected by injections of TiO_2 NPs in trout (Scown et al., 2009). However, the effects of ENMs on the central nervous system of fishes are poorly understood (see discussion in Handy et al., 2011). The brain is known for its lack of tolerance to hypoxia and sensitivity to oxidative stress. In the brain of carp (*Cyprinus carpio*) TiO_2 NPs caused dose-dependent increases in thiobarbituric acid reactive substances (TBARS), a marker of lipid peroxidation (Hao et al., 2009). Changes in TBARS were also reported in whole brain homogenates from rainbow trout exposed to 1 mg l^{-1} TiO_2 NPs (Federici et al., 2007). There are also concerns for the electrical properties of the nervous system, with

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http://dx.doi.org/10.1016/j.aquatox.2012.10.006

Appendix D: Diploma supplement

Diploma Supplement

The University of Plymouth

DIPLOMA SUPPLEMENT

INFORMATION IDENTIFYING THE HOLDER OF THE QUALIFICATION

Surname
Al-Bairuty

First Name(s)
Genan

Date of Birth
10/04/1971

Student Reference Number
10208279

INFORMATION IDENTIFYING THE QUALIFICATION

Name of Qualification and title conferred
PhD

Title of thesis
Histopathological effects of metal and metallic nanoparticles on the body systems of rainbow trout (*Oncorhynchus mykiss*)

Names of Supervisors
Richard Handy

Name(s) of collaborating institution(s)
University of Plymouth

Language of thesis
English

INFORMATION ON THE LEVEL OF THE QUALIFICATION

Level of the Qualification
PhD

Mode of study (full time/part time/mixed mode)
Full time

Official length of the programme
4 years

Completion date
19/6/2013

FURTHER INFORMATION

Professional Status

Official Stamp of the Graduate School

Diploma supplement Appendix

RESEARCH SKILLS

Subject Specific Skills Training completed

Course Title	**Module Code**	**Date Attended**	**Performance Pass/Dist or %**	**Credit rating and level**
English language support for international students feature of academic style	(ELSIS)	12-26th October 2009		
Gas and Liquid Nitrogen Training		29th April 2009		
Postgraduate Research Skills and method coursework	BIO 5124	30th June 2010	76%	20
Training school for young researchers in Impact assessment of nanomaterials in vitro and in vivo testing strategies		12-14th October 2011	Full attendance	Certificate
General Teaching Associates Course	GTA	12-16th April 2010	Full attendance	Certificate
Plymouth consortium Student Associate Scheme	SAS	2010/2011	Full attendance	Certificate
Practical Techniques in the Molecular Biology Workshop	PG Society	16-19th July 2012	Full attendance	Certificate

Verified by Director of Studies

Name …….. Signature …… Date ……

Generic Skills Training completed

Course Title	Joint Research Councils Skills Statement category	Date Attended	Location of Course
Home office licence- 1-3 fish module- licence gained for fish species		30-31st March 2010	University of Plymouth

Verified by Graduate School Skills Development Officer

Name …….. Signature …… Date ……

Other Skills Training completed (e.g UKGRAD School; English/Foreign Language) and/or conferences/meetings attended

Title	Location	Organisers	Date Attended
Preparing effective poster presentations	Rolle building 211	Alison stokes	2/10/2009
Getting started in office 2007	Babbage building 322	Jacqui Hunter	3/10/2009
Greating basic web pages with Microsoft office share point designer 2007	Babbage building 322	Liz McGregor	20/10/2009
Research ethics	Babbage building 005	John Eddison	20/10/2009
Excel 2007- Database and filtering	Babbage building 322	Jacqui Hunter	21/10/2009
A stress management workshop for research student	Roland levinsky 304	Sarah Kearns and other	23/10/2009
Methodology in science	Scott building 001	J. Eddison	23/10/2009
PowerPoint 2007: Enhancing your presentation	Babbage building 322	Gary	27/10/2009
Safety and risk assessment field work	Babbage building 005	Paul Ramsay	27/10/2009
Introduction to pebble pad	Babbage building 322	Liz McGregor	28/10/2009
Excel 2007 Introduction to essential features	Babbage building 322	Gary Prize	29/10/2009
Safety and risk assessment laboratory	Babbage building 410	Andy Foey	30/10/2009
Preparing for your viva	Rolle building 207	Mick Fuller	2/11/2009
Scientific writing	Babbage building 410	P. Ramsay	3/11/2009
Publication strategies	SHC1 (lower)	P. Ramsay	3/11/2009

Creating graphics using paint shop pro photo X2	Babbage building 322	Sarah	4/11/2009
Critical review of writing	Rolle building 008	Pathway leaders	5/11/2009
Excel 2007/ Lookup tables and pivol table reports	Babbage building 322	Jacqui Hunter	11/11/2009
Latex introduction	Rolle building 210	Martin Coath	12/11/2009
Working with literature: electronic resources and advanced searching	Babbage building 106-107	Graham Titley	16/11/2009
Biostatistics 1	Babbage building 005	M. Franco	24/11/2009
Biostatistics 2	Babbage building 106-107	M. Franco	27/11/2009
The transfer process	Rolle building 211	Mick Fuller	27/11/2009
Introduction to endnote- for the science, technology, medical and health disciplines	Babbage Building 112	IT Staff	1/12/2009
Project management and funding environment	Babbage building 005	Ted Henry	1/12/2009
Careers in Biology	SCB 001	M. Burns	4/12/2009
Biostatistics 3	Babbage Building 005	M. Franco	8/12/2009
Poster presentations	SHC1 (lower)	Rad Blackshaw	11/12/2009
Oral presentations	Babbage Building 005	Piero Calosi	15/12/2009
Public communication of science	SCB 001	M. Donkin	18/12/2009
Introduction to qualitative research	Babbage Building 210	Debby Cotton	15/01/2010
Introduction to Spss part 1	Babbage Building 111	Paul Hewson	22/1/2010
Essential tools for the viva: debating skills	Cookworthy Building 404	Stephen Farrow	26/1/2010
Developing Professional Writing Skills	Rolle Building Room 115	IT Staff	3/2/2010
Power point-creating a presentation	Babbage Building Room 322	Jacqui Hunter	10/2/2010
Endnote under clinic	Library Room 114	IT staff	18/2/2010
A Doctor A. S.A.P.	Babbage Building Tamar committee room	Chris Wood	19/2/2010

Going Global	Babbage Building Tamar committee room	Chris Wood	19/3/2010
Preparing to transfer	Rolle building Room 114	Mick Fuller	19/3/2010
Microsoft PowerPoint 2007 Enhancing your Presentation	Babbage Building Room 322	Jacqui Hunter	23/3/2010
Developing Professional Writing Skills	Rolle Building Room 116	Joe Allison	24/3/2010
SPSS part 2	Babbage Building Room 112	Paul Hewson	27/4/2010
Impact factor	Rolle Building Room 117	Chris Wood	30/4/2010
Microsoft excel 2007 conditional formatting & charts	Babbage Building Room 322	Jacqui Hunter	10/6/2010
Effective reading	Smeaton building room 107 B	Eloíse Sentito and John Hilsdon	10/11/2010
A stress management workshop for research students	Babbage Building Room 411	IT.Training	19/11/2010
Introduction to Endnot X3	Babbage Building Room 111	Jayne moss Graham Titley	30/11/2010
Greating Graphics for paintshop pro	Babbage Building Room 322	Sarah (IT training)	3/12/2010
Words: creating form	Babbage Building Room 322	Sarah Barnes	10/12/2010
Microsoft excel conditional formatting and charts	Babbage Building Room 322	Jacqui hunter	2/2/2011
Presenting to an Audience (part 1)	Portland Square Room C4	Sarah Kearns & Terri Rees	9/3/ 2011
Professional writing skill	Rolle building Room 115	Joe Allison	6/4/2011
Writing up and completing the thesis	Roland Levinsky Building, Room 304	John Hilsdon	5/7/2012

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Name …….. Signature …… Date ……

Diploma supplement Appendix (cont)

RESEARCH OUTPUTS

Seminar/Conference/Performance presentations

Title of Paper or Performance	**Title of Meeting & location**	**Poster or Oral paper**	**Date**	**Published* or not published**
Effects of dissolved copper and nanocopper on histopathology and haematopoietic organs of rainbow trout (*Oncorhynchus mykiss*).	Society of Environmental Toxicology and Chemistry, U.K. branch annual meeting, London.	Poster presentation	13-14th September 2010	Published (Abstract)
Histopathological and Haematopoietic Study of the Effect of Dissolved copper and Copper Nanoparticles on some organs of rainbow trout (*Oncorhynchus mykiss*).	3rd Annual Conference, Spirit of Discovery Plymouth's diverse marine and maritime research, University of Plymouth, Roland Levinsky Building, Plymouth, U.K.	Poster presentation	20th December 2010	Published (Abstract)
Effect of dissolved copper and nano copper on histopathology and haematopoietic organs of rainbow trout (*Oncorhynchus mykiss*).	3rd NanoImpactNet Conference, "Building a Bridge from NanoImpactNet to Nanomedical Research" Lausanne, Switzerland.	Poster presentation	14-17th February 2011,	Published (Abstract)
Histopathological study of the effect of bulk or titanium dioxide nanoparticles on some organs of rainbow trout (*Oncorhynchus mykiss*).	6th International Conference on the Environmental Effects on Nanoparticles and Nanomaterials, The Royal Society, London.	Poster presentation	19th-21st September	Published (Abstract)
The effect of pH on copper accumulation from copper nanoparticles and	Ecotoxicology Research and Innovation Centre,	Poster presentation	13th July 2012	Published (Abstract)

$CuSO_4$ in rainbow trout (*Oncorhynchus mykiss*).	University of Plymouth, 2nd Annual, Plymouth lecture theatre in the Portland Square building, Plymouth Campus, Plymouth, U.K			
Effects of waterborne exposure to dissolved copper or copper nanoparticles on the spleen of juvenile rainbow trout (*Oncorhynchus mykiss*).	Society of Environmental Toxicology and Chemistry, SETAC Europe 23rd annual meeting, "Building a better future: Responsible innovation and environmental protection" , Glasgow, U.K.	Poster presentation	12 -16th May 2013	Published (Abstract)
Effects of manufactured nanomaterials on fishes: a target organ and body systems physiology approach	Journal of Fish Biology	Journal paper	79**,** 821-853. 2011	Published
Effects of waterborne copper nanoparticles and copper sulphate on rainbow trout, (*Oncorhynchus mykiss*): Physiology and accumulation	Aquatic Toxicology	Journal paper	116-117, 90-101. 2012	Published
Histopathological effects of waterborne copper nanoparticles and copper sulphate on the organs of rainbow trout (*Oncorhynchus mykiss*)	Aquatic Toxicology	Journal paper	126, 104-115. 2013	Published
Subtle alterations in swimming speed distributions of rainbow trout exposed to titanium	Aquatic Toxicology	Journal paper	126, 116-127. 2013	Published

dioxide nanoparticles are associated with gill rather than brain injury				

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Name …….. Signature …… Date ……

PUBLISHED REFEREED/EDITED PUBLICATIONS

* Give the full bibliographic reference (including Journal ISSN No., DOI (Digital Object Identifier) and Web address if appropriate)

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