INVESTIGATIONS OF WATERBORNE METAL INTERACTONS IN THE GILLS OF RAINBOW TROUT USING SYNCHROTRON-BASED TECHNIQUES

A Thesis Submitted to the College of

Graduate and Postdoctoral Studies

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

In the Toxicology Graduate

Program, University of

Saskatchewan Saskatoon,

Saskatchewan, Canada By

Yusuf O. Saibu

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis. Requests for permission to copy or to make other use of material in this thesis, in whole or part, should be addressed to:

Chair of the Toxicology Graduate
Program Toxicology Centre
University of
Saskatchewan Saskatoon,
Saskatchewan Canada,
S7N 5B3

ABSTRACT

Most of our current understanding of metal toxicity in aquatic organisms has been based on single metal toxicity. However, metals do not exist singly in the aquatic environment, but rather as mixtures. As mixtures, they are subject to complex interactions for uptake and/or intracellular handling. An enhanced understanding of intracellular metal interactions could help in developing predictive models (e.g., biotic ligand model) for assessing the toxicity of metals in mixture. To this end, I adopted a suite of synchrotron-based techniques to investigate the acute interactions of waterborne Zn, Cd and Cu in binary mixtures *in situ* at the gills of a model teleost species, rainbow trout (*Oncorhynchus mykiss*). Gills (the biotic ligand) of freshwater fish are primary site of toxic action for metals during acute waterborne exposures, and thus were chosen as the target tissue for this investigation. Micro X-ray fluorescence imaging (μ-XRF) and micro X-ray absorption near-edge spectroscopy (μ-XANES) were employed to investigate: (i) the spatial distribution and chemical speciation of Zn and its co-localization pattern with other essential elements (Ca, S and Fe), (ii) the effect of competing metals (Cd and Cu) on Zn distribution and speciation in the gills of rainbow trout. In addition, Fourier transform infrared micro-spectroscopy (FTIRM) was employed to examine the effects of Zn, Cd and Cu, singly and in binary mixtures, on biochemical constituents (e.g., proteins, lipids and nucleic acids) of the gills of rainbow trout. Fish (~200g) were exposed to acutely toxic concentrations (1x 96h LC_{50}) of each metal, alone and in binary combination, in dechlorinated municipal water for 24h. Following exposure, gills were dissected and 10 μm thick sections were prepared and analyzed at the VESPERS and Mid-IR beamlines of the Canadian Light Source. My findings indicated that Zn accumulated in high proportions in the primary lamellae (the primary site of mitochondria-rich (chloride) cell localization) of the fish gill. Zinc was also found to

predominantly co-localize with Ca and S, but not with Fe, indicating that Ca and S binding intracellular ligands play a crucial role in Zn handling in the gill. The distribution of Zn in the gill was markedly reduced during co-exposure to Cd, but not to Cu, suggesting a competitive interaction between Zn and Cd for uptake. Exposure to Zn, alone and in combination with Cd or Cu, was found to decrease Ca from the gill tissue. The speciation of Zn in the gill was dominated by Zn-phosphate, Zn-histidine and Zn-cysteine; however, the interactions of Zn with Cd or Cu resulted in the loss of Zn-cysteine. The composition of the biochemical constituents of the fish gill was also found to be altered by the exposure to metals, both individually and in binary mixture, and this was apparent both on the primary and secondary lamellae. These alterations were mainly characterized as degradations of proteins and lipids. Generally, exposure to Cu, alone and in mixture with Cd, was found to induce maximum adverse effects in the fish gill, which was followed by exposure to Cd and Zn alone. Interestingly though, the adverse effects of Cu and/or Cd in the gill were ameliorated by the presence of Zn in the co-exposure. Collectively, my findings indicated that the interactions of Zn and Cd or Cu in the fish gill could occur *via* both common and disparate mechanisms, and their interactions elicit antagonistic toxicity in the fish gill.

DEDICATION

This thesis is dedicated to the loving memory of my mother, Mrs. Sherifat Saibu. Words can not fully describe how much I miss you. Thank you for the motherly love and care you bestowed on me. I couldn't have been me without you. Thank you for nurturing me into a responsible and conscientious man that I have become. May your soul continue to rest in peace.

ACKNOWLEDGEMENTS

I am profoundly grateful to my supervisor Dr. Som Niyogi for taking me as his student and for providing me with the opportunity to pursue my MSc degree in his laboratory. His relentless support and encouragement enabled me to complete this thesis. I would also like to thank my committee members, Dr. Mark Wickstrom, Dr. Derek Peak and Dr. Markus Hecker for their comments, guidance and constructive criticism, which greatly improved the scope of my research. Thanks are also due in great measure to my external examiner, Dr. Juan Ianowski for taking the time to review my thesis and attend my Defense.

The completion of this thesis wouldn't have come to fruition without the financial support from the Natural Sciences and Engineering Research Council of Canada (NSERC), Copper Development Association Inc., Copper Alliance and Niperia. Appreciations are greatly extended to these sponsors. Special thanks to the Toxicology Centre for also providing me financial assistance through the bestowment of the Toxicology Graduate Devolved Scholarship. I would also like to express my gratitude to the Canadian Light Source (CLS) and specifically the staff of VESPERS and Mid-IR beamlines for introducing me to the world of synchrotron and for teaching me how to operate and troubleshoot their beamlines for seamless data collection. In addition, I would like to thank Dr. Peter Blanchard, Dr. Renfei Feng, Dr. Saroj Kumar, and Dr. Rachid Lahlali for their advice in analyzing the data.

It would have been impossible to complete this research without the support of my family. I acknowledge their substantial efforts towards my education. I thank them for being there for me, encouraging and supporting me. I would also like to say thank you to my fiancée, Nafeesah, for her support and patience during the course of writing this thesis. I am also thankful to Ankur Jamwal for being a good partner in progress and for his assistance during running of

my experiments and collection of data at the CLS. Last but not the least, I also wish to express my appreciation to the other members of Niyogi's lab group, Kamran Shekh, Melissa Driessnack and Mohammed Naderi, for their support and encouragement during the course of my program.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAS	Atomic absorption spectroscopy
ATRF	Aquatic Toxicity Research Facility
ATSDR	Agency for Toxic Substances and Disease Registry
BLM	Biotic ligand model
CLS	Canadian Light Source
CTR-1	Copper transporter 1
DMT-1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
DOM	Dissolved organic matter
DWAF	Department of Water Affairs and Forestry
ECaC	Epithelial calcium channel
ENaC	Epithelial sodium channel
EU	European Union
FPA	Focal plane array
FTIR	Fourier transform infrared spectroscopy
FTIRM	Fourier transform infrared microspectroscopy
g	gram
GSH	Glutathione
hr	hour
ICP-MS	Inductive coupled plasma mass spectrometry
IR	Infrared radiation
L	Litre
LC_{50}	Lethal concentration to kill 50% of a population
LCF	Linear combination fitting
mg/L	milligram per litre
MRCs	Mitochondria-rich cells
MRGs	Metal-rich granules

MS-222	Tricaine Methanesulfonate
MT	Metallothionein
OCT	Optimal cutting temperature
OECD	Organisation for Economic Co-operation and Development
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDD	Silicon drift detector
US EPA	United States Environmental Protection Agency
UNEP	United Nations Environmental Programme
VESPERS	Very Sensitive Elemental and Structural Probe Employing Radiation
WHO	World Health Organization
XANES	X-ray absorption near-edge spectroscopy
XFI	X-ray fluorescence imaging
^{o}C	Degrees centigrade
µg/L	Microgram per litre
µg/g	Microgram per gram
µ-XANES	Micro X-ray absorption near-edge spectroscopy
µ-XFI	Micro X-ray fluorescence imaging

NOTE TO READERS

The contents of this thesis have been organized and formatted in a manner that follows the guidelines put forth by the University of Saskatchewan College of Graduate and Postgraduate Studies for a manuscript-style thesis. Chapter 1 is a general introduction, which comprises a brief literature review along with a description of the project objectives. Chapters 2 and 3 of this thesis are written and organized into manuscripts intended for future publication in *Aquatic Toxicology* and *Metallomics*, respectively. Chapter 4 entails a general discussion and the overall conclusions conveyed by the data. Therefore, certain sections of each chapter will contain information that has been stated previously in other areas of this thesis.

The author contributions for each research chapter include:

Yusuf Saibu (University of Saskatchewan, Canada) designed and executed all of the experiments, carried out most of the data analysis, and wrote the manuscript drafts.

Dr. Som Niyogi (University of Saskatchewan, Canada) provided comments and editorial corrections for manuscripts in addition to scientific guidance, direction and funding for this project.

Dr. Derek Peak (University of Saskatchewan, Canada) and Dr. Saroj Kumar (Upssala University, Sweden) provided guidance, corrections and comments pertaining to analysis of the data.

Dr. Renfei Feng (Canadian Light Source) provided assistance with data collection at the Canadian Light Source.

Ankur Jamwal (University of Saskatchewan, Canada) provided assistance with experimental design, as well as sample and data collection.

1.0 GENERAL INTRODUCTION

1.1 Metal Pollution in the Aquatic Environment

The aquatic environment is constantly exposed to a variety of metals, which are released into receiving water bodies via point and non-point sources. Metals are naturally present in the geosphere but in recent times, anthropogenic activities have resulted in increased concentrations of these metals in the environment, including aquatic systems, where they can accumulate and cause toxicity in sensitive aquatic organisms such as fish (Nriagu and Pacyna, 1988).

Pollution of water bodies by metals is an issue of global concern, and this has received a considerable attention in recent times due to their bioaccumulative and persistent properties, toxicity to aquatic organisms, as well as potential harmful effects on humans that rely on aquatic resources for the sustenance of life (Has-Schön et al., 2006). Ambient water quality criteria have been developed in many parts of the world to protect aquatic life. This has evolved from the measurement of total metal concentrations to the more recent approach of employing the biotic ligand model (BLM) (Paquin et al., 2002; Reiley, 2007). The BLM is a mechanistic tool used to predict site-specific acute and/or chronic toxicities of metals in aquatic ecosystems (Di Toro et al., 2001; Niyogi and Wood, 2004; Paquin et al., 2002), and it has been successfully adopted by the USEPA to set freshwater quality criteria for Cu (USEPA, 2007), and by the European Union (EU) for risk assessment of metals such as Cu, Ni, and Zn (Bodar et al., 2005). The BLM combines physiology of metal uptake by the fish gill and water chemistry to predict toxicity, and its underlying concept is that "toxicity occurs as a result of metal accumulation at the physiological active sites of action" (Niyogi and Wood, 2003). These sites of action are also known as the biotic ligands (e.g., fish gill).

At present, the BLM and most other risk assessment methods of metals in the aquatic environment are essentially based on single metal toxicity. However, metals rarely exist singly in

the aquatic environment but rather as complex mixtures (Taylor, 2008; Vijver et al., 2011). For the better protection of aquatic life, it is therefore desirable that metal mixture risk assessment methods be developed. A better understanding of metal mixture toxicity in fish would be a significant step towards improving the risk assessment of metal mixture in the aquatic environment.

The metals focused on in this thesis were zinc (Zn), copper (Cu) and cadmium (Cd). These metals are ubiquitous in aquatic systems and often co-occur together (ATSDR, 2004; UNEP, 2010). Their typical concentrations in freshwater systems range from 0.02 - 50μg/L, 0.2 - 30μg/L and less than 0.5μg/L for Zn, Cu and Cd, respectively (Bodar et al., 2005; Naito et al., 2010; USEPA, 2007). The 96hr median lethal concentrations (LC_{50}) of these metals in rainbow trout (*Oncorhynchus mykiss*), a sensitive teleost often used for regulatory toxicity testing, range from 410 - 7210μg/L, 17 - 5100μg/L, and 2.10 – 1160μg/L for Zn, Cu and Cd, respectively, depending on water chemistry parameters and exposure conditions (USEPA, 2016). In highly contaminated aquatic ecosystems, the concentrations of these metals have been found to be well above twice the lethal levels (96h LC_{50s}) (DWAF, 1998; Oryx, 2000). The release of these metals into the aquatic environment comes from both natural and anthropogenic sources. However, inputs from anthropogenic sources are much more significant. The major anthropogenic release of these metals into the aquatic environments comes from mining and smelting, while release from natural sources comes from volcanic eruption and rock weathering (Nriagu and Pacyna, 1988).

The major factor that determines the bioavailability and toxicity of these metals in aquatic organisms is speciation, i.e. the physical or chemical form in which an element exists. Free metal ions are the most bioavailable and, by extension, the most toxic metal species (Wood, 2011). Speciation of metals in freshwater aquatic systems is highly influenced by water chemistry

variables such as hardness, alkalinity, pH and dissolved organic matter (DOM). Cations that contribute to water hardness such as Ca^{2+} and Mg^{2+} along with other cations such as Na^{+}, K^{+}, and H^{+} offer a protective effect against metal toxicity by competing with metal ions for binding to the biotic ligands. Similarly, alkalinity (i.e. $HCO3^{-}$ and $CO3^{2-}$), which co-varies with hardness and inorganic anions such as chlorides, sulphates, sulphides, carbonates, bicarbonates, also mitigate metal toxicity by complexing metal ions, thereby decreasing the free metal ions available for uptake at the biotic ligands (Wood, 2011). DOM is also another factor that influences metal speciation in the aquatic ecosystems. Specifically, DOM is the greatest single factor that influences Cu speciation (Grosell, 2011). DOM complexes Cu with a high affinity, thereby reducing its uptake at the biotic ligands (Playle et al., 1993; Richards et al., 2001; Wood, 2011). Metals are also usually more toxic at low pH than high pH. Low pH increases the concentration of free metal ions by keeping metals in soluble form, thereby increasing toxicity (Wood, 2011).

1.2 Mechanism of Metal Uptake in Fish Gill

The gill is the primary route of metal uptake in fish during waterborne exposure. It is a multi-functional organ responsible for gaseous exchange, nitrogenous waste excretion, acid-base balance and ionic regulation (Evans et al., 2005). The surface of the gill is composed of negatively charged proteins, which attract positively charged metal ions. These proteins include several ion transporters, channels, carriers, enzymes, and exchangers (Niyogi and Wood, 2003).

Metals such as Zn, Cu, and Cd usually gain entry across fish gills, especially during acute exposure, through a process known as ionic mimicry (Bury et al., 2003; Büsselberg, 1995). This is the process by which metals disguise as essential ions (such as Ca^{2+} and Na^{+}), and thus gain entry across fish gills via the transport pathways of these ions. For example, it has been shown that

Cd and Zn are both Ca analogues, and are able to gain entry across fish gills through the epithelial calcium channel (ECaaaC) (Hogstrand et al., 1996; Niyogi et al., 2008). The basolateral transport of these metals after entry through this channel could take place via the ZNT family for Zn transporters and/or via the Ca^{2+}- ATPase and Na^{+}/Ca^{2+} exchanger for both Zn and Cd (Hogstrand et al., 1996; Verbost et al., 1989) (see Figure 1.1). Similarly, Cu has been shown to mimic Na and is thus able to gain apical entry across fish gills through the epithelial sodium channel (ENaC), and the basolateral efflux occurs via the Cu^{2+} -ATPase (Bury et al., 2003). The promiscuous divalent metal transporter, DMT-1, which is essentially an Fe transporter has also been implicated in the apical transport of Cu and Cd in fish gills (Bury and Grosell, 2003). However, it worthy to note that of these two metals, DMT-1 has a higher affinity for Cd than Cu (Garrick et al., 2006) (see Figure 1.1).

Since Zn and Cu are essential metals, they also have specialized transport systems for their absorption from the water. These transport systems include the ZIP and ZNT families for the apical and basolateral transport of Zn (Hogstrand, 2011), and CTR1 and Cu^{2+} -ATPase for the apical and basolateral transport of Cu, respectively (Grosell, 2011). These transport systems are characterized by high affinity and high specificity for Zn and Cu transport. Uptake via these pathways is usually more pronounced during the conditions of low metal concentrations in aquatic systems (Wood, 2011) (see Figure 1.1).

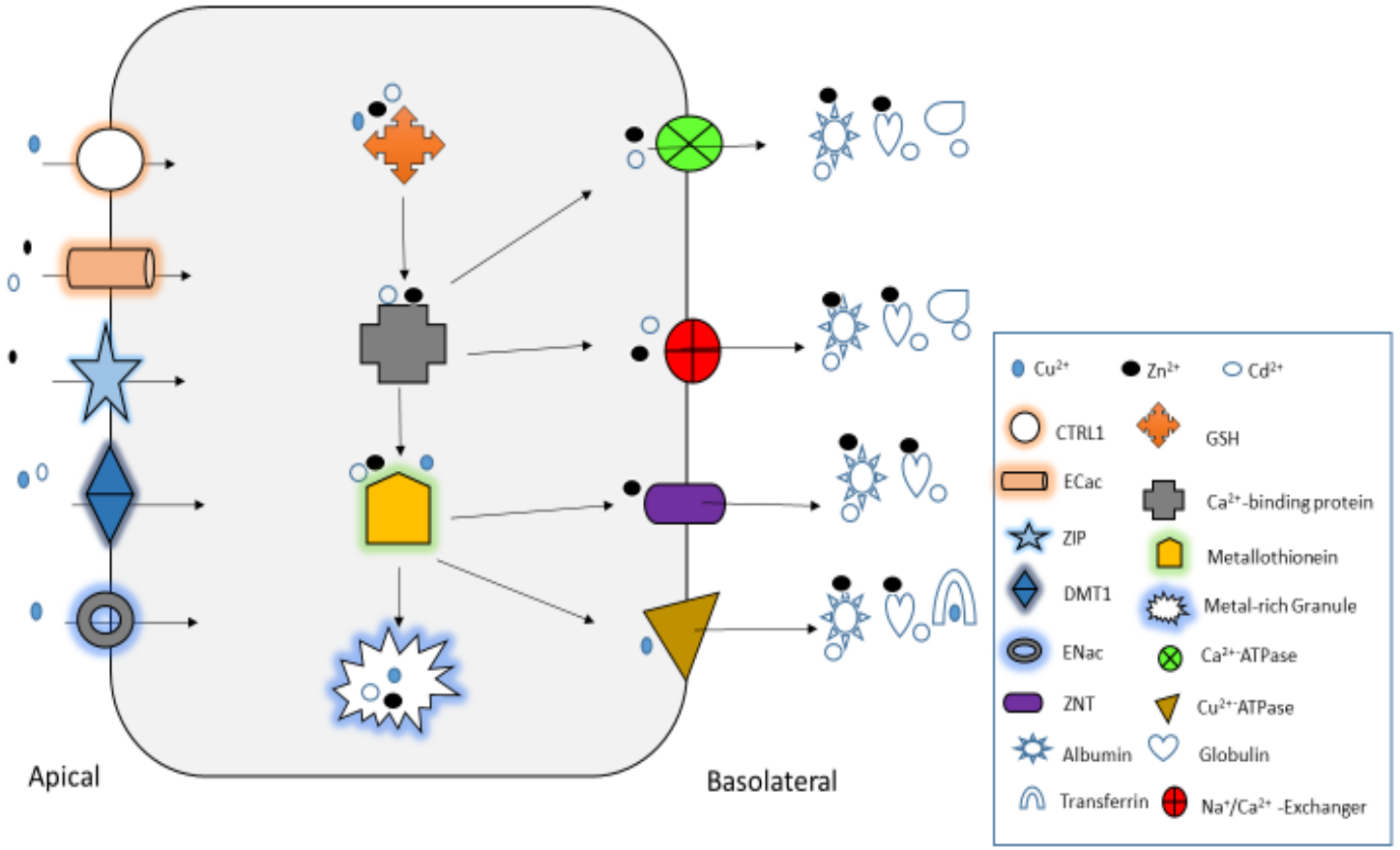

Figure 1.1: A schematic representation of the potential sites of interactions between Zn, Cd and Cu, both at the transport sites of fish gills and within the intracellular environment. Note that some of the mechanisms displayed in the diagram are hypothesized based on information reported in mammalian models. [Modified from (McGeer et al., 2011)]

1.3 Internal Handling of Metals in Fish

Free metal ions are potentially toxic within the intracellular environment, which prefers to be in a reduced state. Thus, free metal concentrations need to be kept low within the cytosol to prevent toxicity. On entry into the epithelial cells, metals are often bound to low molecular weight thiols such as metallothionein, glutathione, and cysteine (Glynn, 1996; Olsson and Hogstrand, 1987), as well as calcium binding proteins such as calbindin (Zalups and Ahmad, 2003). Unbound metals gain entry into the bloodstream where they are transported by plasma proteins to various organs in the fish. Cu has specific carrier protein in the form of ceruloplasmin, which is involved in the distribution of Cu into target tissues of fish (Grosell, 2011). Unlike Cu, Zn does not have a specific carrier protein. Rather, its transport from the plasma to different tissues is mediated by a specific set of Zn transporters whose expression patterns regulate Zn uptake in different tissues (Hogstrand, 2011). Nonetheless, most of the Zn in the plasma binds to albumin (Blindauer et al., 2009), and the rest to α2-macroglobulin (Inagaki et al., 2000). Transport of Cd to various organs in fish is mediated by the carrier protein, transferrin in conjunction with albumin and metallothionein (McGeer et al., 2011).

Metals can be stored in target organs of fish such as gill both temporarily and permanently. Metallothioneins (MTs) and glutathione (GSH) are the two primary metal binding moieties involved in temporary storage, while metal-rich granules (MRGs) are largely involved in the permanent storage and detoxification of metals (Wood, 2011). MTs are cysteine-rich, low molecular weight metal-binding proteins which were first isolated as a Cd-binding protein from horse kidney (Margoshes and Vallee, 1957). Since then, their occurrence has been demonstrated in all vertebrates, including fish (Olsson et al., 1998; Roeva et al., 1999). Apart from being involved in Cd binding, MTs have also been implicated in the binding of other metals such as Cu,

Hg, Ag and Zn (Kägi and Kojima, 1987). MTs are involved in the homeostatic regulation of essential metals such as Zn and Cu (Roesijadi, 1996; Viarengo and Nott, 1993), and also play an important role in the detoxification of non-essential metals such as Cd, Hg and Ag (Amiard et al., 2006).

GSH is a tripeptide consisting of cysteine, glycine, and glutamate. It occurs in copious amount (mM) intracellularly, but its affinity to metals is less than that of MTs (Wood, 2011). In addition to being involved in the storage and binding of metals, MTs and GSH are also parts of the defense system against oxidative stress. They mitigate oxidative stress in the cells by scavenging reactive oxygen species (ROS) (Chiaverini and De Ley, 2010; Ercal et al., 2001).

MRGs are metal binding moieties that complex metals and precipitate them as insoluble phosphate and sulfide. They were initially thought to be present only in invertebrates, but their presence has now also been demonstrated in fish (Goto and Wallace, 2010) (see Figure 1.1)

1.4 Mechanisms of Metal Toxicity in Fish: Importance of the Gill

The gill in fish is perpetually in contact with toxic substances in the water and is often the first organ to respond to metal stress. At acutely toxic concentrations, metals induce pathophysiological responses in fish gills. Such responses include lamellar fusion, necrosis, edematous swelling, cellular lifting, increased water-blood diffusion distance and impeded blood and water flow through the respiratory lamellae. All these pathological responses hinder respiration in fish, eventually leading to death. However, it is worthy to note that these responses are not specific to metals, as other classes of contaminants have been shown to induce the same responses in fish gills (Mallatt, 1985). A specific mechanism of acute toxicity of metals to freshwater fish is through the disruption of ionic transport and homeostasis, and this has severe

implications on fish physiology. The internal environment in freshwater fish is hypertonic to the surrounding external waters and as such they are often faced with the problem of diffusive loss of ions and water gain across the gills through osmosis. To maintain ionic homeostasis, freshwater fish have developed transport systems, which are present on the surface of the gills for the active uptake of essential ions such as Ca^{2+} and Na^{+} from the external waters. In addition, they also produce a large amount of dilute urine. The apical transport of Ca^{2+} across fish gills takes place through the epithelial calcium channel (ECaC), and the basolateral extrusion of these ions into the blood occurs via the Ca^{2+}- ATPase. Similarly, the epithelial sodium channel (ENaC) and the Na^{+}/K^{+}-ATPase are involved in the apical and basolateral transport of Na^{+} respectively. At acutely toxic concentrations of metals, the transport of these ions from the external water through the gill is compromised leading to disruption of ionic homeostasis. These ionoregulatory disturbances ultimately lead to fish death (Niyogi and Wood, 2003). For example, Cu at high concentrations competitively interacts with Na ions for uptake across the ENaC and also inhibits Na^{+}/K^{+} - ATPase, an enzyme which is responsible for the basolateral extrusion of Na^{+} into the blood, resulting in the condition of hyponatremia. This is due to the ability of Cu to attack sulfhydryl group of key enzymes and proteins thus inactivating them. Eventually, fish die as a result of osmoregulatory disturbance (Pelgrom et al., 1995). Acute toxicity of Zn and Cd in freshwater fish, on the other hand, occurs primarily as a result of the disruption of Ca^{2+} homeostasis (hypocalcaemia). This is due to the competitive interaction between Zn and Cd for uptake across the epithelial calcium channel (Hogstrand et al., 1995; Qiu and Hogstrand, 2004). These two metals are also known to inhibit Ca^{2+}-ATPase, which is responsible for basolateral extrusion of Ca ions into the blood (Hogstrand et al., 1996), resulting into hypocalcaemia. In addition, Cd has also been shown to disrupt the uptake of other physiologically important ions such as Na^{+} (Fu et al., 1989; McGeer et

al., 2000), perhaps as a result of the Cd-induced inhibition of the Na^+/K^+- ATPase (Atli and Canli, 2007).

Chronic toxicity of metals in fish, on the other hand, could have several mechanisms, most of which are not well-defined (Wood, 2011). One of the specific mechanisms of chronic toxicity is oxidative stress. Oxidative stress occurs as a result of the imbalance in the production and removal of reactive oxygen species (ROS) (Stohs and Bagchi, 1995). Reactive oxygen species are usually produced as a result of normal body metabolic activities (e.g., oxidative phosphorylation). They can, however, also be induced by pro-oxidants such as metals through a process known as the Fenton reaction. The end-product of this reaction is the generation of reactive oxygen species (ROS) such as superoxide anions, hydroxyl, and peroxide radicals. These chemical species are highly reactive and can cause oxidation of DNA, proteins, and lipids causing damage via DNA adduct formation, protein carbonylation, and lipid peroxidation, respectively (Livingstone, 2003). Organisms have natural defense systems such as glutathione, DNA repair enzymes, and antioxidant enzymes such as catalase and superoxide dismutase to scavenge these reactive species. However, when these defense mechanisms are overwhelmed, oxidative stress occurs, resulting in toxicity (Ercal et al., 2001).

1.5 Interactive Effects of Zn, Cu and/or Cd in Fish

The interactions of metals such as Zn, Cu, and Cd could potentially occur due to their shared mechanism of cellular uptake and internal handling. However, to date very little is known about these interactions. Zn and Cd share uptake pathway in the form of the epithelial calcium channel (ECaC) (see Figure 1.1). It is thus expected that the interactions for uptake between these two metals would be competitive, and this has been demonstrated in a number of studies. For

example, in a short-term (3h) gill-metal binding experiment in rainbow trout, Niyogi et al. (2015) reported that Zn significantly decreased short-term gill-Cd binding in a concentration-dependent manner. In fact, an 80% reduction in Cd-gill binding was observed with the greatest concentration of Zn (~96h LC_{50}) tested relative to the control. Similarly, Cd reduced Zn-gill binding, although the magnitude of reduction (35%) was relatively small compared to the control. The results of this study are consistent with findings by Komjarova and Blust (2009) and Wicklund-Glynn (2001), who also reported that Cd reduced Zn uptake in the gills of zebrafish (*Danio rerio*). It is worthy to note, however, that the greatest reduction in metal-gill binding/gill metal uptake in all these studies was observed at concentrations close to the 96hr LC_{50} of Zn and Cd.

On the other hand, Zn and Cu do not share a common cellular uptake pathway in fish gills, and it is expected that these metals would not interact with each other (see Figure 1.1). Studies that have investigated interactions between waterborne Zn and Cu in fish gills are relatively scarce. In one of the few available studies, Dethloff et al. (1999), reported no significant difference in Zn concentrations in the gills of rainbow trout following exposure to Cu/Zn mixtures, suggesting Cu had no effect on Zn uptake. Conversely, they also reported that Cu concentrations were all elevated in the presence of Zn in the Cu/Zn mixture treatments relative to the Cu only treatment. It is important to note here that the metal concentrations adopted by Dethloff et al. (1999) were sub-lethal, and it remains to be seen if similar results will be observed during exposure to acutely toxic concentrations of metals.

Although the presence of DMT-1 has been demonstrated in fish (Bury et al., 2003; Cooper et al., 2007; Kwong et al., 2010), its involvement in the transport of Cd and Cu in fish gills is yet to be fully established. Nevertheless, available evidence from studies by Komjarova and Bury (2014) suggest that these two metals may share a common uptake pathway, perhaps via DMT-1.

This suggestion is also consistent with findings by Niyogi et al. (2015), who equally reported a competitive interaction of Cd on Cu-gill binding and vice versa in rainbow trout gills. In this study, however, a greater competitive effect of Cu on Cd-gill binding was observed compared to that of Cd on Cu-gill binding. Specifically, Cu was shown to significantly reduce Cd-gill binding at concentrations well below the 96hr LC_{50} of Cu, whereas Cd only significantly decreased Cu-gill binding at concentrations close to lethal levels.

The interactive effects of these metals (i.e., Zn, Cu, and Cd) on fish toxicity (mortality) may, however, be complex and sometimes cannot be accurately predicted from gill-metal binding studies. For example, Niyogi et al. (2015) observed that sub-lethal waterborne concentrations of Zn in the range of 195 -390 μg/L had no protective effect against the acute toxicity of waterborne Cd in *O. mykiss*. Similarly, sub-lethal waterborne Cd concentrations in the range of 9 – 21 $\mu g/L^{-1}$ did not mitigate the acute toxicity of waterborne Zn in *O. mykiss*. In fact, an increase in fish mortality was observed with increasing waterborne Cd concentrations. Since Zn and Cd have been found to competitively interact for the gill binding of each other in fish (Niyogi et al., 2015), it is thus expected that the toxicity of these two metals in mixture would be less-than-additive. However, the interactive effects of Cd and Zn on fish mortality were antithetical to the interactions on their uptake in the gill. In contrast to the findings by Niyogi et al. (2015), Mebane et al. (2012) reported that Cd decreased Zn-induced mortality in cutthroat tout, suggesting an antagonistic interaction between these two metals. Contrasting results have also been reported in the literature on the interactive effects of Zn and Cu in mixture on fish mortality. A less-than-additive toxicity has been reported by Finlayson and Verrue (1982) with Chinook salmon (*O. tshawytscha*), whereas Naddy et al. (2015) and Lynch et al. (2016) reported a more-than-additive toxicity with rainbow trout (*O. mykiss*) and fathead minnow (*Pimephales promelas*), respectively. In summary, these

studies indicate that the general assumption of additivity for metal mixture toxicity may not necessarily be always true.

Most of the metal interaction studies in fish so far have been based on metal uptake/or toxicity. However, metals do not only interact for cellular uptake, but interactions could also potentially take place within the cytosol for binding to intracellular ligands (see Figure 1.1). In particular, very little is known about the interactive effects of metals in mixtures on the biochemical constituents (e.g., protein, carbohydrates & lipids), intracellular metal distribution and/or localization, and speciation in vital fish organs such as the gills. These interactions could have a significant influence on metal toxicity in fish. At present, there is a paucity of studies addressing the mechanistic underpinnings of such interactions within the intracellular environment of fish. Knowledge of such interactions could go a long way in enhancing our understanding of metal mixture toxicity in fish. My study utilized a suite of synchrotron-based techniques to study such interactions of metals in binary mixture (Zn, Cd and/or Cu) in fish gills.

1.6. Applications of synchrotron-based techniques in Biology

Synchrotron-based techniques such as X-ray fluorescence imaging (S-XFI), X-ray absorption near-edge spectroscopy (XANES) and Fourier transform infrared spectroscopy (FTIR) have been used in Biology for the determination of elemental distribution and localization, speciation, and the study of biochemical effects of metal-induced changes, respectively in biological tissues. These techniques offer potentials to enhance our understanding of metal mixture toxicity and interactions in fish.

1.6.1 Synchrotron-based X-ray fluorescence imaging

Synchrotron-based X-ray fluorescence imaging (S-XFI) is a technique used to determine elemental composition and distribution in a specimen (Pushie et al., 2014). The principle of this technique is based on the emission of secondary fluorescent X-rays from samples that have been excited with high energy X-rays. According to the Bohr's atomic model (Figure 1.2), an atom of an element is composed of a nucleus, which is surrounded by electrons arranged in different shells or orbitals. When atoms of an element are excited by X-rays, electrons are ejected from a lower energy orbital. A vacuum is created due to the ejection of these electrons from the lower energy orbital, and electrons from a higher energy orbital fill the lower energy orbital. This process leads to the emission of a photon whose energy is equal to the energy difference between the two orbitals involved in the transition. This phenomenon is referred to as 'fluorescence', and every element has a characteristic energy spectrum at which this occurs (Gräfe et al., 2014). S-XFI is an element-specific and non-destructive technique. Due to this reason, it has been used as a sensitive analytical tool for the detection of the elemental composition of biological tissues. Furthermore, elemental concentrations in the sub μg/g range in biological specimens can be detected with this technique (Punshon et al., 2005).

The spatial distribution of an element within biological tissues can also be examined by using a variant of this technique known as micro X-ray fluorescence imaging (μ-XFI). This form of S-XFI basically uses X-ray beam in the micrometer range to excite the atoms of a sample. Typically, thin sections of a sample are scanned through an X-ray beam. Spectra are then read from a fluorescent detector and correlated to a particular position on the sample. The distribution of the element of interest is often displayed by plotting an image of the element's peak intensity at

each pixel position. The results are presented as false colored images showing areas of high and low concentration of the element of interest (Figure 1.3.).

The use of μ-XFI could offer insights into the preferential sites of elemental accumulation in biological tissues, as well as the potential modes of transport or toxicity mechanisms of these elements (Punshon et al., 2005). The application of μ-XFI can also be complemented with other synchrotron-based techniques such as micro X-ray absorption near-edge spectroscopy (μ-XANES). The combination of these two techniques is often referred to as synchrotron-based X-ray microprobe. In this case, a defined region of a sample is mapped with μ-SXFI, and 'hot spots', which correspond to sites of high elemental accumulation in the sample, are selected for μ-XANES analysis. The combination of these two techniques could offer a great deal of chemical information on the probed samples.

S-XFI is one of the most widely used synchrotron-based techniques in Biology. In particular, it has found an increasing use in understanding metal distribution in biological tissues. Some metals are essential components of the biological systems as they play an important role in cellular and physiological functions (Blackstock and Weir, 1999). Essential metals such as Cu, Zn, and Fe are important co-factors of enzymes and proteins. S-XFI has been used to show that homeostatic disruption of these metals may be involved in the pathology of neurodegenerative diseases such as Parkinson's disease (Dexter et al., 1990; Ide-Ektessabi et al., 2004), Alzheimer's disease (Lovell et al., 1998), Amyotrophic lateral sclerosis (Szczerbowska-Boruchowska, 2008), as well as the development of prostate and breast cancer (Costello & Franklin, 2006; Farquharson et al., 2008).

S-XFI has also been applied in plant sciences to characterize the uptake, localization and distribution mechanisms of metals such as Cu in a Cu-tolerant plant, *Commelina commulis* (Shi et

al., 2011), Cd in a species of hyperaccumulating willow plant, *Silax giglia* (Harada et al., 2010), Cu, Zn and Ni in the roots of hydrated cowpeas (Kopittke et al., 2011), as well as the effects of Cu, Zn and Mn on metal interactions in the Mn hyperaccumulator plant, *Phytolacca Americana* (Zhao et al., 2012).

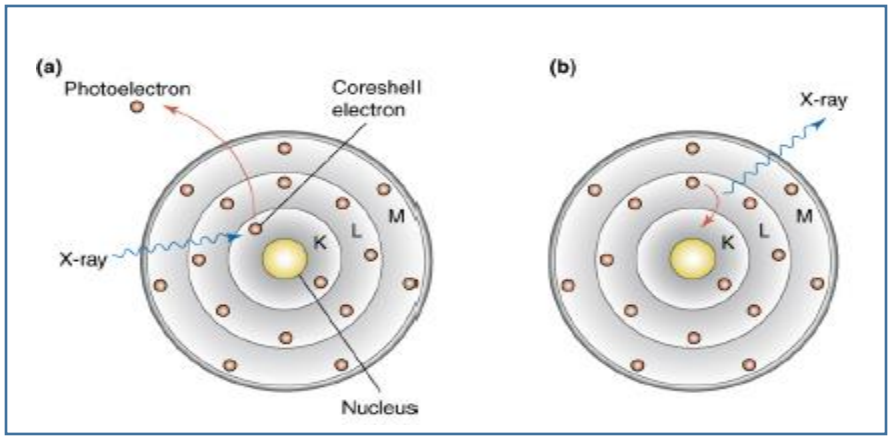

Figure 1.2: Schematic diagram of the Bohr's model of atom showing the principle of X-ray fluorescence. (a) A core-shell electron is ejected from the atom due to X-ray excitation. (b) Vacancy created is filled by an electron from a higher orbital [Adapted from (Fahrni, 2007)]

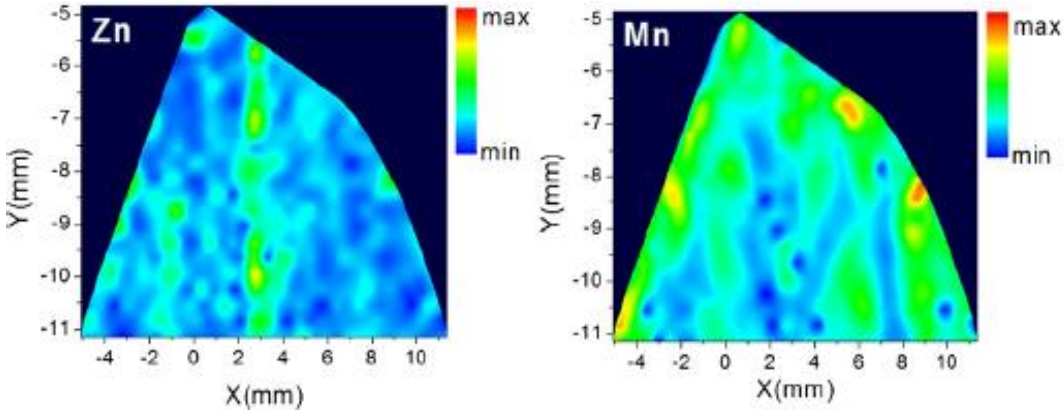

Figure 1.3: μ-XFI maps showing the distribution profiles of Zn and Mn in the leaf tissues of *Phytolacca americana* [Adapted from (Zhao et al., 2012)]

S-XFI has similarly been applied on aquatic organisms to characterize the uptake, localization and distribution of Hg in zebrafish larvae (Korbas et al., 2012), As and Zn in daphnia (Caumette et al., 2012; De Samber et al., 2008), Hg and Se interactions in zebrafish larvae (MacDonald et al., 2015), Cu in marine microalgae (Adams et al., 2016), as well as characterization of various elements in the tropical sponge, *Spheciospongia vagabunda* (Padovan et al., 2012), marine bivalves (Jones et al., 2009; Thorn et al., 1995), and marine protists (Diaz et al., 2009; Twining et al., 2008, 2003). To date, XFI has not been used to explore the interaction of metals in mixture in fish gills.

1.6.2 X-ray Absorption Near-edge Spectroscopy

X-ray absorption near-edge spectroscopy is a synchrotron-based technique which is used to reveal chemical information such as oxidation states and speciation of a probed atom of a sample. This technique operates on the principle that when atoms of a sample are excited with high energy X-ray, core electrons are ejected, thereby generating a characteristic absorbance. Upon continuous scanning with the high-energy X-ray, a sharp or abrupt increase in absorption occurs. This increase is known as absorption edge, and it is element specific (Yano and Yachandra, 2009). The edges are named K, L, or M absorption edges depending on the shell where the electrons are ejected. However, it is worthy to note that the K-edge is usually used for most biological samples due to its ease of measurement. A typical example of an XANES spectrum is shown in Figure 1.4.

One of the popular techniques used for XANES analysis is linear combination fitting (LCF). This is the process by which the relative contributions of chemical forms (speciation) are determined by reconstructing the XANES spectra of the probed element from a various combination of reference standards.

XANES is an analytical technique that offers a couple of advantages. It is a non-destructive technique and requires only minimal sample treatment. It is also element-specific, as each element has a unique absorption edge. Furthermore, this technique can be used to probe samples in different forms such as aqueous, freeze-dried, dissolved or precipitated forms (Yano and Yachandra, 2009).

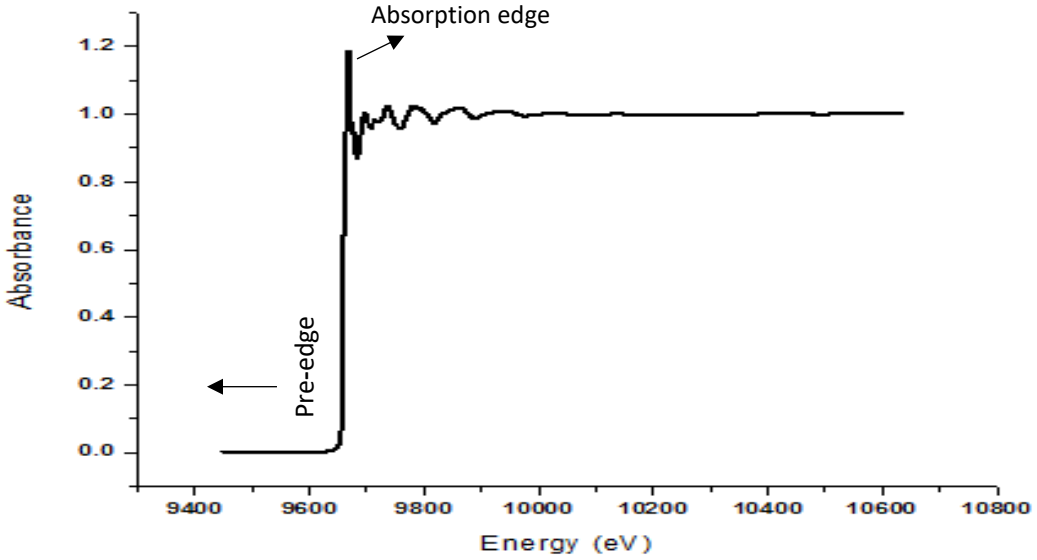

Figure 1.4: XANES spectrum of zinc carbonate showing the pre-edge and absorption edge.

The bioavailability and the toxicity of metals are hugely dependent on their speciation. Knowledge of speciation could help to decipher the roles of metal-binding ligands involved in metal detoxification and/or metal transport. XANES has been applied in numerous studies in aquatic toxicology to determine speciation of Zn in the livers of rainbow trout (Beauchemin et al., 2004), Se in various tissues of rainbow trout (Misra et al., 2012), Cu and Zn in oysters (Tan et al., 2015), As in Daphnia (Caumette et al., 2012), as well as Cu in a marine microalgae (Adams et al., 2016). Similarly, this technique has been applied in plant sciences to understand metal detoxification mechanisms in various species of metal hyperaccumulating plants (Fukuda et al., 2008; Harada et al., 2010; Polette et al., 2000; Sahi et al., 2007; Salt et al., 2002; Sharma et al.,

2004). To the best of my knowledge, this technique has not previously been used to study metal speciation in fish gills.

1.6.3 Fourier Transform Infrared Spectroscopy

Fourier infrared spectroscopy (FTIR) utilizes the infrared radiation of the electromagnetic spectrum to study changes in the structure of molecules. The energy of molecules typically consists of electronic, vibrational and rotational energy. FTIR spectroscopy is concerned with the vibrational motion of these molecules upon the absorption of infrared radiation. This vibrational transition can be used to characterize changes in molecular structure and composition (Marcelli et al., 2012)

The infrared (IR) spectrum is divided into three regions: near-IR (12500-4000 cm^{-1}), mid-IR (4000-400 cm^{-1}), and far-IR (400-5 cm^{-1}). The mid-IR is the region where the greatest absorption of organic compounds takes place, and thus most FTIR studies are often conducted within the wavelength of this region (Marcelli et al., 2012)

FTIR has enjoyed wide usage over the last few years in biological research for detecting changes in the structure and molecular composition of biomolecules such as proteins, nucleic acids, carbohydrates and lipids (Dumas and Miller, 2003). This is made possible because these biomolecules have characteristic functional groups with distinct vibrational modes corresponding to specific peaks of infrared light frequencies (Settle, 1997). Since the vibrational motion of every molecule is unique, IR spectroscopy can be used for the fingerprinting or identification of these molecules (Movasaghi et al., 2008). The IR spectrum of biological samples is typically composed of bands contributed by different functional groups of lipids, proteins, carbohydrates and nucleic acids (Le Naour et al., 2012). The alteration in intensity, position and width of infrared light absorption by these functional groups has been used to characterize molecular changes and

structure of biomolecules in a wide range of biomedical and biological research (Mantsch and Chapman, 1996; Yee et al., 2004). Different functional groups of biomolecules are assigned band numbers based on the peaks of absorption of infrared radiation. An example of such assignment is shown in Table 1.1. A typical IR spectrum of a biological sample consists of the fingerprint region (600-1450 cm^{-1}), the amide I and amide II region (1500-1700 cm^{-1}), the higher wavenumber region (2550-3500 cm^{-1}), which corresponds to stretching vibrations such as C-H, N-H and O-H and the lower wave number region, which denotes bending and carbon skeleton fingerprint vibrations. Collectively, these regions constitute the biochemical fingerprint of any biological sample (Walsh et al., 2008). An example of an IR spectrum of a biological sample is shown in Figure 1.5.

The most studied bands in biological tissues are those of the lipids, proteins, carbohydrates, and nucleic acids. The amide I and amide II bands correspond to the protein band, and the amide I band is particularly sensitive to secondary protein structure. Four types of secondary protein structures have been identified from the amide I band from past studies. They include the α helix [1662 -1645 cm^{-1}], the β sheet [1637-1613 cm^{-1}], the β turn [1682-1662 cm^{-1}] and the unordered random coil [1645-1637cm^{-1}] (Byler & Susi, 1986; Dieter, 2000).

For studies on biological samples, FTIR spectroscopy is often combined with microscopy. This technique is referred to as Fourier transform infrared microspectroscopy (FTIRM). The coupling of FTIR with microscopy not only allow changes in the structure and composition of biomolecules to be characterized from spectral features but also spatially resolved images of the changes in chemical composition and structure of these biomolecules can be revealed up to the micrometer range. The results are often displayed as pseudo color images showing the distribution of a particular biomolecule across a target area of a tissue (Marcelli et al., 2012)

Table 1.1: Band assignments of the FTIR spectra of the control liver tissues of *Labeo rohita* [Adapted from (Palaniappan and Vijayasundaram, 2008)]

S/N	Peak position (cm^{-1})	Definition of spectral assignments
1	3500	O-H stretch of the hydroxyl group
2	3293	Amide A: mainly N-H stretching of proteins
3	3081	Amide B: N-H stretching of proteins
4	2958	CH_3 asymmetric stretch: mainly lipids
5	2926	CH_2 asymmetric stretch: mainly lipids
6	2872	CH_3 symmetric stretch: mainly lipids
7	2854	CH_2 symmetric stretch: mainly lipids
8	1659	Amide I: mainly C-O stretching of proteins
9	1534	Amide II: N-H bending and C-N stretching of proteins
10	1451	CH_2 bending: mainly lipids
11	1388	COO^- symmetric stretch: fatty acids and amino acids
12	1230	PO_2^- asymmetric stretch: mainly nucleic acids
13	1170	CO-O-C asymmetric stretching: glycogen and nucleic acids
14	1081	PO_2^- symmetric stretch: mainly nucleic acids
15	1044	C-O stretching: polysaccharides

Several studies have adopted the FTIR technique to characterize changes in the structure and composition of biomolecules of various biological samples exposed to different classes of contaminants, including metals. For instance, this technique has been used to elucidate biochemical changes in a marine alga exposed to Cd (D'Souza et al., 2008), in fish gills exposed to Zn (Palaniappan et al., 2010), cyanide (Chu et al., 2001) and in gills and livers of fish exposed to As (Palaniappan and Vijayasundaram, 2008). However, this approach has not been previously used to study the metal mixture interactions in fish gills.

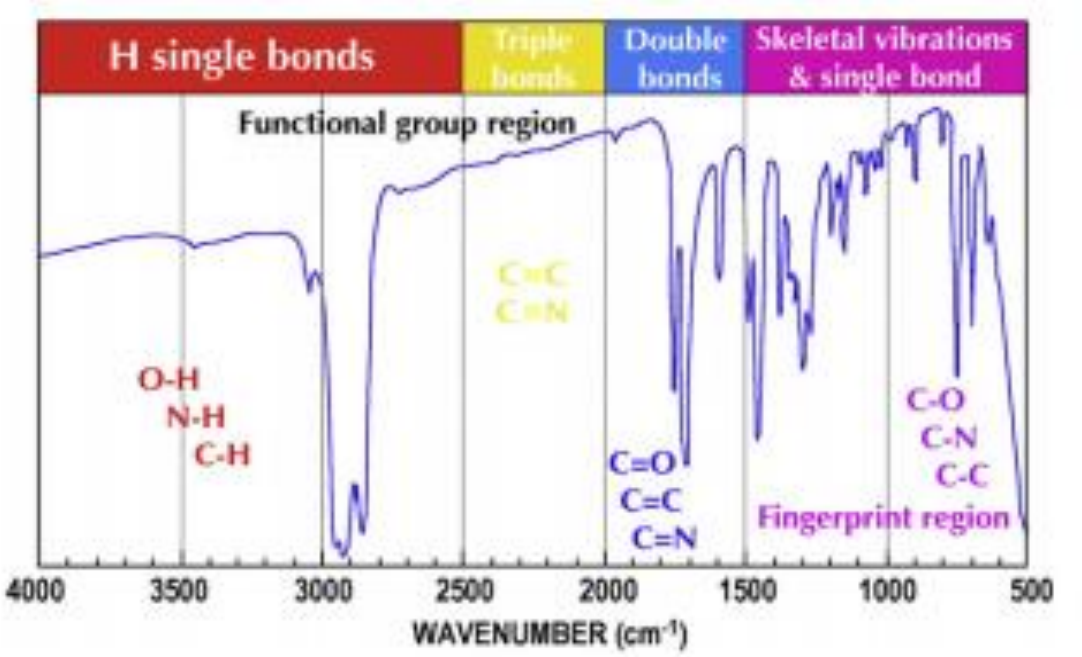

Figure 1.5: FTIR spectrum of a biological sample showing the vibrational modes of different functional groups (Marcelli et al., 2012)]

FTIR coupled with microscopy (otherwise known as FTIRM) has also been complemented with S-XFI on the same biological samples to correlate metal distribution with organic component distribution, especially in biomedical research. For example, Wang et al. (2005) made use of both techniques to characterize prion protein structure and metal accumulation in scrapie-infected ganglion tissue of hamsters. Both techniques were also adopted by Miller & Dumas (2006) to characterize the *in situ* elemental and biochemical composition in the brain tissues of individuals suffering from Alzheimer's disease (AD).

1.7 Rationale for the research

Fish are widely used for assessing the toxicity of metals in aquatic ecosystem. The gill is the primary route of entry for waterborne metals in fish, and the acute toxicity of metals to fish has

been attributed to metal binding to fish gills. This is the underlying assumption for predictive models currently being used for site-specific risk assessment of metals in natural waters such as the BLM (Niyogi and Wood, 2004)

At present, the BLM and most other risk assessment methods of metals in the aquatic environment are essentially designed to assess single metal toxicity, despite the fact that metals exist in mixtures in the natural environment and are subject to complex interactions (as described in section 1.5). Understanding metal interactions in fish gill – the initial site of toxic action of metals in fish – would go significantly improve our knowledge of metal mixture toxicity in fish, and this may also provide input towards the development of metal mixture BLMs.

Most studies that have investigated metal interactions in fish so far have focused on the uptake and/or toxicity of metals; however, the mechanistic underpinnings of these interactions remain largely unknown. Virtually little is known about the interactive effects of metals on metal distribution and/or localization, speciation and biochemical constituents (i.e. carbohydrates, proteins, and lipids) in fish. Furthermore, little is known about how these interactions could affect the co-localization of metals with essential elements (such as Ca, S and Fe). Over the last couple of years, the advent of synchrotron-based techniques such as S-XFI, XANES, and FTIR has enabled us to study metal distribution, speciation, and molecular fingerprinting of metal-induced changes in biological tissues with greater insights (Beauchemin et al., 2004). However, research in this field, especially in context with environmental sciences, has been focused mostly on plants with little or scanty literature on fish and invertebrates (Punshon et al., 2005). In addition, most of these studies have only investigated a single metal at a time. Although these studies provided new information, they are not particularly useful in understanding the cellular and physiological basis of metal mixture toxicity in fish.

1.8 Research Objectives and Hypotheses

1.8.1 Objectives

The overall goal of this research was to enhance our understanding of metal mixture interactions in fish gills following short-term exposure (24hrs) to acutely toxic concentrations (96h LC_{50}) of metals. Specifically, the objectives were:

a) To use μ-XFI to examine the interactive effects of Cu or Cd on the spatial distribution of Zn in rainbow trout gills.

b) To examine the co-localization patterns of Zn with essential elements such as Ca, S and Fe under the exposure conditions described in (a)

c) To use μ-XANES to examine Zn speciation in the gills of rainbow trout, and how it is influenced during co-exposure with Cu or Cd.

d) To use FTIR to characterize changes in the biochemical composition (i.e., proteins, lipids, carbohydrates & nucleic acids) of rainbow trout gills exposed to waterborne Zn, Cd, or Cu, singly and in binary mixtures.

1.8.2 Hypotheses

This research was based on the main hypothesis that metals interact during uptake, distribution, and binding to intracellular ligands. Thus, I expected metals with shared uptake sites at the gill, e.g., Zn and Cd, to have competitive interaction on the uptake and/or distribution of each other. In addition, the toxicity of these metals would be less than additive. Similarly, I expected metals with different uptake sites, e.g., Zn and Cu or Cu and Cd, to have no interactive effects on the uptake and/or distribution of each other. Toxicity of these metals in mixture would therefore be additive or more than additive.

2.0 INVESTIGATION OF THE SPECIATION AND DISTRIBUTION OF ZINC IN THE GILLS OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) USING SYNCHROTRON-BASED X-RAY MICROPROBE: INTERACTIONS WITH COPPER OR CADMIUM

This chapter will be submitted to *Aquatic Toxicology* under joint authorship with Som Niyogi, Derek Peak, Ankur Jamwal, and Renfei Feng.

2.1 Preface

In this chapter (2), the *in situ* distribution of zinc (Zn) in the gills of rainbow trout was characterized under acute (96h LC_{50}) exposure to waterborne concentrations of Zn, singly and in binary combination with cadmium (Cd) or copper (Cu). The co-localization/correlation of other essential elements such as calcium (Ca), sulphur (S) and iron (Fe) with Zn was also examined. This chapter also reveals the preferential sites of Zn accumulation in rainbow trout gills, as well as explains the underlying biochemical properties that could be responsible for Zn accumulation at these preferential sites. One of the factors that influence metal toxicity is speciation. This chapter concludes by investigating the speciation of Zn in the gills of rainbow trout and how it is influenced during co-exposure with Cu or Cd. The speciation results will offer insights into how fish cope with excess Zn burden in the gills during acute exposure.

2.2 Introduction

Zinc (Zn), Copper (Cu), and cadmium (Cd) are three of the most common metals in the aquatic environment and usually co-occur together (ATSDR, 2004; UNEP, 2010). These metals occur naturally in the environment, but in recent times, their concentrations have been elevated in the aquatic ecosystems due to anthropogenic activities such as mining and smelting (Nriagu and Pacyna, 1988). Zn is an essential metal in biological organisms, constituting a co-factor for more than 300 metalloenzymes (Passerini et al., 2007). It is only second to iron as the most ubiquitous metal in the human body (Vallee, 1986). Although Zn is an essential metal, it can also be potentially toxic at very high concentrations beyond the threshold of metabolic requirements. The primary mechanism of the acute toxicity of Zn to fish occurs as a result of the disruption of Ca^{2+} homeostasis (Hogstrand et al., 1995; Qiu and Hogstrand, 2004). The chronic effects of Zn exposure

in fish include reduced growth and survival (Adhikari and Mohanty, 2011; Schamphelaere, 2004; Woodling et al., 2002), reduced reproductive output (Brungs, 1969; Holcombe, 1978) and impairment of Ca^{2+} uptake (Hogstrand et al., 1995; Sappal et al., 2009).

Copper is an essential metal which is required in trace amounts in biological organisms for the maintenance of metabolic activities. It is an important cofactor of enzymes such as cytochrome c oxidase, which is required for cellular respiration in aerobic organisms, as well as catalase, glutathione reductase and superoxide dismutase, which are all anti-oxidant enzymes that form part of the body defence mechanisms against oxidative stress (Lee et al., 2001; Solomon and Lowery, 1993). However, despite its essentiality, it can also be toxic at concentrations above metabolic requirements. The acute toxicity of Cu in freshwater fish arises primarily as a result of the disruption of Na^{+} homeostasis (Grosell and Wood, 2002). Chronic exposure to high concentrations of Cu could result in a wide range of pathophysiological and toxicological effects in fish. These include reduced growth and survival (Hansen et al., 2002; Niyogi et al., 2006), respiratory distress (De Boeck et al., 1995), oxidative stress (Eyckmans et al., 2011), impaired swimming performance (Beaumont et al., 2000, 1995), inhibition of Na^{+} and Cl^{-} transport (Laurén and McDonald, 1985; Niyogi et al., 2006) and elevated ammonia excretion (Grosell et al., 2004; Laurén and McDonald, 1985).

Cadmium, on the other hand, is a non-essential metal which has no known biological function in organisms. The only instance where essentiality has been demonstrated for this metal is in the marine diatom, *Thalassiosira weissflogii,* where it replaces Zn at the catalytic site of carbonic anhydrase enzyme (Lane et al., 2005). Cadmium acts as a Zn and Ca analogue, and causes acute toxicity to freshwater fish via the disruption of Ca^{2+} homeostasis (Niyogi and Wood, 2004). Chronic effects of Cd exposure in fish are diverse and range across impaired growth and survival

(Hansen et al., 2002; Mebane et al., 2008), disruption of Na^+ and Ca^{2+} homeostasis (McGeer et al., 2000), suppression of immune functions (Zelikoff et al., 1995), oxidative stress (Cao et al., 2010), tissue and skeletal damage (Benaduce et al., 2008; Wangsongsak et al., 2007) and endocrine disruption (Foran et al., 2002; Lizardo-Daudt et al., 2008)

The gill is the primary route of entry for these metals in fish when exposed via water, and the acute toxicity of metals to fish has been attributed to metal binding to the fish gills. This assumption is the basis of mechanistic risk assessment models such as the biotic ligand model (BLM). According to this model, the gill is a biotic ligand or physiological site of action where metal accumulation results into acute toxicity (Niyogi and Wood, 2003). In addition to being the main route of entry for waterborne metals, the gill is also responsible for other functions in fish such as gaseous exchange, ionic regulation, maintenance of acid-base balance and nitrogenous waste excretion (Evans et al., 2005). The basic structure of the gill consists of a filament or primary lamellae from where leaf-like structures called secondary lamellae project. There are two major epithelial cells in the gill; these are the mitochondria-rich cells (MRCs) and the pavement cells (PVCs) (see Figure 2.2). The MRCs are primarily involved in the uptake of ions (Ca^{2+}) and metals (e.g., Cd) in freshwater fish (Galvez et al., 2006). The pavement cells, on the other hand, play an important role in gaseous exchange in the gills (Evans et al., 2005)

Due to the debilitating effects metals (e.g., Zn, Cu, and Cd) could have on aquatic organisms, as well as on humans who rely on these aquatic resources for the sustenance of life, ambient water quality criteria have been developed in many parts of the world for the protection of aquatic life. Ambient water quality criteria refer to the maximum concentrations of metals that will protect about 95% of the resident population of an aquatic ecosystem (Wood, 2011). Most of these criteria have, however, been based on single metal toxicity studies, in spite of the fact that

metals rarely occur singly in the aquatic environment but rather as mixtures. Interactions of metals in mixture may occur due to their shared uptake and/or handling pathways, resulting in less than additive or more than additive toxic effects in aquatic organisms. A better understanding of metal interactions in the gill (the biotic ligand) could help to predict the toxicity of metal mixtures in fish.

Studies that have examined metal interactions in fish are very few. In fact, most of them have been based on metal uptake and/or accumulation as endpoints (Kamunde and MacPhail, 2011; Komjarova and Blust, 2009; Niyogi et al., 2015). There is virtually little or no information on the mechanistic underpinnings of these interactions and how these interactions could potentially affect metal distribution, co-localization and speciation within the intracellular environment of the fish. Traditionally, metal toxicity in aquatic organisms has been correlated to metal accumulation at critical target sites of action. This has been measured historically in biological tissues with analytical techniques such as atomic absorption spectroscopy (AAS) and inductive-coupled plasma mass spectroscopy (ICP-MS). These techniques involve the homogenization of tissues, followed by measurement of accumulated metals or metal bound fractions. The pitfall of these techniques, however, lies in the fact that they are destructive and do not reveal the *in situ* distribution of these metals nor their co-localization with essential elements (such as Ca, S, and Fe) within the intracellular milieu of organisms (Ralle and Lutsenko, 2009). Furthermore, these techniques are also fraught with contamination by artifacts, which could lead to erroneous conclusions (Tan et al., 2015). In addition, speciation, which is an important factor which influences metal toxicity, cannot also be determined using these techniques.

Synchrotron X-ray microprobe consists of two complementary techniques: micro X-ray fluorescence imaging (μ-XFI) and micro X-ray absorption near-edge spectroscopy (μ-XANES)

The advent of these techniques has enhanced the study of the *in situ* distribution and speciation of metals, respectively in biological tissues. These two techniques are element- specific and non-invasive. The high brightness of synchrotron light, which these techniques rely on, allows samples to be examined in their natural states (*in situ*) with a high spatial and spectral resolution (Lombi and Susini, 2009; Luo and Zhang, 2010). Most studies that have adopted these techniques for biological research have, however, been focused on plants and clinical sciences with scanty literature on fish or invertebrates (Punshon et al., 2005). Furthermore, most of these studies investigated speciation or distribution of a single metal at a time, neglecting the fact that metals could potentially interact.

The main aim of this study, therefore, was to use synchrotron-based techniques to enhance our understanding of metal interactions in fish gills – the site of toxic action of metals - following short-term exposure (24hrs) to acutely toxic concentrations (96h LC_{50s}) of Zn, Cu, and Cd. Specifically, the objectives of this study were three-fold:

a) To use μ-XFI to examine the spatial distribution of Zn in the gills of rainbow trout (*Oncorhynchus mykiss*), and how it is modulated during co-exposure to Cd or Cu.
b) To examine the correlation and co-localization of Zn with Ca, S, and Fe, and how it is modulated during co-exposure to Cd or Cu.
c) To use μ-XANES to determine the speciation of Zn in the gills of *O. mykiss*, and to also determine how this speciation is influenced during co-exposure with Cu or Cd.

We hypothesized that metals with shared uptake sites at the gill (e.g., Zn and Cd) would have competitive interaction on the uptake and/or distribution of each other. Similarly, we assumed that metals with apparently different uptake sites at the gill (e.g., Zn and Cu) would have no interactive effects on the uptake and/or distribution of each other.

2.3 Materials and Methods

The experimental protocol described in this study was in accordance with the Canadian Council for Animal Care Guidelines and was approved by the Animal Care Committee of the University of Saskatchewan.

2.3.1 Experimental procedure

Juvenile rainbow trout (*O. mykiss*) weighing approximately 200g were purchased from the Lucky Lake Fish Farm (Saskatchewan) and reared at the Aquatic Toxicology Research Facility (ATRF) of the University of Saskatchewan. The fish were maintained at a photoperiod of 16h light:8h dark and a water temperature of 12 ± 1^{o}C. They were fed with a commercial diet once daily at a ratio of 2% body weight. Prior to the start of the experiment, the fish were acclimated to the exposure water (dechlorinated Saskatoon City tap water; Ca^{2+} = 44, Mg^{2+} = 18, Na^{+} = 26, K^{+} = 3, Cl^{-} = 11, SO_4^{2-} = 50, hardness = 160, alkalinity = 110 (both as $CaCO_3$), dissolved organic carbon (DOC) = 2.5 (all in mg/L), pH = 7.9) (Driessnack et al., 2016) for 21 days. Fish were not fed a day prior to the start of the experiment and throughout the experimental duration. The experimental treatments consisted of four groups: control (no added metals in the exposure water), Zn (1mg/L), Zn (1mg/L) + Cd (20µg/L), and Zn (1mg/L) + Cu (100µg/L). Fish were exposed individually to nominal concentrations of waterborne metals (as mentioned above) for 24 hrs in 20L of exposure water under static non-renewal exposure condition. Each exposure was replicated three (3) times. The fish were continuously supplied aeration throughout the exposure duration. Metal concentrations were added as metallic salts of $ZnSO_4.7H_20$, $CdNO_3.4H_2O$ and $Cu(NO_3)_2.5H_20$ (all purchased from Sigma-Aldrich, MO, USA) for Zn, Cd, and Cu respectively. The concentrations of metals employed for this study were equivalent to the 96hr median lethal concentration (LC_{50}) of each individual metal in rainbow trout, as reported by Alsop et al (1999), Niyogi et al. (2008),

and Taylor et al. (2000) under similar water chemistry conditions as used in the present study. For the measurement of dissolved metal concentrations, water samples were collected at the start and at the end of the exposure, filtered using a 0.45μm nylon syringe filter (Nalgene, NY, USA), and acidified with 0.2% HNO_3 (Trace metal grade; VWR, ON, Canada). They were subsequently stored at 4^{o}C until analysis. The actual (dissolved) metal concentrations for the exposure were verified by measuring the metal levels using a graphite furnace atomic absorption spectrometer (AAnalyst 800, Perkin Elmer, CT, USA). The actual concentrations of water samples were within ±10% of nominal concentrations. At the end of the exposure period, fish were euthanized with an overdose of Tricaine Methanesulfonate (MS-222) and gill baskets were excised. They were thoroughly cleaned in nanopure water and blotted dry with kim wipes. Gill tissues were thereafter placed in tissue cassettes, fixed in 10% neutral buffered formalin (VWR, ON, Canada) for 24hrs, and then stored in 70% ethanol for later processing.

2.3.2 Tissue processing and sectioning

An automated tissue processor (MUP1, Modular Vacuum Processor) was used to process the gill tissues. Briefly, tissues were dehydrated through a series of graded ethanol (70-100%) and then fixed in paraffin. The paraffin blocks were longitudinally sectioned along the axis of gill filaments into 5 μm thick sections using a rotary microtome (HM330; Heidelberg, Germany). Tissue sections were then fixed on pre-cleaned suprafrost slides (VWR, ON, Canada), and dried on a slide warmer. Adjacent sections from the same tissues were also fixed on lexan sheet (thickness; 100μm) for μ-XFI and μ-XANES analyses at the Canadian Light Source (CLS). Tissues deposited on the suprafrost slides were then de-paraffinized, rehydrated and stained using Meyer's hematoxylin and eosin, following the OECD recommended protocol (OECD, 2009). Once slides were stained and coverslips affixed, they were examined using a Zeiss Axioplan

Fluorescence Microscope and photographed using an AxioCamICc1 (Colour, 1.4 MP) digital camera. Three different gill sections, each prepared from an individual fish, were used for μ-XFI and μ-XANES analyses. Tissue sections were photographed in several non-overlapping areas for the identification of areas to use for these analyses.

2.3.3 μ-XFI and μ-XANES Data Collection

The μ-XFI and μ-XANES data were acquired at the VESPERS (Very Sensitive Elemental and Structural Probe Employing Radiation from a Synchrotron) beamline of the Canadian Light Source (CLS). The storage ring of the CLS operates at an energy of 2.9 GeV and a current range of 250-150mA. The features of the VESPERS beamline have already been described extensively by Feng (2007). Briefly, this beamline consists of a microprobe which employs hard X-rays in the energy range of 2-20keV to illuminate a microvolume of samples. Two kinds of beam operate on this beamline; these are the monochromatic beam, with a bandpass of 0.01%, 0.1%, and 10%, as well as a polychromatic beam (pink). The spot size of the beam at this beamline ranges from 2 x 2μm to 5 x 5μm.

For the μ-XFI data, the gill samples were scanned using a polychromatic (pink) beam with a spot size of 4 x 4μm, a step size of 5μm and a dwell time of 5s per data point. Gill sections deposited on the lexan sheets were mounted on a sample holder and attached to a motorized stage. Synchrotron beam (pink) was incident at an angle of 90^{o} to the gill samples, while a 4-element vortex silicon drift detector (SDD), incident at an angle of 45^{o} and positioned at a distance of 50mm away from the gill samples was used to collect fluorescent signals. The incident X-ray energy was 20 keV and this allowed the simultaneous collection of the kα fluorescent signals of elements ranging from S, Ca, Fe, Cu to Zn. An illustration of the set-up for data collection at the VESPERS beamline is described in Figure 2. 2. Regions of the gill tissue consisting of the primary

and secondary lamellae, with a map size of approximately 200μm by 200μm were selected for the elemental mapping of all gill samples. Three replicates of gill tissues preparations from each treatment were used for μ-XFI analysis in order to ensure reproducibility of data.

Hotspots of Zn localization from the μ-XFI maps were used to collect Zn K-edge μ-XANES data from the gill samples. A Zn foil was used for energy calibration prior to the collection of Zn μ-XANES data, with the first assigned inflection point to be 9659 keV. This Zn reference point was consecutively measured with all gill samples and reference standards throughout the experiment. The μ-XANES data were collected with a Si (220) double crystal monochromator with the use of a monochromatic beam incident at an angle of 90^{o} to gill samples. Data were collected in fluorescence mode both on the gill samples and dilute Zn reference standards. A transmission mode was used to collect data on concentrated Zn reference standards. These reference standards were diluted with boron nitride (BN) in order to eliminate self-absorption.

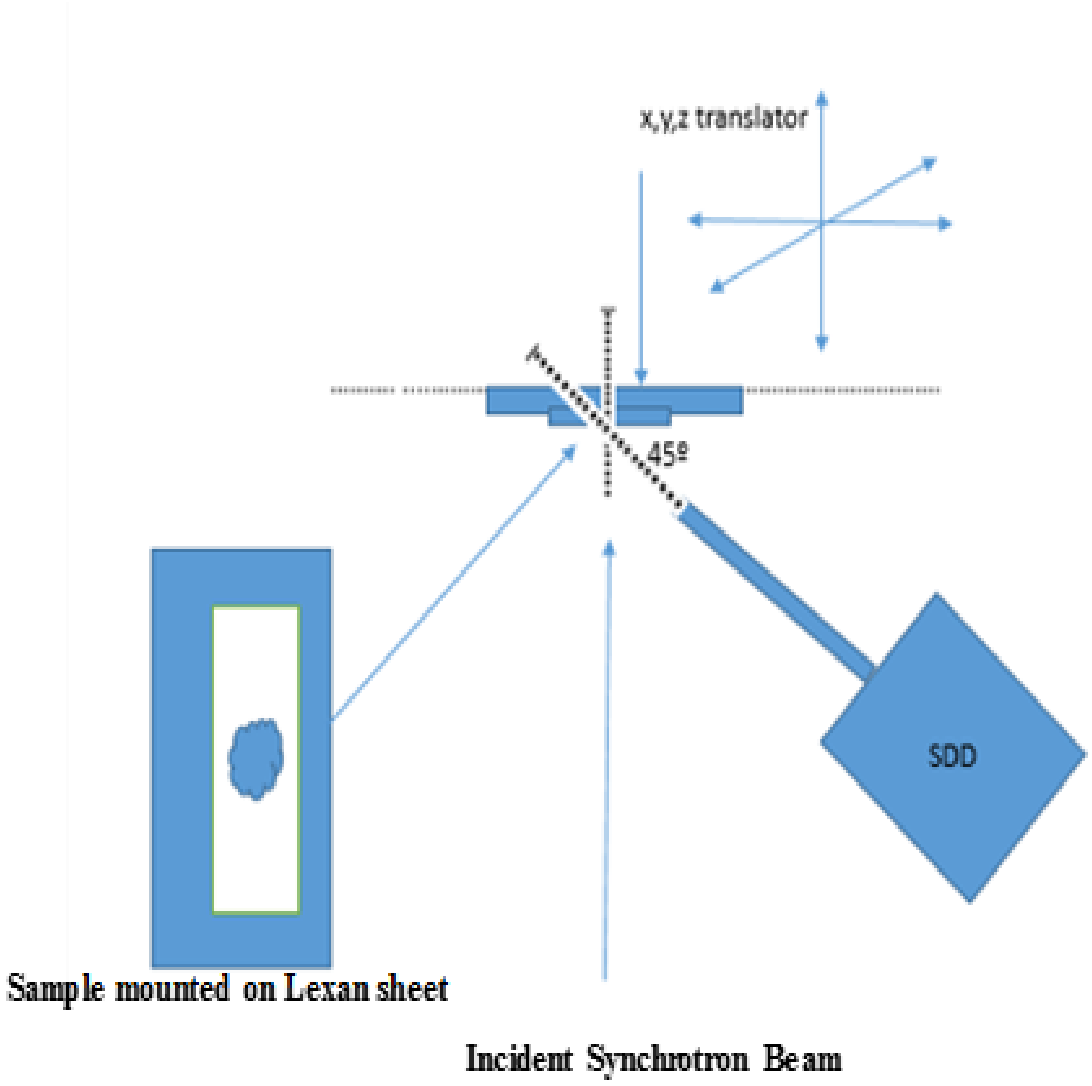

Figure 2.1: Experimental set-up at the VESPERS beamline depicting sample, X-ray source, and detector arrangement [Modified from (Desouza et al., 2013)]

A total of eleven (11) Zn reference standards was used for this study. These include zinc oxide (ZnO), zinc hydroxide ($Zn(OH)_2$), zinc sulphide (ZnS), zinc sulphate monohydrate ($ZnSO_4.H_2O$), Zn-cysteine, Zn-histidine, zinc carbonate ($ZnCO_3$), zinc phosphate ($Zn_3(PO_4)_2$), zinc chloride ($ZnCl_2$), carbonic anhydrase, and aqueous Zn (50mM). ACS grades of the mineral standards were purchased from a commercial vendor (Sigma-Aldrich, MO, USA) except for Zn-histidine, Zn-cysteine, and aqueous Zn. These three standards were prepared as described in Tan et al. (2015), Beauchemin et al. (2004), and Hamilton et al. (2016), respectively. All reference standards were finely ground in an agate mortar and then spread thinly on kapton tape for spectrum collection. In order to maximize the number of Zn fluorescence counts from the gill samples, the distance of the detector to the gill samples was set as 35mm. Scans (8-10) were run on the gill samples with a dwell time of 5s and on the reference standards with a dwell time of 3s.

2.3.5 Data Analysis

The μ-XFI data were plotted using the filled contour map feature of the SigmaPlot v.12 statistical software (Sigmastat Software Inc., California, USA). Normalized intensities of elements of interests from each treatment were plotted at each pixel position on the x and y axis. The μ-XFI maps are presented as pseudo color images, with red colour showing areas of high elemental concentrations and blue colour depicting areas of low elemental concentrations. Color scales were normalized for each element of interest across all treatments for ease of comparison. Correlation between two elements of interest was done by plotting the normalized intensities of the two elements in a scatter plot and calculating the Pearson correlation coefficient (r) value.

The μ-XANES data were analyzed with Athena software (Ravel and Newville, 2005). Data reduction consisted of alignment of individual scans to the energy of the Zn foil, which was used for calibration, merging of individual scans, and normalization. The relative contributions of the

chemical species of Zn across the experiment treatments were deconvoluted using least square linear combination fitting (LCF). The fitting range was set as -20 to 80 eV relative to the Zn edge at 9659 eV. A maximum of three combinations of standards was used for the fitting.

2.4 Results and Discussion

Synchrotron-based X-ray fluorescence imaging is a sensitive and element-specific analytical technique, which can be used to determine the elemental composition of a specimen. This is made possible due to the fact that each element has a characteristic or distinct energy at which it emits its fluorescence. A representative picture showing the structure of a control gill sample is displayed (Figure 2.2). An example of the XFI spectrum showing the elemental composition of one of the gill samples is displayed (Figure 2.3).

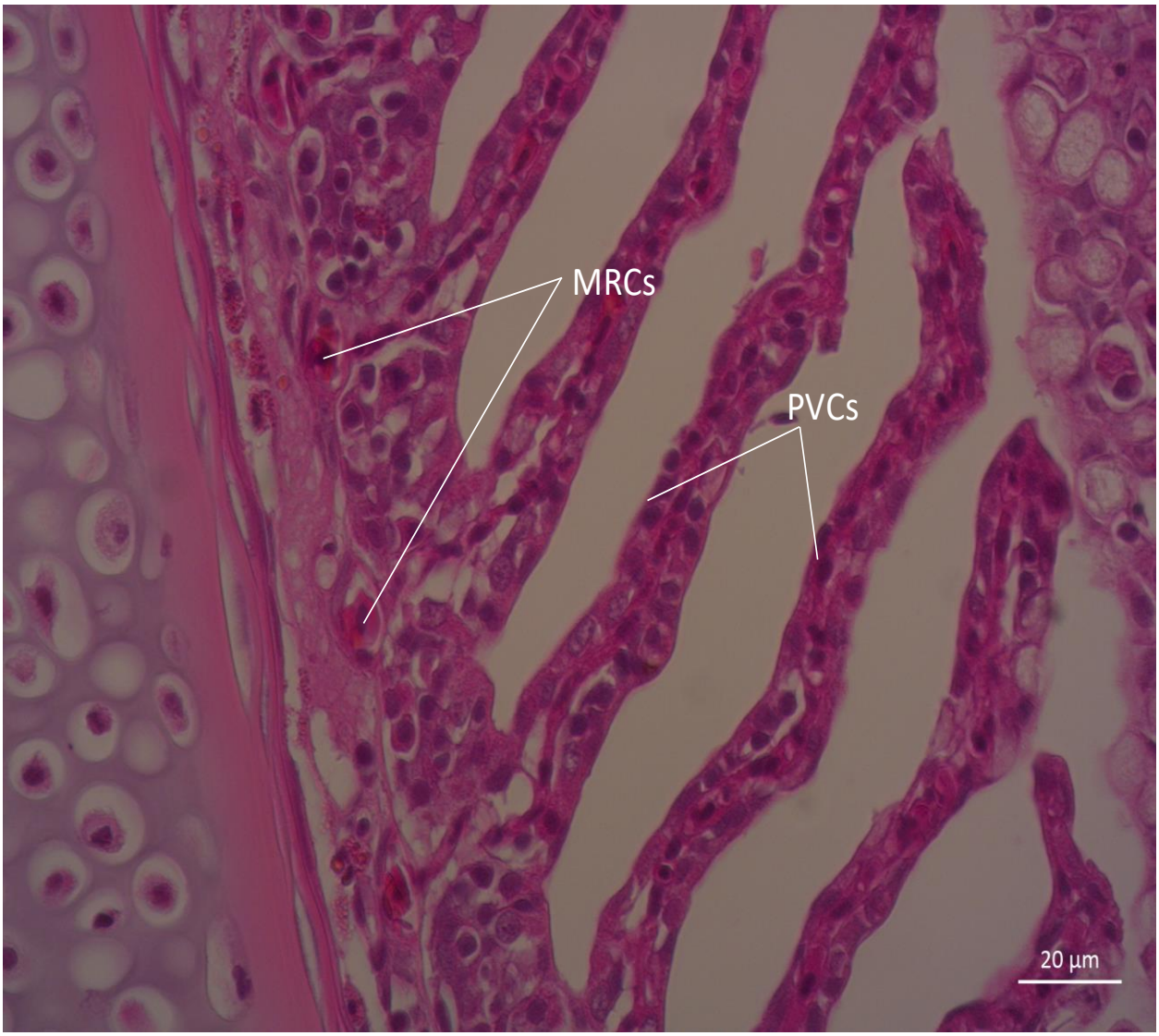

Figure 2.2: Longitudinal section of the gill of rainbow trout (*Oncorhynchus mykiss*) from a representative sample (control) stained with hematoxylin & eosin. Magnification;400X. PL = Primary lamellae, SL =secondary lamellae, MRCs= Mitochondria-rich cells, PVCs = Pavement cell. Scale:20µm

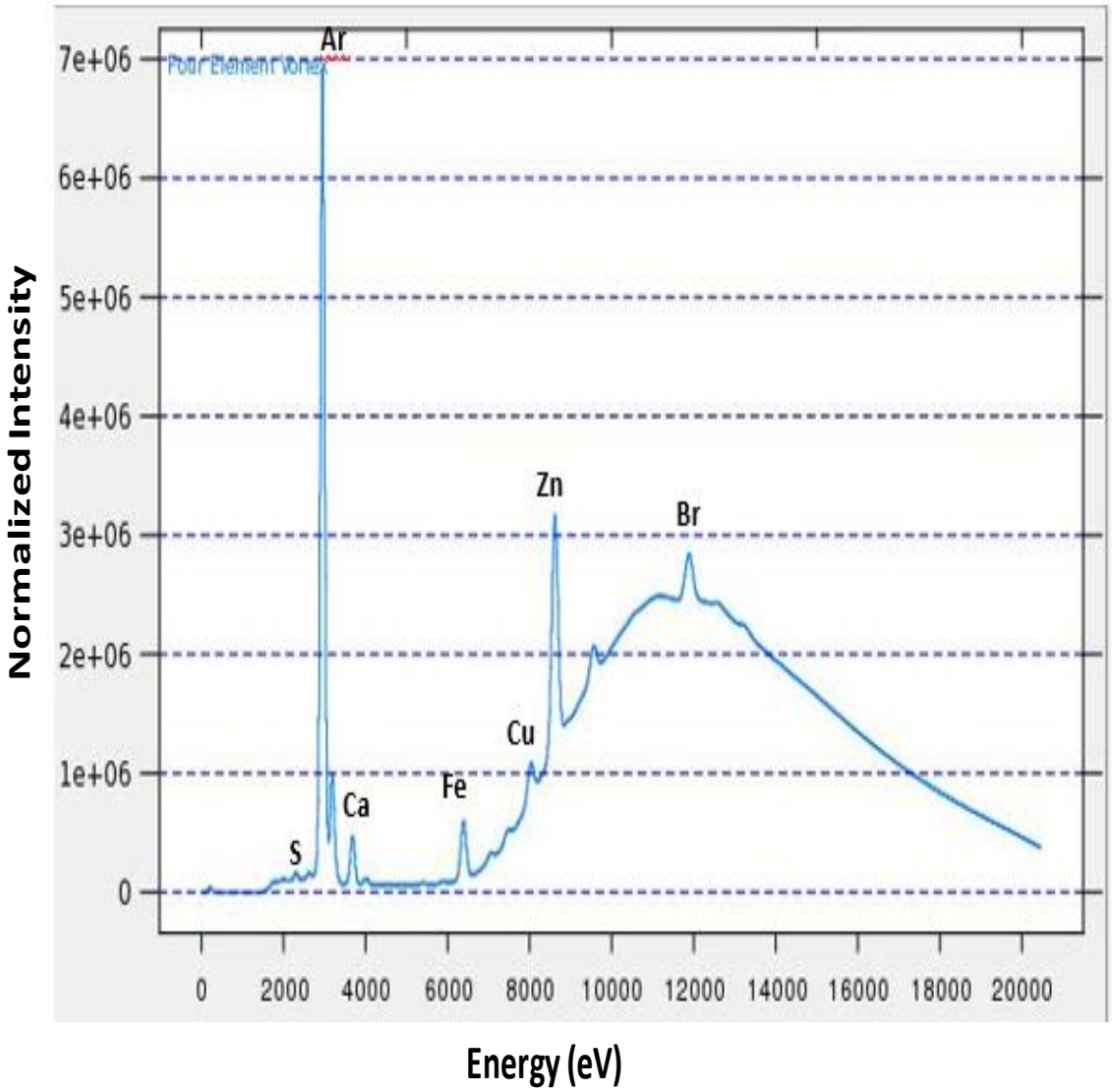

Figure 2.3: An XFI spectrum from a rainbow trout gill exposed to Zn showing the integrated intensities and the kα peak of the elements present. Note that the high peak of argon in the spectrum is from the atmosphere.

2.4.1 Zn Distribution and its Interactions with Cd or Cu

Zn fluorescence was observed in all treatments, including that of the control gill tissues (Figure 2.4). This result was not surprising as Zn is known to be an essential metal in fish and a background concentration is expected to be present. Zn fluorescence as observed in the other treatments (Zn, Zn +Cd, and Zn + Cu) also suggested that Zn exposure resulted in marked increase in Zn levels in the gills (Figure 2.4). Furthermore, there was a similar pattern of Zn localization in all the experimental treatments (Figure 2.4). Zn localization was found to be highest at the base of the primary lamellae compared to the secondary lamellae. The reason for the high accumulation of Zn on the base of the primary might be due to the prevalence of mitochondria-rich cells (MRCs) on these sites of the gill. MRCs (refer to Figure 2.2) are cells that are primarily involved in ion (Ca^{2+}) uptake in the gills of freshwater fish (Ishihara and Mugiya, 1987; Payan et al., 1981; Perry and Flik, 1988). Apart from being involved in ion uptake, these cells have also been implicated in the active uptake of metals such as Cd across fish gills from water (Galvez et al., 2006). In fact, the expression of many metal transporters, including that of Zn (e.g., ECaC), have been demonstrated in these cells (Pan et al., 2005). MRCs are numerous on the base of the primary lamellae, and during exposure of fish to lethal concentrations of metals (e.g. Zn), these cells proliferate (Evans et al., 2005). The proliferation of MRCs acts as a mechanism to counteract disruption of ion (Ca^{2+}) transport in fish caused by the exposure to acutely toxic metal concentrations (in this case; Zn) (Perry and Laurent, 1993). The proliferation of these cells, inadvertently, also likely resulted in an increase in the uptake of Zn. This explains the probable reason for the preferential accumulation of Zn at the base of the primary lamellae as observed in the results. This observation is also supported by findings by Spry and Wood (1988) and Tuurala

and Soivio (1982), who similarly reported an increase in the uptake of Zn in rainbow trout gills with increased MRCs proliferation.

Despite the similarities observed in the pattern of Zn localization, the uptake and/or distribution of Zn was, however, different across treatments. Zn fluorescence was found to substantially decrease in the Zn + Cd treatment compared to the Zn treatment alone, indicating reduced Zn uptake in the presence of Cd (Figure 2.4C). This result suggested a competitive interaction between Zn and Cd for uptake across fish gills. Zinc and Cd occupy the same group of the periodic table and share similar chemical characteristics when they are in their free ionic form (Zn^{2+} and Cd^{2+}) (Brzóska and Moniuszko-Jakoniuk, 2001). In addition, they both have been shown to act as a Ca analogue (Hogstrand et al., 1996; Niyogi et al., 2004). They can thus both gain entry across the epithelial calcium channel (ECaC) of fish gills by disguising as Ca^{2+} ions. It has also been shown in a number of biological processes that Cd can displace Zn from its intracellular binding sites (Endo et al., 1997; Gachot and Poujeol, 1992). The observations from this study are also in agreement with findings by Niyogi et al. (2015) and Komjarova and Blust (2009), who similarly showed that Cd had an inhibitory effect on branchial Zn uptake in rainbow trout and zebrafish, respectively. The co-exposure of Zn with Cu, however, did not have any significant effect on Zn fluorescence compared to the Zn treatment only (Figure 2.4D). This result may be attributed to the fact that Zn and Cu have different apical uptake routes in fish gill. The apical uptake of Zn across fish gills is known to take place either through the ECaC and/or the ZIP family of transporters (Hogstrand et al., 1996; Niyogi et al., 2008; Qiu et al., 2005), and that of Cu occurs through the ENaC and/or or Ctrl-1 (Bury et al., 2003; Craig et al., 2010; Minghetti et al., 2008). Thus, the non-interaction of these two metals as observed in this study may be due to the fact that they have different routes of uptake in fish gills. Unlike interactions between Zn and Cd, studies

that have examined Zn and Cu interactions in fish gills are limited. Nonetheless, Niyogi et al. (2015) also found no effect of acute waterborne Cu on short-term (3h) branchial Zn uptake in rainbow trout. In addition, Dethloff et al. (1999) reported that Cu did not produce any significant effect on Zn concentrations in the gills of rainbow trout following exposure to Cu/Zn mixtures. It is also worthy to note that the metal concentrations adopted for this study were sub-lethal, and it remains to be seen if similar findings will be observed during exposure at lethal metal concentrations. In contrast, findings by Komjarova and Blust (2008) showed that Cu had an inhibitory effect on Zn uptake in the water flea, *Daphnia magna*. This observation could have been due to the fairly low metal (sub-lethal) concentrations of Zn and Cu used in their exposure. Another reason for their observation could also be due to potential interactions of Zn and Cu through Ca uptake pathways or through the DMT-1. For example, Alsop and Wood (2011) have shown that Cu could inhibit Ca^{2+} uptake in zebrafish, perhaps via the ECaC (which also transports Zn). In addition, the DMT-1 is a metal transporter that could also transport both Zn and Cu, although its affinity for Zn is low compared to Cu. Thus, there is a potential for these two metals, i.e., Zn and Cu, to competitively interact via the ECaC and/or DMT-1 uptake pathways; however, these assumptions remain controversial, and further research is required to clarify this ambiguity.

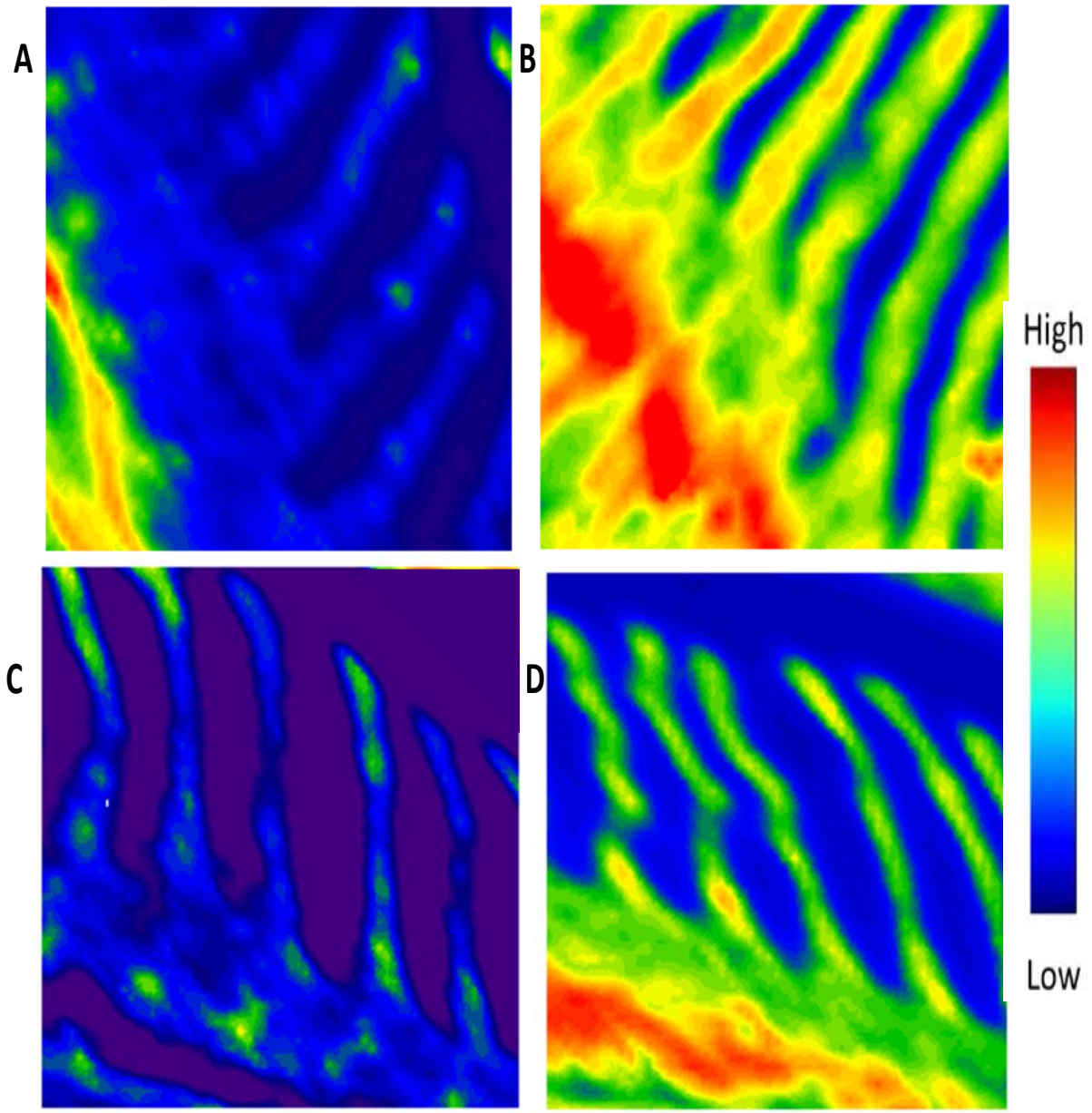

Figure 2.4: Representative normalized μ-XFI maps showing the spatial distribution of Zn from (A) Control treatment (B) Zn treatment (C) Zn + Cd treatment (D) Zn + Cu treatment. Fluorescence signal intensity is shown as a color scale.

2.4.2 Distribution of Ca and its modulations during exposure to metal(s)

High fluorescence of Ca was observed in the control treatment, suggesting that Ca is an important constituent of the fish gill (Figure 2.5A). Interestingly, though, there was a marked loss of Ca fluorescence in all of the other treatments relative to the control (Figures 2.5B, 2.5C & 2.5D). As described before, both Zn and Cd are Ca analogues, and they cause acute toxicity in fish by inducing disruption of Ca homeostasis via the competitive inhibition of Ca uptake by ECaC, as well as non-competitive inhibition of Ca^{2+}-ATPase. Thus, the reduction in Ca fluorescence as observed in the Zn and Zn + Cd treatments relative to the control may simply imply competitive interaction of these metals (i.e., Zn and Cd) on Ca uptake in fish gills. Furthermore, reduction in Ca fluorescence in the fish gills during exposure to Zn (alone) and Zn + Cd, might have also occurred, at least in part due to the displacement of Ca from cytosolic Ca binding proteins (e.g., Calmodulin and Calbindin) by Zn and/or Cd. In the present study, Zn + Cu treatment was also found to markedly decrease Ca fluorescence (uptake) – an effect which was most likely caused predominantly by Zn in the exposure. Since we observed that Cu did not influence the distribution of Zn in the rainbow trout (Figure 2.4), it is logical to assume that Cu had very little, if any, additional effect on Ca distribution in the fish gill during combined exposure to Zn and Cu.

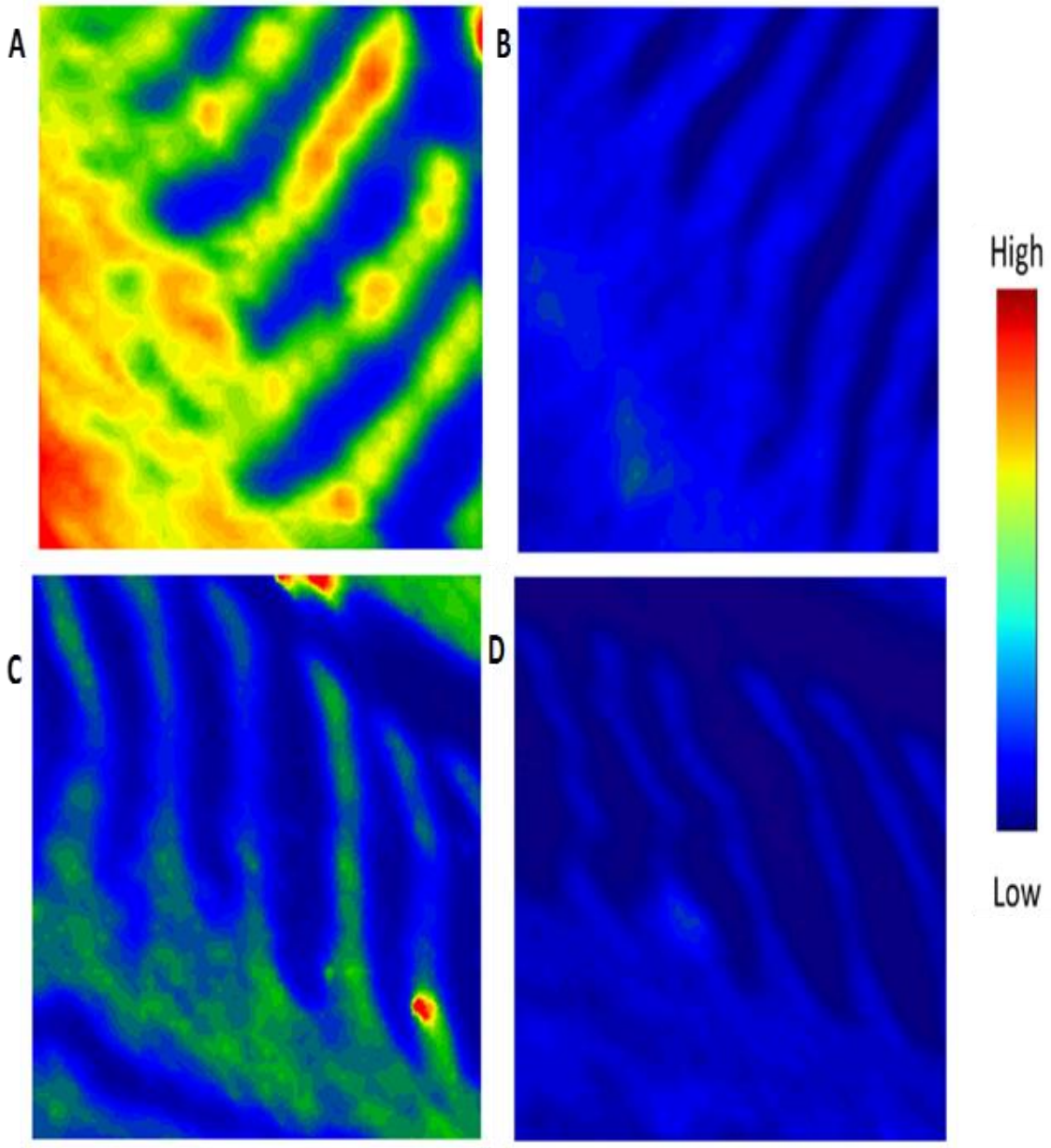

Figure 2.5: Representative normalized μ-XFI maps showing the spatial distribution of Ca from (A) Control treatment (B) Zn treatment (C) Zn + Cd treatment (D) Zn + Cu treatment. Fluorescence signal intensity is shown as a color scale.

2.4.3 Co-localization of Zn with Ca, S, and Fe

The correlation of the distribution of one element with another can be used as a reliable indicator of their co-localization patterns in biological tissues. In this study, Zn was found to have a strong correlation with Ca and S (Figure 2.6 and Table 2.1) in gill tissues across all of the treatments. These results suggest that Zn was co-localized with Ca and S – thereby indicating the important role of Ca-binding and S-enriched intracellular ligands in the transport and/or internal handling of Zn in the gill cells. Conversely, a low positive correlation of Zn with Fe was observed (Figure 2.6 and Table 2.1), suggesting Fe transporting or regulating proteins were not likely involved in internal handling of Zn. Although it has been reported that Zn and Fe may share uptake pathway via the DMT-1 (which is essentially an Fe transporter), however, Zn has the lowest affinity for this transporter compared to other divalent metals (Garrick et al., 2006).

As described previously, Zn is an analogue of Ca, and it is known to interact with apical ECaC and basolateral Ca^{2+}ATPase in the gill cells (Hogstrand et al., 1996). In addition, Zn has strong affinity for binding to calcium-binding proteins e.g., calbindin (see Figure 1.1) (Bauer et al., 2008; Hempe and Cousins, 1991). Thus, the strong co-localization of Zn with Ca as observed in this study might have also occurred due to the binding of Zn to these calcium moieties. Furthermore, Zn is known to have a strong preference for binding to S-enriched ligands (e.g., cysteine-rich metallothionein, histidine/cysteine containing zinc finger proteins) (Rainbow, 1997) (see Figure 1.1), and thus a strong correlation of Zn with S as observed in this study is also to be expected. The correlation patterns of Zn with these essential elements (Ca, S, and Fe) in the trout gills did not appear to be significantly influenced by metal mixture treatments except that of Zn and Ca in the combined exposure of Zn + Cd (based on the correlation coefficients presented in Table 2.1). The relative lower correlation of Zn and Ca in the Zn + Cd treatment compared to that

in the Zn only treatment might have been caused by the displacement of Zn by Cd from the Ca-binding intracellular ligands.

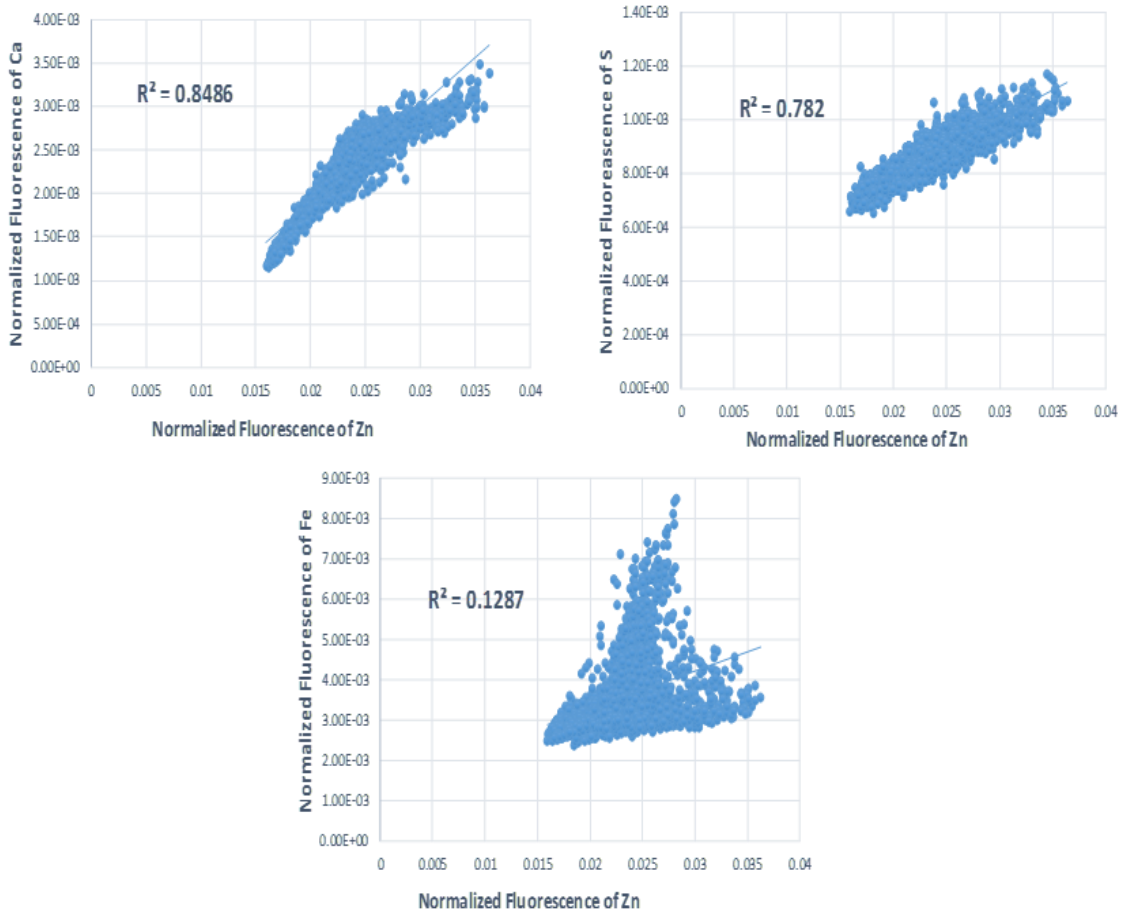

Figure 2.6: Correlation plots showing the correlation (localization) of Zn with (A) Ca, (B) S, and (C) Fe in the gill of rainbow trout exposed to Zn alone. Pearson correlation coefficient values of each plot are displayed as insets.

Table 2.1: Pearson correlation coefficients showing correlation of Ca, S, and Fe with Zn across the different experimental treatments. NB: S correlation with Zn in the Zn + Cu treatment is not included due to noisy fluorescence signals of S recorded in this treatment.

Treatments	Zn	Zn + Cd	Zn + Cu
Ca	0.849	0.674	0.809
S	0.782	0.753	-
Fe	0.129	0.363	0.214

2.4.4 Speciation of Zn and how it is influenced across experimental treatments

Another objective of this research was to examine the speciation of Zn in the gills of rainbow trout and how it is influenced during co-exposure with Cd or Cu. Speciation is an important factor which influences metal toxicity and knowledge of speciation would significantly help in elucidating mechanisms of metal detoxification in fish. The XANES spectral features of the reference standards (Figure 2.7) used in this study were similar to those reported in previous studies (Beauchemin et al., 2004; Tan et al., 2015). The XANES spectra of the experimental gill samples are also presented in Figure 2.8.

Analysis with least square linear combination fitting (LCF) revealed subtle changes in Zn speciation across the experimental treatments. Three dominant Zn species, comprising of Zn-phosphate, Zn-cysteine and Zn-histidine were observed in the Zn treatment, whereas the speciation of Zn in the Zn + Cd and Zn + Cu treatments was better explained by two species (Zn-histidine and Zn-phosphate), suggesting a similar mechanism of Zn detoxification across these mixture treatments. Zn is an element that usually exists in three kinds of coordination geometry: octahedral, tetrahedral, and trigonal bipyramidal (Shay and Mangian, 2000). It is to be noted that the coordination of Zn in all these three Zn reference standards (i.e., Zn-phosphate, Zn-cysteine, and Zn-histidine) is in the form of tetrahedral symmetry, consisting of one Zn^{2+} atom binding to three Zn ligands. This also suggests that Zn was coordinated in a similar manner across the experimental treatments. The relative proportions of Zn species in the Zn treatment were 51.9% Zn-phosphate, 34.8% Zn-histidine, and 13.3% Zn-cysteine. However, with the Zn + Cd treatment, the relative fractions consisted of 52.7% Zn-phosphate and 47.3% Zn-histidine. The proportions of Zn species in the Zn + Cu treatment were made up of 46.8% Zn-phosphate and 53.2% Zn-histidine (Table 2.2). From these results, it is evident that Zn-phosphate and Zn-histidine were the two dominant

Zn species, although their proportions differed slightly across treatments. It is important to also note here that although LCF is a valuable technique for deconvoluting the contribution of elemental species from a combination of reference standards, it may also be limited by the available standards in the library. The results displayed here, therefore, are the best representation of the data based on the available standards in our library.

To the best of our knowledge, this is the first study to investigate Zn speciation in fish gills, and as such, there is a paucity of literature to compare our results. Nonetheless, findings from a few available studies on Zn speciation in marine invertebrates such as oysters are generally in agreement with our findings. For example, Tan et al. (2015) investigated the speciation of Zn in two species of oysters (*Crassostrea hongkongensis* and *Crassostrea sikamea*), which were collected from a metal-contaminated estuary and a reference site. These oyster species have the ability to bioaccumulate huge concentrations of Zn in their tissues. In fact, Zn concentrations as high as 23,000 $\mu g\ g^{-1}$ have been reported in the tissues of some of these oysters (Weng and Wang, 2014). XANES was employed by Tan et al. (2015) to understand the speciation and detoxification mechanisms of Zn in the gills, mantle, and digestive glands of *C. hongkongensis* and *C. sikamea*. Their findings indicated that the speciation of Zn in the gills was similar in both oyster species. Zn in the gill tissues of contaminated oysters was found to be predominantly complexed to O and N containing ligands in contrast to S-enriched ligands, which were the dominant species in normal oysters from pristine reference sites. Specifically, the speciation of Zn in the gills of contaminated *C. hongkongensis* was explained by Zn-phosphate and Zn-histidine. These two Zn ligands constituted about 51.1% and 48.9% respectively of total Zn species, whereas in *C. sikamea,* Zn-cysteine (33.8%) in addition to Zn-phosphate (30.2%) and Zn-histidine (36.0%) made up the total Zn species. It is worthy to note however that Tan et al. (2015) did not specifically isolate the gill

tissues in *C. sikamea*, and the XANES analysis was done on a mixture of gill and mantle tissues and the results reported for *C. sikamea*, therefore, represent Zn speciation in these two combined tissues. Beauchemin et al. (2004) also investigated Zn speciation in the livers of rainbow trout exposed to sublethal concentrations of waterborne Zn. Their findings revealed that Zn speciation in both control and Zn-exposed fish was predominantly in the form of Zn complexed with cysteine. Their finding, despite the fact that it is contrary to our results, however, is not surprising as the liver is known to be rich in metallothionein, a S-enriched protein which has a high affinity for Zn binding. In addition, chronic Zn exposure is known to increase metallothionein production in the liver in order to enhance Zn-detoxification capacity in fish (Roch and McCarter, 1984). The discrepancy of our observations with that of Beauchemin et al. (2004) may also be a reflection of the fact that Zn detoxification mechanisms could be organ-specific. For example, Misra et al. (2012) reported notable differences in selenium (Se) speciation in different organs (e.g., gill *vs* liver) of rainbow trout during sublethal exposure to dietary Se.

Thiolate species such as metallothionein and glutathione are usually involved in the complexation of metals in aquatic organisms and are also believed to be induced by Zn (Wood, 2011). However, in the present study, Zn was not found to be complexed with cysteine in high proportions in the trout gills. Rather, non-thiolate species such as Zn-phosphate and Zn-histidine were observed to be the predominant forms of Zn species in the gills. In fact, an attempt was made to incorporate Zn-cysteine into the fitting of the Zn + Cd and Zn + Cu treatments, but the relative proportions of this ligand in these treatments were not more than 2%, suggesting that this species was probably not present in high proportion in the gill tissue. Based on the classification of metals proposed by Nieboer and Richardson (1980), Zn, Cd ,and Cu are borderline metals, with affinities for binding to S-enriched ligands, especially cysteine-rich thiols, in the order of Cu>Cd> Zn. Thus,

the loss of Zn-cysteine species in the Zn + Cd and Zn + Cu treatments might have occurred due to the displacement of Zn bound with cysteine by Cd or Cu. It has also been reported in several studies that Zn has a high affinity for histidine than cysteine (Gregory et al., 1993; Nieboer and Richardson, 1980; Rulíšek and Havlas, 2000; Shay and Mangian, 2000). Thus, the relatively high proportions of Zn-histidine than Zn-cysteine in the gills as observed in the present study were probably the result of preferential binding of Zn with histidine. Intracellular phosphate moieties are often involved in the detoxification of metals in aquatic organisms (Goto and Wallace, 2010). This detoxification strategy involves the sequestration of metals as phosphates into insoluble metal-granules for long-term storage, and the findings of our study suggest that this pathway plays an important role in Zn detoxification in the fish gill during acute Zn exposure. It is important to note that Zn-phosphate was the only phosphorus containing reference standard used in this study, and its high proportions across different experimental treatments may not actually be all in phosphate form, and may also include Zn complexed with a variety of other phosphorus containing intracellular ligands. It would have also been worthwhile to examine Zn speciation in the gills of control fish in our study. However, this could not be performed because the concentration of Zn in the control gill was not sufficiently high enough to obtain XANES spectra with high signal to noise ratio. Overall, these observations from the present study indicated that the internal handling of Zn rainbow trout gill essentially involves complexation of free Zn ion with O (Zn-phosphate), N (Zn-histidine), and S (Zn-cysteine) containing cytosolic ligands. However, during co-exposure of Zn with Cd or Cu, Zn complexed with cysteine can be replaced by Cd or Cu, which may alter the toxicity in fish acutely exposed to the binary mixture of Zn +Cd, or Zn + Cu, relative to Zn only.

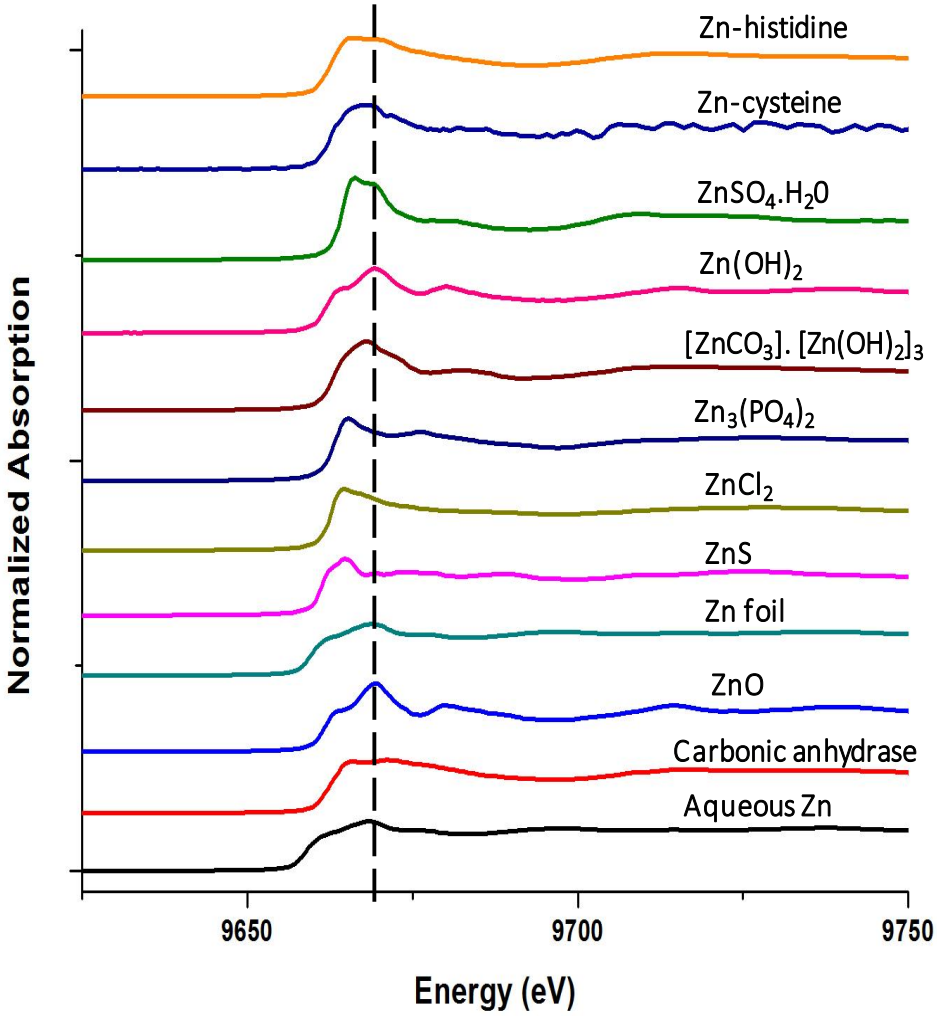

Figure 2.7: Representative Zn K-edge XANES spectra of reference standards used in this study. Dotted vertical line is displayed to show variation in the near edge.

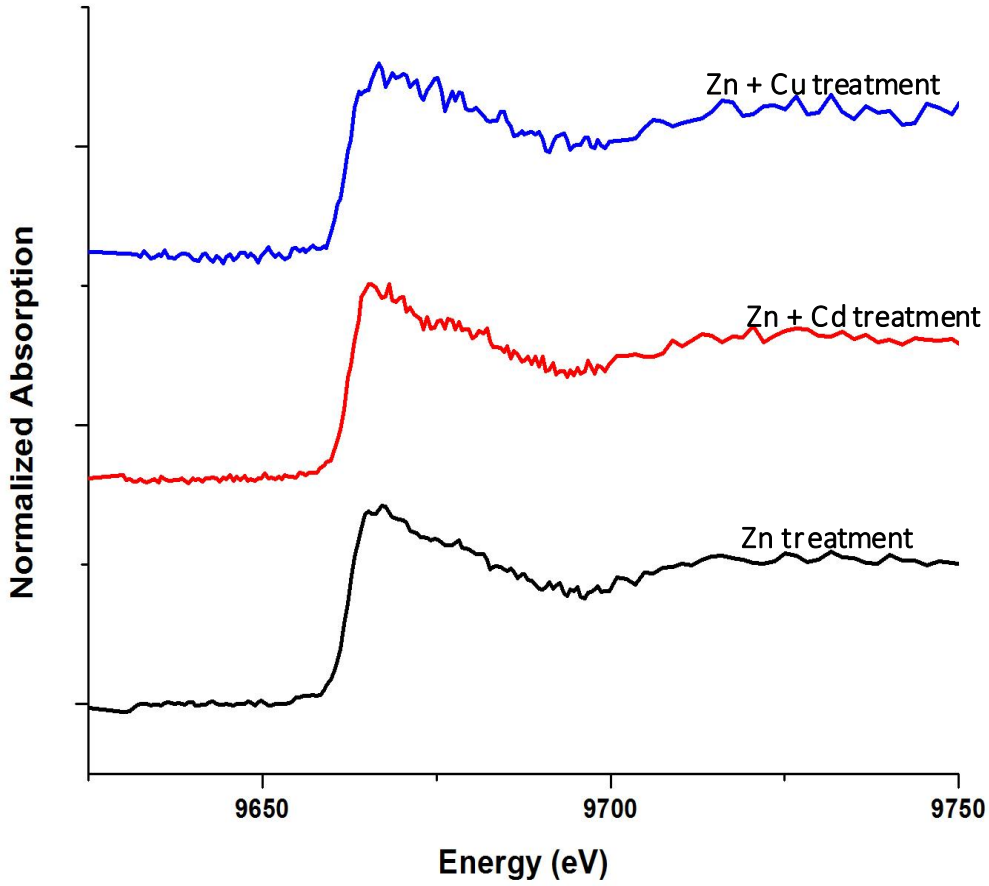

Figure 2.8: Representative Zn K-edge XANES spectra of experimental samples

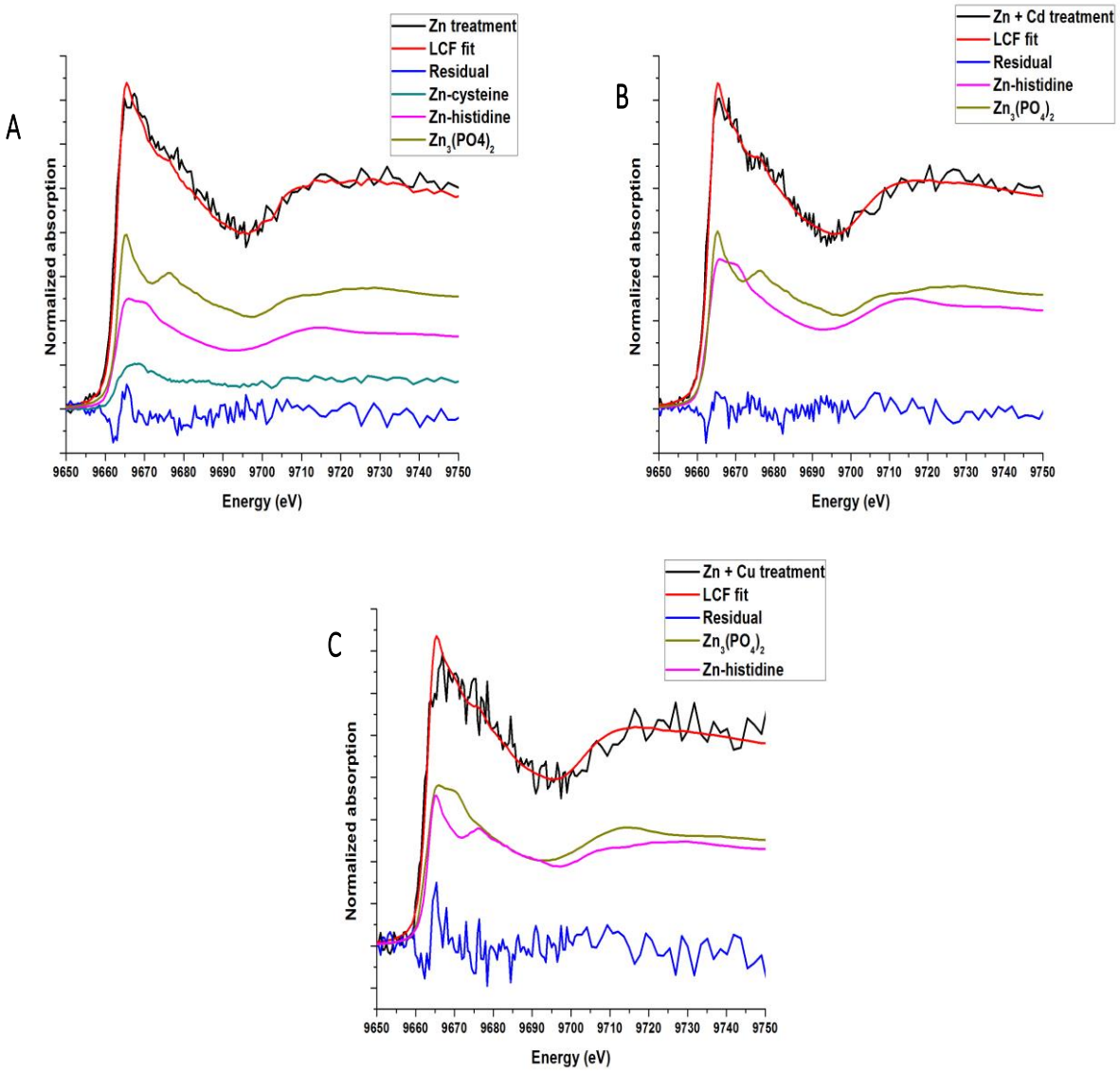

Figure 2.9: Linear combination fitting of Zn K-edge XANES in experimental treatments using three model compounds. (a) Zn treatment (b) Zn + Cd treatment (c) Zn + Cu treatment. Residuals of the fits are displayed as offsets. Numerical results are shown in Table 2.2.

Table 2.2: Proportion of Zn species across experimental treatments assessed by linear combination fitting of Zn K-edge XANES. R-factor is a statistical measure of how good a fit is. The smaller the R-factor, the better the fit.

Treatments	% Zn phosphate	% Zn-histidine	% Zn-cysteine	R-factor
Zn	51.9	34.8	13.3	0.0099
Zn + Cd	52.7	47.3	-	0.0066
Zn + Cu	46.8	53.2	-	0.0242

2.5 Conclusions

This study provided novel insights into the distribution, localization, and speciation of Zn in the fish gill, and how these properties are influenced by the interactions of Cd or Cu. The base of the primary lamellae – which corresponds to areas of MRCs localization - was found to be the major site of Zn accumulation in the fish gill. MRCs have been implicated in the active uptake of metals in fish gills, and the preferential accumulation of Zn at the base of the primary lamellae as observed in this study further corroborated this fact. Zinc was found to co-localize mainly with Ca and S in the fish gill during exposure to waterborne Zn, suggesting Ca-binding and S-enriched ligands play a critical role in the handling of excess Zn in the gill tissue. Cadmium, but not copper, was found to have a competitive effect on Zn distribution in the gill tissue, which is consistent with the fact that they are both transported in the fish gill *via* a common pathway (e.g., Ca transport pathway). Acute Zn exposure, alone or in the presence of Cd or Cu, resulted in the loss of Ca from the gill tissue, further corroborating that Zn acts as a Ca antagonist in the fish gill. Zinc in the fish gill was found to be present predominantly in the form of Zn-phosphate, Zn-histidine, and Zn-cysteine during acute exposure to waterborne Zn. Co-exposure of Zn + Cd, or Zn + Cu resulted in the loss of Zn-cysteine in the gill tissue, indicating that both Cd and Cu can displace Zn from the Zn-cysteine, possibly because of their relative high affinity for binding to cysteine than Zn.

3.0 INTERACTIVE EFFECTS OF METALS (ZINC, COPPER AND CADMIUM) IN BINARY MIXTURES ON THE BIOCHEMICAL COMPOSITION OF THE GILLS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

This chapter will be submitted to *Metallomics* under joint authorship with Som Niyogi, Saroj Kumar and Derek Peak.

3.1 Preface

This chapter utilized Fourier transform infrared micro-spectroscopy (FTIRM) to investigate changes in biochemical composition of fish gills following exposure to acutely toxic concentrations (96h LC_{50s}) of waterborne metals. Biomolecules such as proteins, lipids, and nucleic acids are essential components of biological organisms, including fish. This chapter will provide valuable information which would complement the findings of the chapter 2 of this thesis.

3.2 Introduction

The gill is an important organ in fish. It performs vital functions such as gaseous exchange, acid-base balance, nitrogenous waste excretion, and osmoregulation. In addition, the gill is also the site of toxic action of pollutants in fish, including metals. The gill of the fish is in perpetual contact with water contaminated with various metals, and it is often the first organ to respond to metal exposure (Evans, 1987). Exposure of fish to waterborne metals can induce structural changes in the gill. The structural changes are mostly pathophysiological responses and they include lamellar fusion, edematous swelling, necrosis, hypertrophy, and hyperplasia. These responses may negatively impact the normal functioning of the gills, and this may lead to suffocation and subsequent fish death (Mallatt, 1985). Apart from these pathophysiological effects, waterborne metal exposure can also result in changes in the biochemical and molecular composition of the biomolecules such as proteins, lipids, and nucleic acids, which are all essential components of all biological systems in the gill tissue of fish.

Over the last few years, Fourier infrared transform spectroscopy (FTIR) has emerged as a viable technique to examine the biochemical changes induced in various biological organisms as a result of contaminant exposure. FTIR utilizes the absorption of infrared radiation by a biological

sample to evaluate changes in organic macromolecules. This is made possible because each biomolecule vibrates at a characteristic frequency or wavelength. The changes in the peak and absorption intensities of these biomolecules can thus be used to characterize biochemical and molecular changes in samples (Marcelli et al., 2012). FTIR is a sensitive and non-invasive technique that allows the examination of tissues in their natural state with little or no chemical pre-treatment (Davis and Mauer, 2010). Most studies that have utilized FTIR to study metal-induced biochemical changes in fish gills have essentially based on single metal exposure such as Zn and As (Palaniappan et al., 2010; Palaniappan and Vijayasundaram, 2009). However, in the natural environment, metals do not exist singly, but rather as complex mixtures. The interactions of metals in mixture could influence their uptake, as well as their internal handling, resulting in less-than-additive, or more-than-additive toxicity in exposed organisms. At present, there is a general lack of information on the mechanistic underpinnings of the interactive effects of metals in mixtures on the biochemical components (e.g., proteins, lipids, and nucleic acids) of vital organs of the fish such as the gill. In addition, all of the previous studies (e.g., Palaniappan et al. 2010; Palaniappan and Vijayasundaram, 2009) that focused on the effects of metals on the composition of biomolecules in fish tissues were conducted using bulk FTIR spectroscopy. This approach provided valuable information on biochemical effects of metals in tissues, but it is not particularly useful in providing spatially resolved information on the changes in the distribution of these functional groups following metal exposure. The main aim of this research, therefore, was to use FTIR coupled with microscopy (FTIRM) to enhance our understanding of the toxic interactions of metals in mixture in fish gills following short exposure (24hrs) to lethal concentrations (96h LC_{50s}) of Zn, Cd, and Cu. Specifically, the objectives of this research were two-fold:

a) To characterize the effects on vital macromolecules (e.g., proteins, lipids, and nucleic acids) in the gills of rainbow trout (*Oncorhynchus mykiss*) exposed to acutely toxic waterborne Zn, Cd or Cu concentrations, singly and in binary mixtures.

b) To examine the relative changes in the spatial distribution of proteins, lipids, and nucleic acids in the gills of *O. mykiss* under the exposure conditions described in (a).

We hypothesized that since the uptake and handling of Zn and Cd occur via the same pathways in the fish gill (see Chapter two), the mixture of Zn and Cd would elicit less than additive effects. Similarly, we also assumed that since the mechanisms of Cu uptake and handling differ to that of Zn or Cd (see Chapter two), the mixture of Zn and Cu, or Cu and Cd would produce additive or more than additive effects.

3.3 Materials and Methods

The experimental protocol described in this study was in accordance with the Canadian Council for Animal Care Guidelines and was approved by the Animal Care Committee of the University of Saskatchewan.

3.3.1 Animal model

Juvenile rainbow trout (*O. mykiss*) weighing approximately 200g were obtained from the Lucky Lake Fish Farm (Saskatchewan) and reared at the Aquatic Toxicology Research Facility (ATRF) of the University of Saskatchewan. The fish were maintained at a photoperiod of 16h light:8h dark and a water temperature of 12 ± 1^{o}C. They were fed with commercial diets once daily at a ratio of 2% body weight. Prior to the start of the experiment, the fish were acclimated to the exposure water (dechlorinated Saskatoon City tap water; Ca^{2+} = 44, Mg^{2+} = 18, Na^{+} = 26, K^{+} = 3, Cl^{-} = 11, SO_4^{2-} = 50, hardness = 160, alkalinity = 110 (both as $CaCO_3$), dissolved organic carbon

(DOC) = 2.5 (all in mg/L), pH = 7.9) (Driessnack et al., 2016) for 21 days. The fish were fasted a day prior to the start of the experiment and throughout the experimental duration**.**

3.3.2 Experimental procedure

The experimental treatments consisted of four groups: control (no added metals in the exposure water), Zn (1mg/L), Zn (1mg/L) + Cd (20µg/L), and Zn (1mg/L) + Cu (100µg/L). Fish were exposed individually to nominal concentrations of waterborne metals (as mentioned above) for 24 hrs in 20L of exposure water under static non-renewal exposure condition. Each exposure was replicated three (3) times. The fish were continuously supplied aeration throughout the exposure duration. Metal concentrations were added as metallic salts of $ZnSO_4.7H_20$, $CdNO_3.4H_2O$ and $Cu(NO_3)_2.5H_20$ (all purchased from Sigma-Aldrich, MO, USA) for Zn, Cd, and Cu respectively. The concentrations of metals employed for this study are equivalent to the 96hr median lethal concentration (LC_{50}) of each individual metal in rainbow trout, as reported by Alsop et al (1999), Niyogi et al. (2008), and Taylor et al. (2000) under similar water chemistry conditions as used in the present study. For the measurement of dissolved metal concentrations, water samples were collected at the start and at the end of the exposure, filtered using a 0.45µm nylon syringe filter (Nalgene, NY, USA), and acidified with 0.2% HNO_3 (Trace metal grade; VWR, ON, Canada). They were subsequently stored at 4^oC until analysis. The actual (dissolved) metal concentrations for the exposure were verified by measuring the metal levels using a graphite furnace atomic absorption spectrometer (AAnalyst 800, Perkin Elmer, CT, USA). The actual concentrations of water samples were within ±10% of nominal concentrations. At the end of the exposure period, fish were euthanized with an overdose of Tricaine Methanesulfonate (MS-222) and gill baskets were excised. They were thoroughly cleaned in nanopure water and blotted dry with kim wipes. Gill tissues were thereafter placed in tissue cassettes, fixed in 10% neutral buffered

formalin (VWR, ON, Canada) for 24hrs. Following fixation, gill tissues were cryoprotected in 30% sucrose solution for 48hrs. They were then placed in cryomolds and flash frozen in optimal cutting temperature (OCT) medium using liquid nitrogen. Thereafter, they were kept at -80^{0}C until sectioning. Longitudinal sections of gill tissues were cut at an optimal cutting temperature of -20^{0}C using a cryotome (Leica, ON, Canada). Tissues were sectioned at a thickness of 12µm, deposited on CaF_2 windows (25mm x 1mm; Crystran, Poole, UK) and air dried at room temperature. Adjacent sections from the same gill tissues were also deposited on normal microscope slides (VWR, ON, Canada) and stained according to the procedures described in Section 2.3.3.

3.3.3 FTIRM Data Collection

FTIR imaging and spectroscopy data were acquired on a global source FTIR spectrometer coupled to a Hyperion 3000 microscope (Bruker Optics Inc., Milton, ON, Canada). The microscope was equipped with a 64 x 64 pixels Focal Plane Array (FPA) detector. For imaging, a 15x objective was used with image spatial resolution of 15µm x 15µm. Large areas of gill samples consisting of the primary and secondary lamellae were imaged. Data were collected in transmission mode in the mid-IR range of 4000-800 cm^{-1} at a spectral resolution of 4 cm^{-1}, with co-addition of 256 scans. A background image from a blank substrate was also collected with 256 scans co-added. A 2x2 pixel binning was employed with all data collection.

3.3.4 Data analysis

The primary and secondary gill lamellae of all the replicates of the experimental groups were analyzed separately. Spectra were extracted from several points of the primary and secondary lamellae using the OPUS software (version 7.0, Bruker Optics Inc., Billerica, MA), and were baseline corrected using a concave rubberband baseline correction of 10 iterations points. They

were also smoothed using a smoothing point of 9 points. Spectra taken from the primary and secondary lamellae of each replicate of each experimental group to get the overall spectrum of the primary and secondary lamellae of each experimental treatment. Thus, spectroscopic analysis was done on the average spectra of three replicates of each treatment. The imaging software, Cytospec (version 1.2.04), was used to generate FTIR functional group (proteins, lipids and nucleic acids) images. Images were normalized to the same intensity scale for ease of comparison across treatments.

3.4 Results

The fish gill IR spectrum is quite complex and consists of contributions from proteins, lipids, carbohydrates, and nucleic acids components. These biomolecules have distinct peaks corresponding to specific infrared light frequency or wavelength. Figure 3.1 shows the averaged FTIR spectrum of a representative rainbow trout gill tissue.

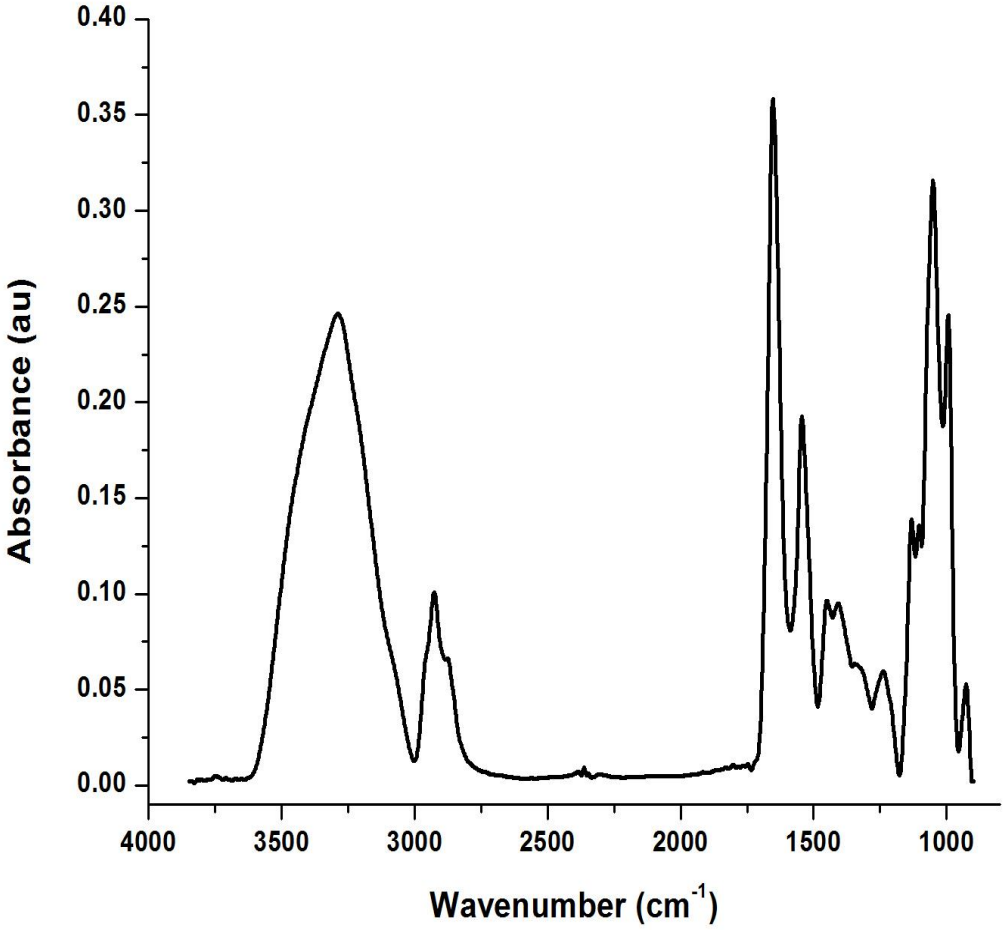

Figure 3.1: A representative FTIR absorbance spectrum of a control rainbow trout gill tissue. Bands assignments are displayed in Table 3.1

Table 3.1: Tentative band assignment of the FTIR spectrum of rainbow trout gill tissue based on data provided in previous studies (Chu et al., 2001; Davis and Mauer, 2010; Palaniappan and Pramod, 2010)

S/N	Peak position (cm^{-1})	Tentative assignment
1	3288	Amide A, mainly N-H stretching of proteins with negligible contribution from O-H stretching
2	2961	CH_3 asymmetric stretching, mainly lipids with little contribution from proteins and nucleic acids
3	2927	CH_2 asymmetric stretching, mainly lipids with little contribution from proteins and nucleic acids
4	2893	CH_3 symmetric stretching, mainly lipids with little contribution from proteins and nucleic acids
5	2876	CH_2 symmetric stretching, mainly lipids with little contribution from proteins and nucleic acids
6	1735	C=O stretch of lipid esters
6	1653	Amide I, C=O stretching of proteins
7	1544	Amide II, N-H bending and C-N stretching of the polypeptides and proteins backbone
8	1451	CH_2 bending, mainly lipids with little contribution from proteins and nucleic acids
9	1409	COO^- symmetric stretching, mainly fatty acids and amino acids from proteins
10	1238	PO_2^- asymmetric stretching of nucleic acids with little contribution from phospholipids
11	1132	C-O-C asymmetric stretching of glycogen and nucleic acids
12	1108	C=O from carbohydrates
13	1052	PO_2^- symmetric stretching of phospholipids
15	900-600	"Fingerprint region"

3.4.1 Lipids

There are many FTIR bands that can be used to monitor lipids in biological samples. Two of such bands include the CH_2 asymmetric (2922cm^{-1}) and CH_2 symmetric (2872cm^{-1}) stretching vibrations. The changes in these bands across the different experimental treatments are displayed in Figure 3.2. Another band, which is also indicative of the lipids status in biological samples is the bending vibration of the CH_2 group (1453cm^{-1}). The changes in this band across the different experimental treatments are displayed in Figure 3.3. The infrared images showing the changes in the spatial distribution of lipids across the different experiments are displayed in Figure 3.4.

In the present study, exposure to metal(s) resulted in an overall increase in the absorption of the asymmetric and symmetric CH_2 groups in both the primary and secondary lamellae of fish gills compared to the control (Figure 3.2). This increase in absorption was more prominent for Cu than either Cd or Zn in the primary lamellae of the fish gill (Figure 3.2A). Furthermore, the intensities of the lipid bands were similar in the Zn + Cu and Zn + Cd treatments in the primary lamellae, although both were lower than the Cu + Cd treatment. Out of the three mixture treatments, exposure to Cu + Cd resulted in the highest overall increase in absorption of the asymmetric and symmetric CH_2 groups (Figure 3.2A). In contrast, in the secondary lamellae, the increase in absorption of these bands in the single metal treatments was in the order of Cu > Zn > Cd (Figure 3.2B), whereas for the mixture treatments, the overall increase in the absorption of these bands was in the order of Cu + Cd > Zn + Cd > Zn + Cu (Figure 3.2B). Interestingly, co-exposure of Zn + Cd or Zn + Cu reduced the overall absorption of the lipid band (3000-2800cm^{-1}) relative to Cd or Cu only exposure. On the other hand, co-exposure to Cu + Cd resulted in an increase in the overall absorption of the lipid band (3000-2800cm^{-1}), which was greater than that in the individual treatment to Cd, but not Cu (Figure 3.2A and Figure 3.2B).

The lipid band arising from the bending vibration of the CH_2 group was also affected by exposure to metal(s). This was evident from the bifurcation of this band, which was centered at about 1454cm^{-1} in the control, into two halves in the Cu (1462cm^{-1} and 1446cm^{-1}) and Cd treatments (1446cm^{-1} and 1453cm^{-1}) of the primary lamellae of the fish gill. These changes were, however, not observed in the primary lamellae of the Zn, Zn + Cu and Zn + Cd treatments. Similar to the effect observed in the Cu or Cd only treatment, co-exposure to Cu + Cd also resulted in the bifurcation of the CH_2 bending vibration band into two halves (1464cm^{-1} and 1451cm^{-1}) in the primary lamellae of the fish gill (Figure 3.3A). On the other hand, the peak of the CH_2 lipid band in the Zn, Zn + Cd, Zn + Cu and Cu + Cd treatments of the secondary lamellae of the fish gill was not bifurcated, but it shifted to a new wavenumber (1457cm^{-1}) compared to the control, which had a peak at 1453cm^{-1}. Moreover, the peak of the Cu only and Cd only treatments was also bifurcated into two halves (1462cm^{-1} and 1446cm^{-1}) in the secondary lamellae (Figure 3.3B).

The spatial distribution of lipids within the region of 3000-2800 cm^{-1} also revealed that exposure to metal(s) noticeably increased lipid accumulation in fish gills and is in general agreement with the observations from the FTIR spectra analysis. Compared to the control, increased lipid accumulation in fish gills was mostly evident in the Cu, Cd and Cu + Cd treatments, respectively (Figure 3.4).

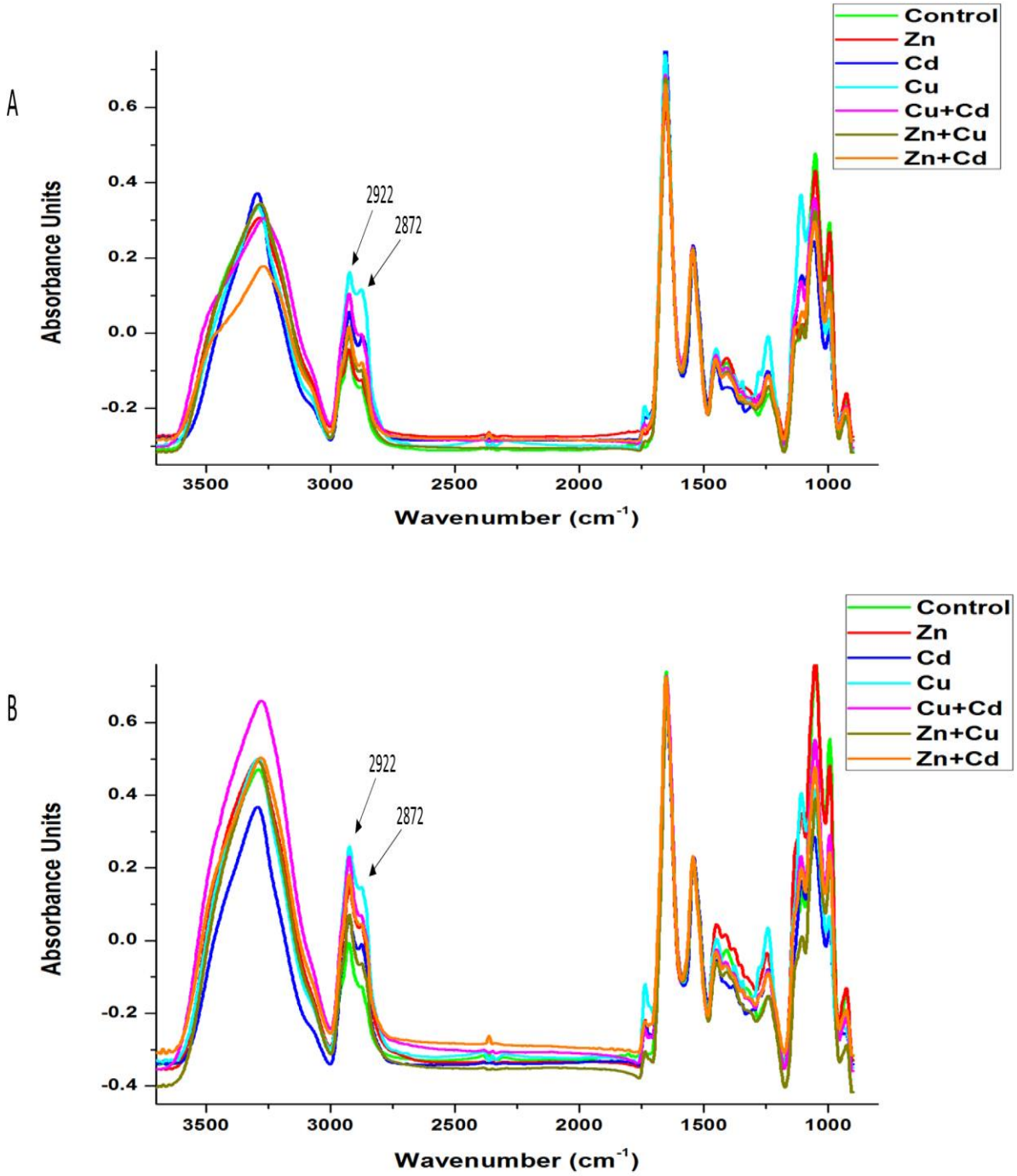

Figure 3.2: The average FTIR spectra (n=3) of different experimental treatments showing the changes in the lipid region (3000-2800cm^{-1}) in the primary lamellae (A) and secondary lamellae (B) of rainbow trout gill. Spectra were normalized with respect to the amide II band (1595-1485cm^{-1}).

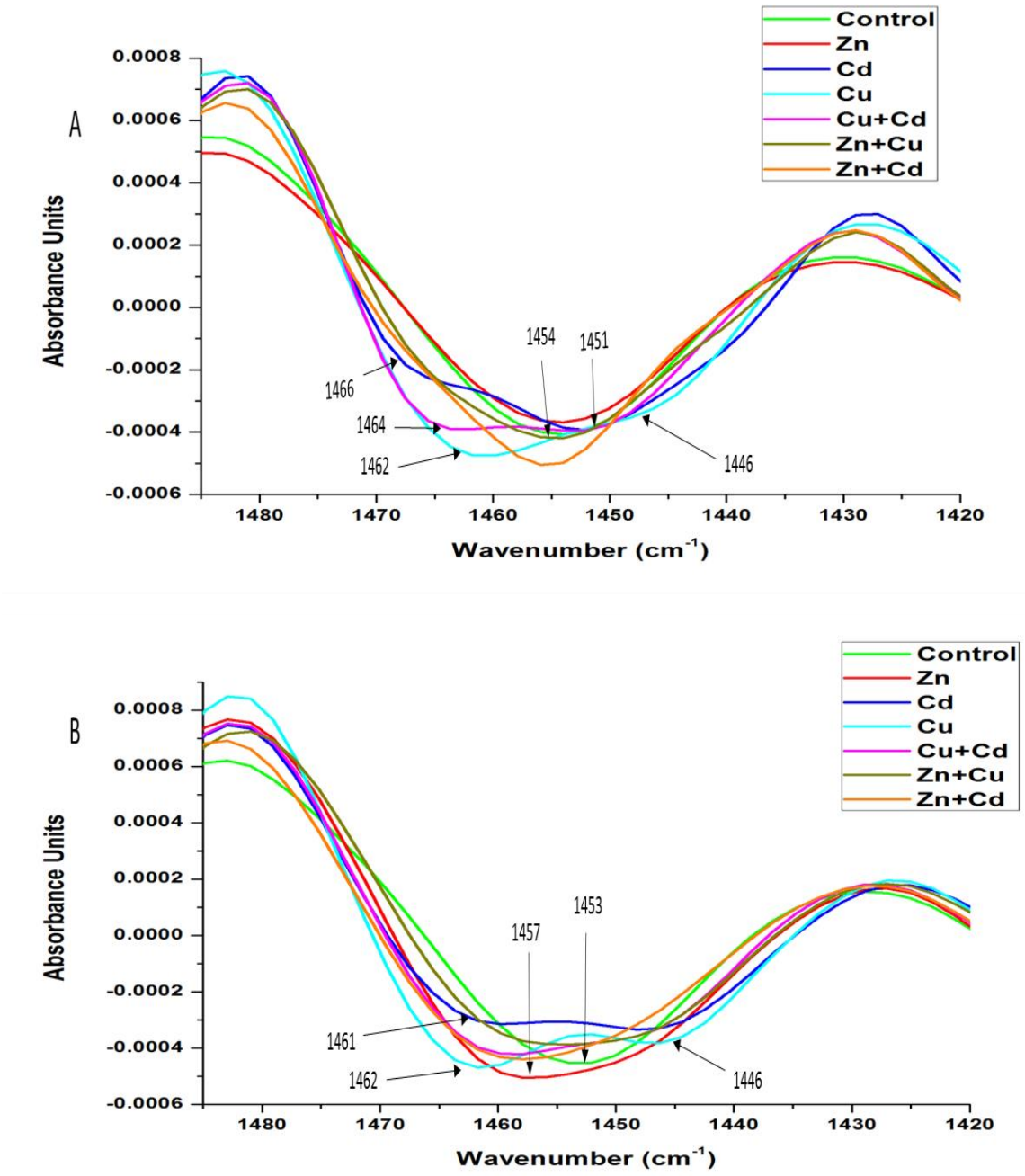

Figure 3.3: The average FTIR spectra (n=3) of different experimental treatments showing the changes in the bending CH_2 band of lipids in the primary lamellae (A) and secondary lamellae (B) of rainbow trout gill. Spectra were converted to second derivatives and normalized with respect to the amide II band (1595-1485cm^{-1}).

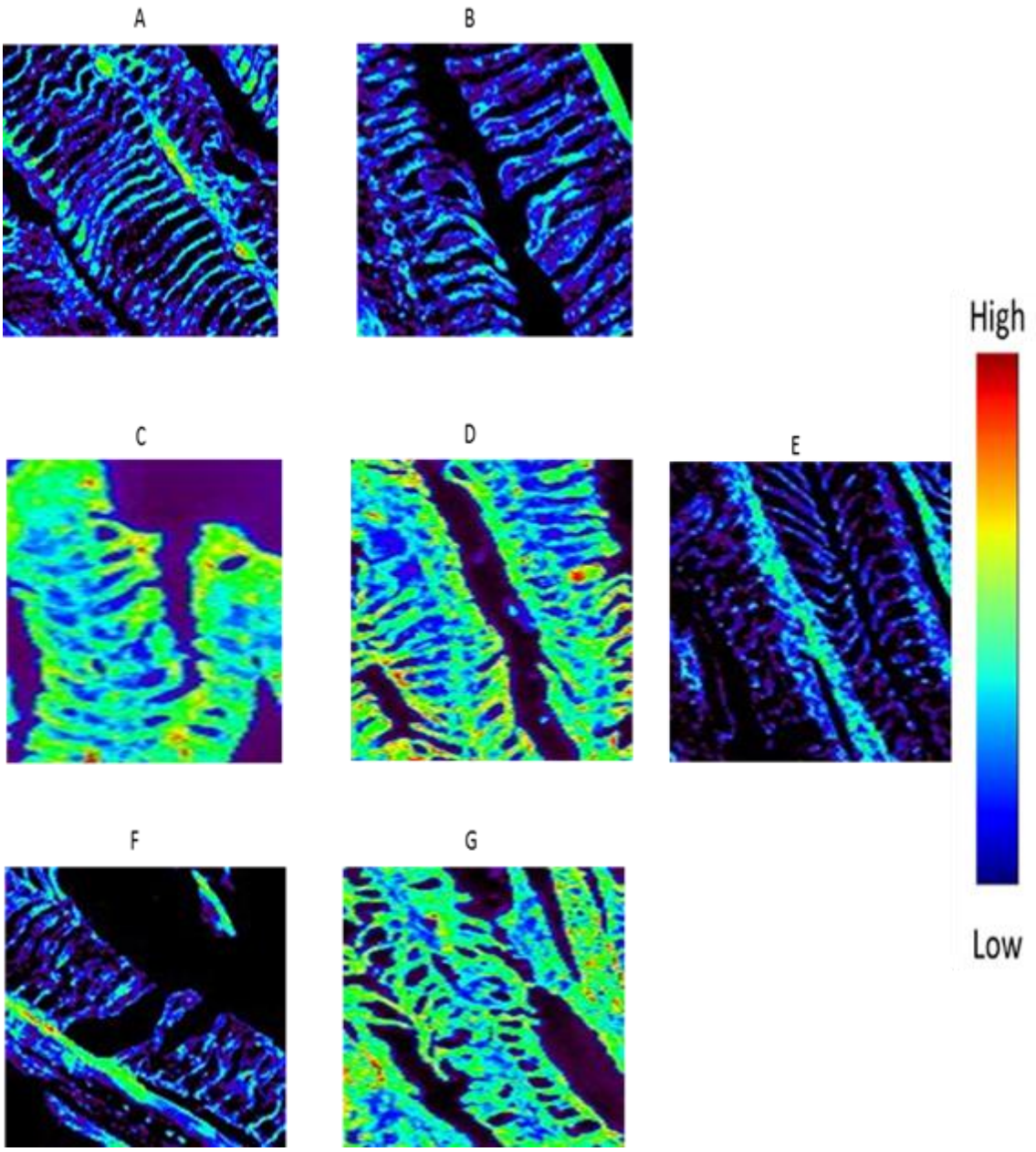

Figure 3.4: Representative infrared micrographs of rainbow trout gill (magnification: 15X) showing the distribution of lipids (3000 -2800cm^{-1}) across different experimental treatments. All maps are normalized to the same scale for ease of comparison. (A) Control (no added metals), (B) Zn (1mg/L), (C) Cu (100µg/L), (D) Cd (20µg/L), (E) Zn (1mg/L) + Cu (100µg/L), (F) Zn (1mg/L) + Cd (20µg/L), and (G) Cu (100µg/L) + Cd (20µg/L).

3.4.2 Proteins

There are 8 bands in the mid-IR region, which are indicative of cellular proteins in biological tissues. These include the amide A, amide B, and amide I-VI, in decreasing order of wavenumber. Out of these bands, the amide I (1700 -1600cm^{-1}) and the amide II (1595-1485cm^{-1}) are the two most prominent protein bands and are often used to analyze conformational changes in the structure of proteins (Barth, 2007). The changes in these bands across the different experimental treatments are displayed in Figure 3.5. The infrared images showing the spatial distribution of proteins (amide I) across the different experimental treatments are displayed in Figure 3.6.

The analysis of the changes in the proteins of the primary and secondary lamellae of the experimental treatments showed a very interesting finding. Exposure to waterborne Cu, Cd or Zn alone resulted in the reduction of the protein content of the primary lamellae of the fish gill (Figure 3.5A). Protein content reduction is evident by the decrease in the intensity of the peak of the amide I band (1653cm^{-1}) of these treatments compared to the control. It is also important to note here that Cu caused the maximum reduction of protein component in the primary lamellae of the fish gill followed by Cd and Zn, respectively. Protein content reduction was also apparent in the mixture treatments. Co-exposure to Cu + Cd produced the highest reduction in protein content out of the three mixture treatments tested in the present study. However, the intensity of the amide I band of the Zn alone, and Zn + Cu treatments were almost the same, and relatively lower than those in the Cu + Cd treatment group (Figure 3.5A). The protein content of the secondary lamellae of the fish gill was also altered by exposure to metal(s). For the single metal treatments, the decrease in protein content was highest with exposure to Cu. However, the infrared absorption spectra of the Zn, and Cd alone treatments were similar (Figure 3.5A). The decrease in the protein content in the

secondary lamellae for the mixture treatments was similar to the trend observed in the primary lamellae (i.e., Cu + Cd > Zn + Cd > Zn + Cu). The trends observed in the amide I band of the primary and secondary lamellae of the fish gill across the different experimental treatments were essentially the same for the amide II band, which has a peak at about 1545cm^{-1}. It is important to note here that the decrease in the intensities of amide I and amide II bands of protein induced by Cu or Cd alone treatments was alleviated during their co-treatment with Zn. In contrast, co-treatment of Cu + Cd resulted in a partial recovery of the intensities of amide I and amide II bands of proteins relative to that in the individual treatment of Cu, but not Cd (Figure 3.5A and Figure 3.5B).

The spatial distribution of proteins (amide I) in the Zn, Zn + Cu and Zn + Cd treatments did not appear to be significantly different from the control. The base of the primary lamellae of the fish gill appeared to be the major site of protein localization in these treatments. The intensity of proteins in the Cu, Cd, and Cu + Cd treatments were, however, markedly reduced compared to the control (Figure 3.6).

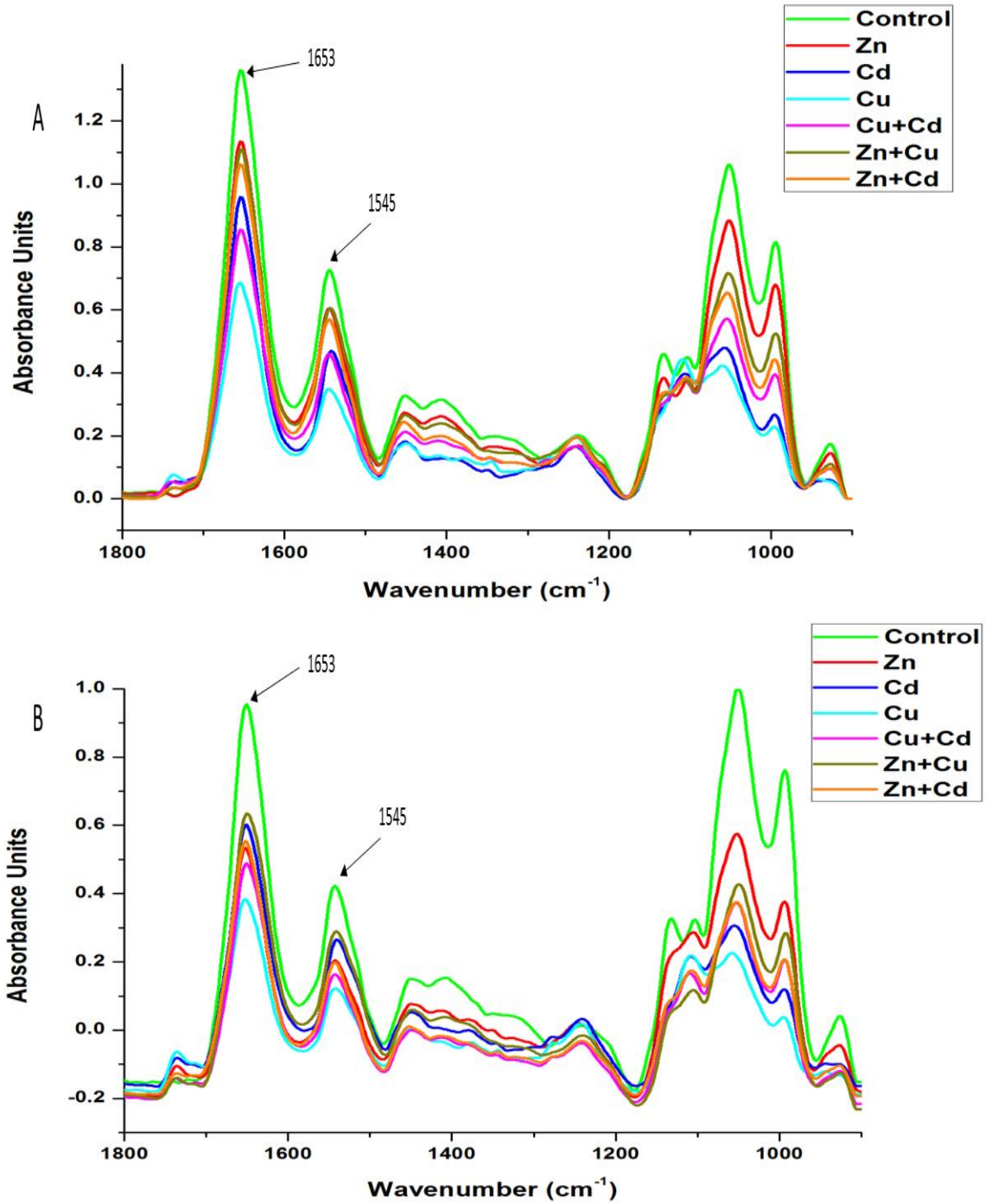

Figure 3.5: The average FTIR spectra (n=3) of different experimental treatments showing the changes in the protein region (1800 -1600 cm^{-1}) in the primary lamellae (A) and secondary lamellae (B) of rainbow trout gill. Spectra were normalized with respect to the lipid band (3000-2800cm^{-1}).

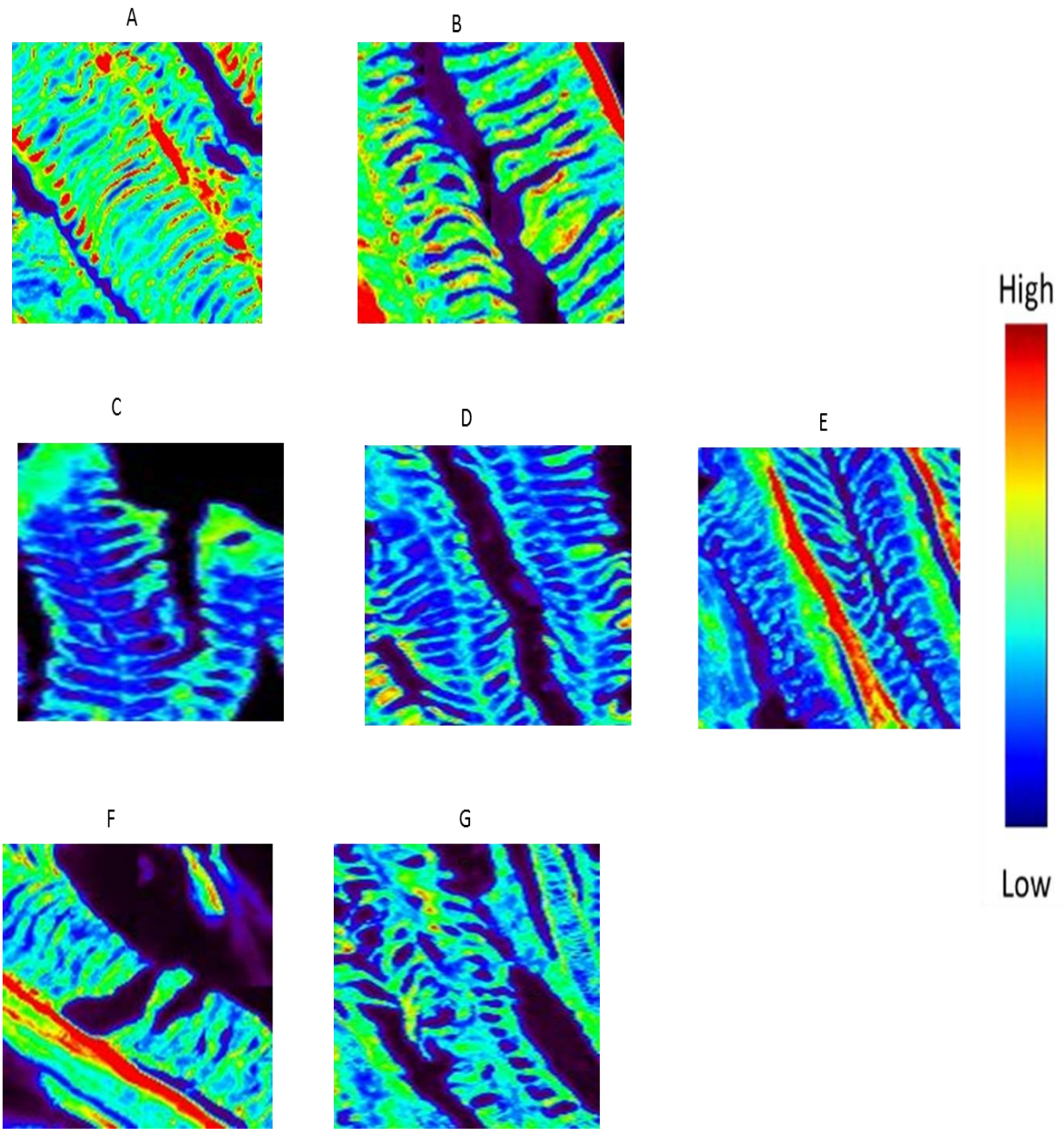

Figure 3.6: Representative infrared micrographs of rainbow trout gill (magnification: 15X) showing the distribution of proteins (1700 -1600cm^{-1}) across different experimental treatments. All maps are normalized to the same scale for ease of comparison. (A) Control (no added metals), (B) Zn (1mg/L), (C) Cu (100µg/L), (D) Cd (20µg/L), (E) Zn (1mg/L) + Cu (100µg/L), (F) Zn (1mg/L) + Cd (20µg/L), and (G) Cu (100µg/L) + Cd (20µg/L).

3.4.3 Nucleic acids

One of the FTIR bands which is indicative of the nucleic acids status of biological organisms is the asymmetric phosphate (PO_2^-) band (1239 cm^{-1}). The changes in this band across the different experimental treatments are displayed in Figure 3.7. The images showing the spatial distribution of nucleic acids across the different experimental treatments are also displayed in Figure 3.8.

A pronounced increase in the intensity of asymmetric phosphate band was noticed in both primary and secondary lamellae of the rainbow trout gill following exposure to Cu alone (Figure 3.7A and 3.7B; Figure 3.8). The intensity of phosphate band in all of the other metal(s) treatments was similar to the control, and did not appear to differ from one other in the primary lamellae (Figure 3.7A). In the secondary lamellae, however, the intensity of phosphate band was somewhat elevated in the Cd alone and Cu + Cd treatments relative to the control, nonetheless it was still lower than the Cu only treatment (Figure 3.7B; Figure 3.8).

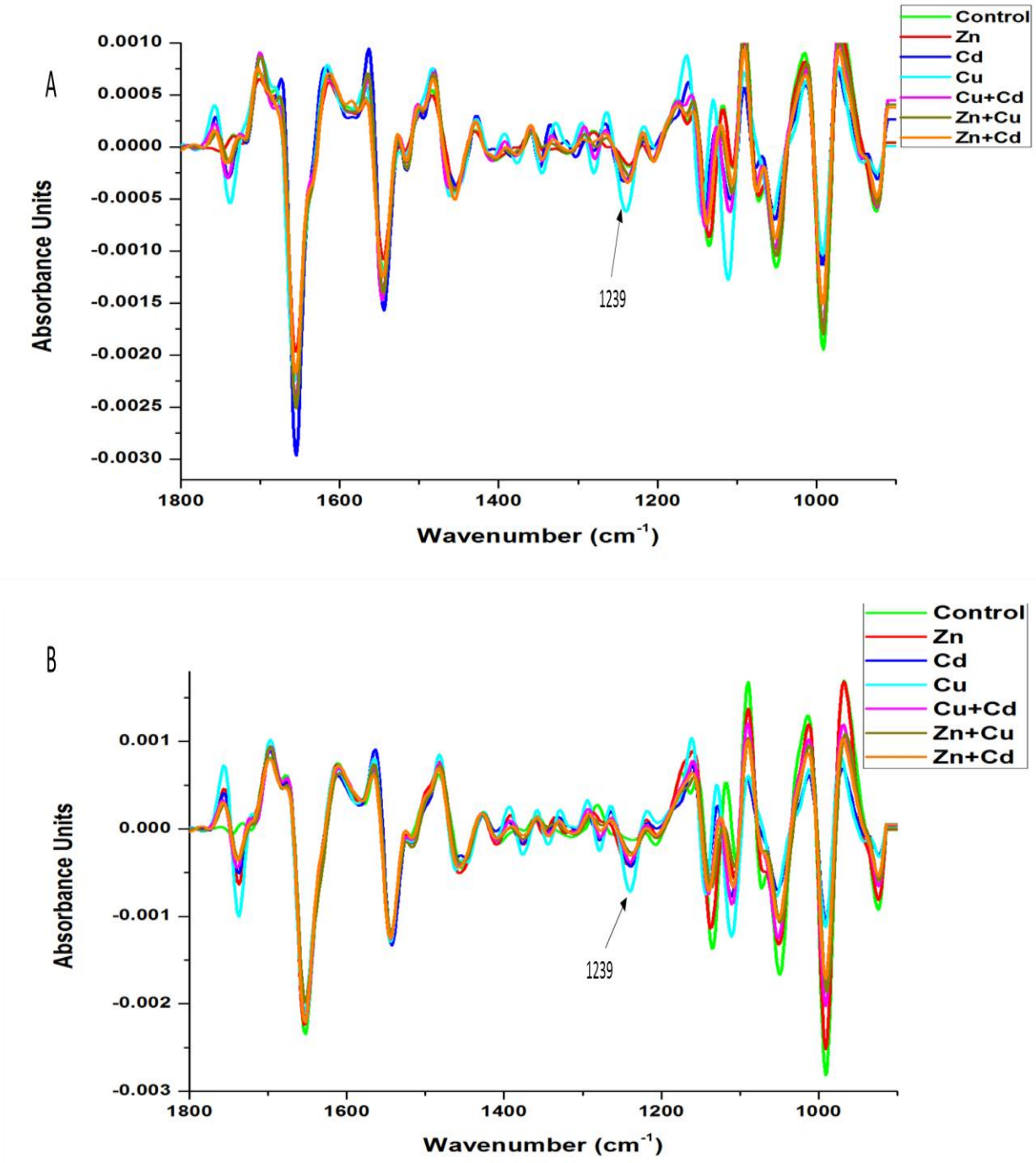

Figure 3.7: The average FTIR spectra (n=3) of different experimental treatments showing the changes in the nucleic acids (1300 – 1200 cm^{-1}) in the primary lamellae (A) and secondary lamellae (B) of rainbow trout gill. Spectra were converted to second derivatives and normalized with respect to the amide II band (1595 - 1485 cm^{-1}).

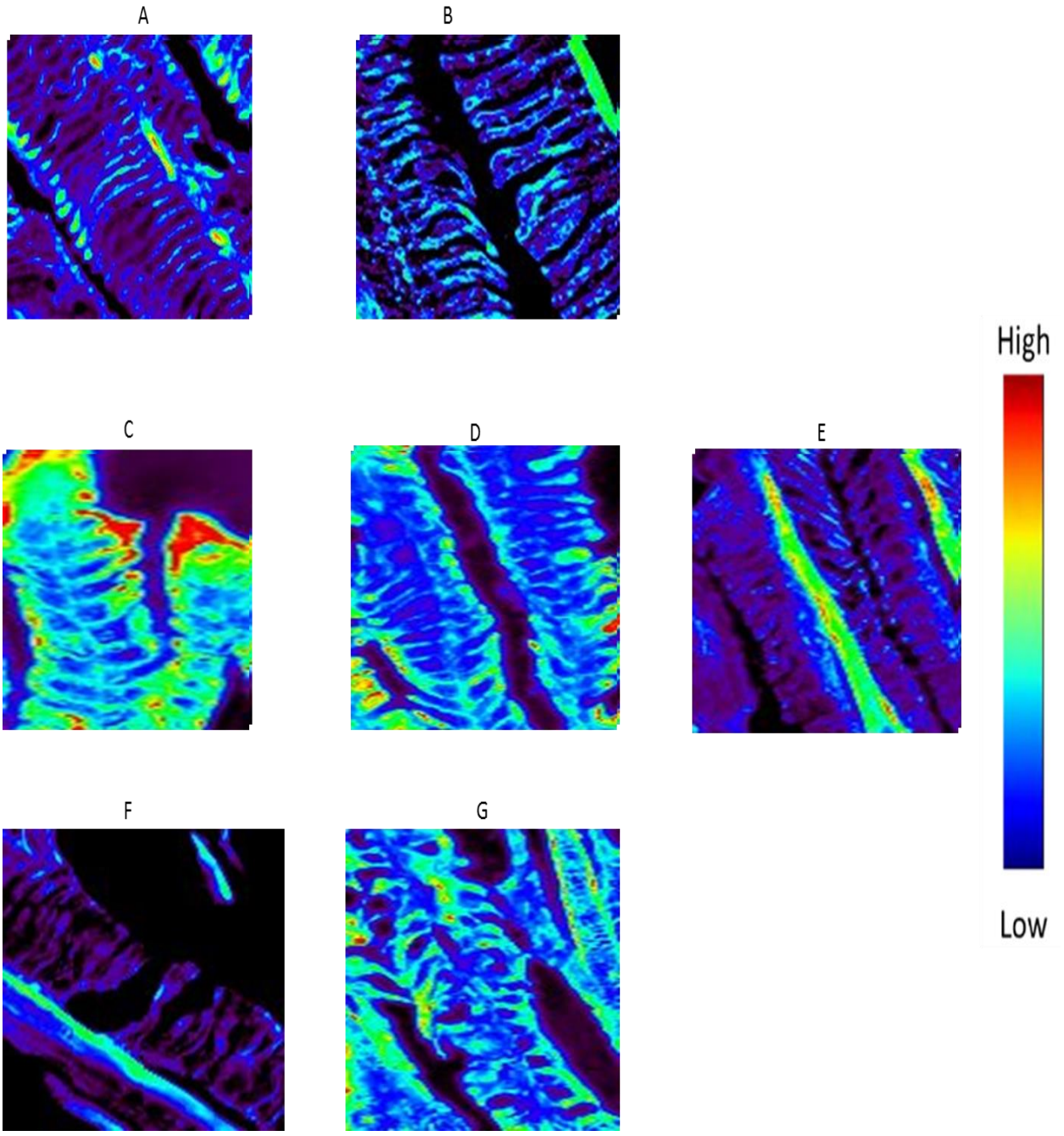

Figure 3.8: Representative infrared micrographs of rainbow trout gill (magnification: 15X) showing the distribution of nucleic acids (1300 -1200cm^{-1}) across different experimental treatments. All maps are normalized to the same scale for ease of comparison. (A) Control (no added metals), (B) Zn (1mg/L), (C) Cu (100µg/L), (D) Cd (20µg/L), (E) Zn (1mg/L) + Cu (100µg/L), (F) Zn (1mg/L) + Cd (20µg/L), and (G) Cu (100µg/L) + Cd (20µg/L).

3.5 Discussion

Some metals such as Zn and Cu are essential components of all biological organisms, including fish as they are involved in the maintenance of cellular and metabolic functions. Although these metals are essential, they could also be toxic at concentrations above the threshold of metabolic requirements. Metals such as Cd, on the other hand, are non-essential and have no known biological relevance in fish (McGeer et al., 2011). The results of this study demonstrated that exposure of fish to these metals at acutely toxic concentrations both singly and in binary mixture could have deleterious effects on the structure and composition of gill biomolecules. The overall increase in the infrared absorption of the lipids bands within the region of 3000-2800cm^{-1} as observed in the present study suggests increased lipid accumulation as a result of metal exposure. It has been demonstrated in a number of mammalian studies that increase in the intensity of these bands may reflect less lipophilic environment due to fragmentation of lipoproteins, and this could have severe impacts on membrane fluidity (Anastassopoulou and Theophanides, 1990; Arsov and Quaroni, 2007; Liu et al., 2002; Toyran et al., 2007). Among the single metal exposures used in the present study, the increase in lipid accumulation was most prominent with Cu compared to either Cd or Zn. An increased lipid accumulation was also observed in the Cu + Cd treatment. However, co-exposure to Zn + Cu and Zn + Cd reduced the extent of lipid accumulation observed in the Cu or Cd only treatments. This could imply that Zn may have an antagonistic effect on Cu or Cd induced alteration of the lipid components of the fish gills. Furthermore, the bifurcation of the bending vibration of the CH_2 group observed in the Cu and Cd treatments is an indicator of lipid peroxidation. This bifurcation of band was also present in the Cu + Cd treatment but not in the Zn + Cd and Zn + Cu treatments. These results indicate that Zn can ameliorate Cu or Cd induced lipid peroxidation in the fish gill.

Reduction in the intensities of the amide I and amide II bands in the experimental treatments as observed in the present study suggests degradation or loss of protein levels in the fish gill as result of increased protein oxidation (Cakmak et al., 2006) . Among the individual metals tested in this study, Cu was found to cause the maximum degradation of proteins in rainbow trout gills, and this effect was also evident with Cd, albeit to a slightly lower degree. Similarly, Zn exposure also decreased protein content of the gills, although the extent of protein degradation was moderate compared to that of Cu or Cd. On the other hand, all three mixture treatments reduced the protein content of the fish gill relative to the control, but the degree of protein degradation was moderate in the Zn + Cu or Zn + Cd treatment compared to the Cu + Cd treatment. In fact, the infrared absorption of the amide I and amide II bands of proteins in the Zn + Cu and Zn + Cd treatments were comparable to that of Zn alone, suggesting that Zn may elicit a protective effect on Cu or Cd-induced protein degradation in the fish gill. This protective effect was more evident in the primary lamellae than the secondary lamellae of the fish gill, and this could be due to the fact that Zn preferentially accumulated on the base of the primary lamellae (as described in Chapter 2).

The increase in the infrared absorption of the phosphate band in the fish gill may imply an increase in the relative content of nucleic acids and/or upregulation of cellular activities (i.e., DNA/RNA ratio - a metabolic strategy to cope with the increased metal accumulation (Ci et al., 1999). Similar to the trends observed with lipid and protein bands, Cu produced the maximum increase in the infrared absorption of phosphate band in the gill compared to other treatments, and this was followed by Cu + Cd and Cd, respectively. This indicates that the increased intensity of the phosphate band could be attributed mainly to Cu and/or Cd exposure, and might have occurred as a compensatory response to the increased protein and lipid degradation induced by these metals.

Taken together, the results in this present study indicate that Zn may have a protective effect on Cu or Cd induced alteration of the biochemical components of the fish gill. A factor which could account for the interactions of Zn with Cu or Cd on the fish gill biomolecules as observed in this study is the antioxidant property of Zn. The anti-oxidant properties of Zn have long been demonstrated in mammals (Bray and Bettger, 1990; Powell, 2000). In biological organisms, oxidative stress occurs as a result of the imbalance in the production and removal of reactive oxygen species (ROS) by antioxidant defense systems (Stohs and Bagchi, 1995). ROS are usually produced in all biological organisms as a result of metabolic activities (e.g., oxidative phosphorylation). They can also be induced by pro-oxidants such as metals (Jomova et al., 2012). There are basically two ways by which metals can induce oxidative stress in living organisms. Firstly, redox-active metals such as Fe, Cu, Cr, and Co can induce oxidative stress by catalyzing the production of free radicals and reduced oxygen species such as superoxide anions and hydrogen peroxide through a reaction known as the Fenton reaction. This could lead to cellular damage of proteins, lipids, and DNA (Valko et al., 2005). Secondly, metals such as Cd, Hg, and Pb, on the other hand, could also induce oxidative stress in biological organisms by depleting the pool of thiol compounds such as glutathione and metallothionein (Valko et al., 2005). These thiol compounds are able to suppress the catalytic ability of Cu and other transition metals to participate in the Fenton reaction by chelating them (Jomova et al., 2012). Unlike most transition metals, Zn does not readily participate in the Fenton reaction. This is because it is not redox-active, as it has a completely filled d shell with 10 d electrons. Zinc is an essential component of antioxidant enzymes such as Cu/Zn- SOD (superoxide dismutase). Zinc could also offer protection against oxidative stress by inducing the synthesis of metallothionein, which can bind metals with pro-oxidant abilities such as Cu and Cd. Zn could also induce the synthesis of other thiol compounds,

which are capable of scavenging hydroxyl radicals and singleton oxygen (Berg and Shi, 1996; Bray and Bettger, 1990; Dondero et al., 2005). It is to be noted as well that the antagonistic effects of Zn against Cd, but not Cu, could also be attributed to the possible reduction in Cd gill burden due to the competitive interaction between these two metals for uptake (see Chapter 2). On the other hand, although Zn is both an essential metal and an antioxidant, it can also be toxic at high concentrations. In the present study, exposure to Zn alone, and in mixture with Cd or Cu was found to cause a moderate degree of alteration in the composition of gill biomolecules in fish. Although this observation may contradict the notion of Zn being an antioxidant, it is important to note that acutely toxic concentrations of Zn were used in this study, hence some degree of cellular damage is to be expected.

To the best of our knowledge, this is the first study to examine the interactive effects of metals in binary mixtures on both the integrity and spatial distribution of the major biomolecules in the fish gill. To date, only a couple of previous studies have been carried out to examine the effects of single metals (Zn and As) on the integrity of biomolecules using bulk FTIR spectroscopy (Palaniappan et al., 2010; Palaniappan and Vijayasundaram, 2009). The bulk FTIR spectroscopy is an invasive technique, and does not allow *in situ* examination of the bio-molecular components and their spatial distribution in tissues. Nonetheless, Palaniappan et al. (2010) and Palaniappan and Vijayasundaram (2009) evaluated the effects of waterborne Zn and As in the gills of the carp (*Labeo rohita)* following an exposure period of 14 days. Consistent with our findings, they also reported that both Zn and As exposures significantly decreased the intensity of protein bands in the gills of *L. rohita*. In addition, they also observed alterations in the composition of lipids in *L. rohita* gills. Contrary to the observations in the present study, they found a decrease in the absorption of the lipid bands, implying a reduced lipid content in the gill following exposure to Zn

or As. One probable reason that could be attributed for this disparity is the difference in exposure duration and/or concentrations of metals between these studies. For example, Palaniappan et al. (2010) employed a Zn exposure concentration of 9.8 mg/L in their study, which was about 10-fold greater than the Zn concentration used in the present study. Exposure to a higher concentration of Zn over an extended period of time might have caused a decrease in lipid content due to the usage of lipids to sustain the energetic cost of metal detoxification in the fish gill.

It is important to note here that although the exposure concentrations of metals (Zn, Cu and Cd) used in this study were high, the levels are still environmentally relevant. For example, Zn concentrations of more than 1000µg/L have been reported in some freshwaters (Luoma and Rainbow, 2008). Similarly, concentrations as high as 100µg/L have also been reported in some aquatic systems for Cu and Cd, respectively (USEPA, 2007; WHO/UNEP, 1989). Overall, the results of this study imply that the interactions of Zn and Cd, or Zn and Cu during short-term (24 hr) waterborne exposure may elicit antagonistic effects in the fish gill. Since gill is considered to be the main site of toxic action for metals during acute waterborne exposure (Niyogi and Wood, 2004), such interactions may ultimately lead to less than additive overall toxicity in fish exposed to Zn and Cd, or Zn and Cu in binary mixture. At present, the information on the toxicity of metals in mixtures in aquatic organisms are extremely limited. Nonetheless, evidence of antagonistic effects of Zn and Cd, and Zn and Cu in binary mixtures on fish mortality during acute exposure has been reported in the literature (Finlayson and Verrue, 1982; Mebane et al., 2012). On the other hand, the findings of this study also suggest that the interactions of Cu and Cd in the gill may induce additive or greater than additive overall toxicity in fish. This assumption is supported by Finlayson and Verrue (1982), who reported an additive toxicity of Cu and Cd in binary mixture during acute exposure in Chinnok salmon (*Oncorhynchus tshawytscha*).

3.6 Conclusions

This study demonstrated that the composition and spatial distribution of vital biomolecules in the fish gill could be critically altered as a result of short-term acute exposure to waterborne Zn, Cu and Cd, alone and in binary mixtures. This alteration can occur both in the primary and secondary lamellae of the fish gill, and it mainly involves increased lipid accumulation and protein degradation. Interestingly, this study also revealed that Zn could elicit an antagonistic effect on the adverse biochemical changes induced by Cu or Cd in the fish gill, which might ultimately translate into less than additive toxicity in fish exposed to acutely toxic levels of Zn and Cd, and Zn and Cu in mixture. In addition, the findings of this study also suggest that the biochemical interactions of Cu and Cd in the fish gill could likely lead to additive or more than additive toxicity in fish during acute waterborne exposure.

4.0 GENERAL DISCUSSION

The overall goal of my thesis was to improve our mechanistic understanding of metal mixture interactions in the fish gill. The gill is considered to be main site of toxic action in fish during acute waterborne exposure to metals. The framework of the biotic ligand model (BLM), which is currently being used by many jurisdictions (e.g., USEPA, EU) for assessing site-specific toxicity of metals in aquatic ecosystems, is based on gill binding characteristics of metals. To date, the BLM has been successfully employed to assess the toxicity of single metals. However, there is a clear need to further develop the BLM approach in order to make it capable of assessing metal mixture toxicity in aquatic organisms including fish, as they are usually exposed to a mixture of metals in the natural environments. To this end, my thesis provides important novel insights into the interactions of metals (Zn, Cu and Cd) in the fish gill, which may ultimately help in the contemporary effort to design metal-mixture BLMs.

In my thesis, I used a suite of synchrotron based analytical techniques (μ-XFI, μ-XANES, and FTIRM) to investigate the interactions of Zn, Cu and Cd in binary mixtures during acute waterborne exposures in the gills of a model sensitive teleost, rainbow trout (*Oncorhynchus mykiss*). One of the most novel aspects of my work is the application of these techniques, which allowed me to study the interactions of these metals *in situ,* and also to analyze alterations in metal distribution as well as metal-induced biochemical changes spatially across the gill tissue.

In Chapter II, I demonstrated that chloride (mitochondria-rich) cells are likely to be the main site of Zn accumulation, and also Zn predominantly co-localizes with Ca and S in the gill tissue. My findings also indicated that Zn is largely complexed by phosphate, followed by histidine and cysteine moieties in the gill tissue. This suggested that the metal-rich granules are the main sites of zinc detoxification in the fish gill. Co-exposure to Cd was found to displace Zn spatially

across the gill, and exposure to Zn, both alone and in combination with Cd, induced loss of Ca from the gill. In contrast, co-exposure to Cu did not influence the distribution of Zn in the gill tissue, and it also did not affect the strong co-localization pattern of Zn and Ca. These findings suggested that Cd and Zn interact with each other, at least partially, through shared uptake and handling pathways (Ca antagonism), whereas Cu does not affect the uptake of Zn as well as the dynamics of Zn and Ca in the fish gill. Interestingly however, my findings also indicated that both Cu and Cd might displace Zn bound with thiols (cysteine) in the fish gill. Overall, the research presented in Chapter II suggests that Cd and Cu may interact with Zn *via* both common and disparate pathways in the fish gill.

In Chapter III, I evaluated the biochemical changes (protein and lipid peroxidation, and metabolic upregulation) in the fish gill during exposure to Zn, Cd and Cu, both singly and in binary mixtures. In general, exposure to Cu, alone and in combination with Cd, was found to induce maximum degradations of lipid and proteins in the fish gill, which was followed by exposure to Cd alone. Exposure to Zn alone caused the least adverse effects among the three metals examined, more importantly though the presence of Zn in binary metal exposures was found to ameliorate the adverse effects of Cu and/or Cd on the lipid and protein moieties of the fish gill. This ameliorative effect of Zn can be attributed to its competitive interaction for uptake with other metals such as Cd, the antioxidant property of Zn, and/or its ability to upregulate thiol synthesis and thereby metal-detoxification capacity. Although the evidence of antagonism between Zn and Cd, and Zn and Cu on the overall toxicity (lethality) in fish during acute waterborne exposure exists, this study provides new insights into the mechanisms underlying such antagonism.

Metals in mixture can cause less than additive or more than additive toxicity due to their interactions via shared pathways of uptake and metabolism in aquatic organisms. My research has

demonstrated that synchrotron-based analytical techniques can be employed successfully to gain critical and novel insights into the mechanisms by which metals interact with each other in target organs (e.g., gill) affecting their uptake, distribution, and toxic effects. The limitation of the research presented in this thesis, however, lies in the fact that the data were mainly qualitative in nature, and future investigations should focus on generating data that can be used to quantify the effects of metal-mixture interactions at the fish gill. It is also important to note here that metal mixture toxicity is likely to be influenced by the dose ratio of each metal in the mixture (Niyogi et al., 2015), and future studies should focus on investigating on how the interactive mechanisms identified in the present study are modulated by the relative changes in the concentration of each metal in the exposure. This type of information will be crucial for the development of metal mixture BLMs. In addition, future studies should also investigate the mechanistic interactions of metal mixtures in aquatic organisms under long-term chronic exposure scenarios. The mechanisms of acute and chronic toxicity of individual metals are often different, and thus the pathways involved in the chronic interactions of metals in mixture can be quite different than those observed in my study.

REFERENCES

Adams, M., Dillon, C., Vogt, S., Lai, B., 2016. Copper Uptake, Intracellular Localization and Speciation in Marine Microalgae Measured by Synchrotron Radiation X-Ray Fluorescence and Absorption. Environmental 50, 8827–8839.

Adhikari, S., Mohanty, M., 2011. Effect of waterborne boron and molybdenum on survival, growth and feed intake of Indian major carp, *Cirrhinus mrigala* (Hamilton). Chem. Ecol. 28, 113–121.

Alsop, D., Wood, C.M., 2011. Metal uptake and acute toxicity in zebrafish: Common mechanisms across multiple metals. Aquat. Toxicol. 105, 385–393.

Anastassopoulou, J., Theophanides, T., 1990. Raman studies of model vesicle systems. Appl. Spectrosc. 44, 523–525.

Arsov, Z., Quaroni, L., 2007. Direct interaction between cholesterol and phosphatidylcholines in hydrated membranes revealed by ATR-FTIR spectroscopy. Chem. Phys. Lipids 150, 35–48.

ATSDR, 2004. Toxicological Profile for Copper. Atlanta, GA.

Barth, A., 2007. Infrared spectroscopy of proteins. Biochim. Biophys. Acta - Bioenerg. 1767, 1073–1101.

Bauer, M.C., Nilsson, H., Thulin, E., Frohm, B., Malm, J., Linse, S., 2008. Zn^{2+} binding to human calbindin D(28k) and the role of histidine residues. Protein Sci. 17, 760–767.

Beauchemin, S., Hesterberg, D., Nadeau, J., McGeer, J.C., 2004. Speciation of Hepatic Zn in Trout Exposed to Elevated Waterborne Zn Using X-ray Absorption Spectroscopy. Environ. Sci. Technol. 38, 1288–1295.

Beaumont, M.W., Butler, P.J., Taylor, E.W., 2000. Exposure of brown trout, *Salmo trutta*, to a sublethal concentration of copper in soft acidic water: effects upon muscle metabolism and

membrane potential. Aquat. Toxicol. 51, 259–272.

Beaumont, M.W., Butler, P.J., Taylor, E.W., 1995. Exposure of brown trout, *Salmo trutta*, to sub-lethal copper concentrations in soft acidic water and its effect upon sustained swimming performance. Aquat. Toxicol. 33, 45–63.

Benaduce, A.P.S., Kochhann, D., Flores, É.M.M., Dressler, V.L., Baldisserotto, B., 2008. Toxicity of cadmium for silver catfish *Rhamdia quelen* (Heptapteridae) embryos and larvae at different alkalinities. Arch. Environ. Contam. Toxicol. 54, 274–282.

Berg, J.M., Shi, Y., 1996. The Galvanization of Biology: Appreciation for the Roles of Growing Zinc. Science (80-.). 271, 1081–1085.

Blackstock, W.P., Weir, M.P., 1999. Proteomics: quantitative and physical mapping of cellular proteins. Trends Biotechnol. 17, 121–127.

Blindauer, C.A., Harvey, I., Bunyan, K.E., Stewart, A.J., Sleep, D., Harrison, D.J., Berezenko, S., Sadler, P.J., 2009. Structure, properties, and engineering of the major zinc binding site on human albumin. J. Biol. Chem. 284, 23116–23124.

Bodar, C.W.M., Pronk, M.E.J., Sijm, D.T.H.M., 2005. The European Union risk assessment on zinc and zinc compounds: the process and the facts. Integr. Environ. Assess. Manag. 1, 301–319.

Bray, T.M., Bettger, W.J., 1990. The physiological role of zinc as an antioxidant. Free Radic. Biol. Med. 8, 281–291.

Brungs, W., 1969. Chronic toxicity of zinc to the fathead minnow, *Pimephales promelas* (Rafinesque). Trans. Am. Fish. Soc. 3, 272-279.

Brzóska, M.M., Moniuszko-Jakoniuk, J., 2001. Interactions between cadmium and zinc in the organism. Food Chem. Toxicol. 39, 967–980.

Bury, N.R., Grosell, M., 2003. Waterborne iron acquisition by a freshwater teleost fish, zebrafish *Danio rerio*. J. Exp. Biol. 206, 3529–3535.

Bury, N.R., Walker, P.A., Glover, C.N., 2003. Nutritive metal uptake in teleost fish. J. Exp. Biol. 206, 11–23.

Büsselberg, D., 1995. Calcium channels as target sites of heavy metals. Toxicol. Lett. 82, 255–261.

Cakmak, G., Togan, I., Severcan, F., 2006. 17. Estradiol induced compositional, structural and functional changes in rainbow trout liver, revealed by FT-IR spectroscopy: A comparative study with nonylphenol. Aquat. Toxicol. 77, 53–63.

Cao, L., Huang, W., Liu, J., Yin, X., Dou, S., 2010. Comparative Biochemistry and Physiology , Part C Accumulation and oxidative stress biomarkers in Japanese flounder larvae and juveniles under chronic cadmium exposure. Comp. Biochem. Physiol. Part C 151, 386–392.

Caumette, G., Koch, I., Moriarty, M., Reimer, K.J., 2012. Arsenic distribution and speciation in *Daphnia pulex*. Sci. Total Environ. 432, 243–250.

Chiaverini, N., De Ley, M., 2010. Protective effect of metallothionein on oxidative stress-induced DNA damage. Free Radic. Res. 44, 605–613.

Chu, H.L., Liu, T.Y., Lin, S.Y., 2001. Effect of cyanide concentrations on the secondary structures of protein in the crude homogenates of the fish gill tissue. Aquat. Toxicol. 55, 171–176.

Ci, Y.X., Gao, T.Y., Feng, J., Guo, Z.Q., 1999. Fourier transform infrared spectroscopic characterization of human breast tissue: implications for breast cancer diagnosis. Appl. Spectrosc. 53, 312–315.

Cooper, C.A., Shayeghi, M., Techau, M.E., Capdevila, D.M., MacKenzie, S., Durrant, C., Bury, N.R., 2007. Analysis of the rainbow trout solute carrier 11 family reveals iron import ≤ pH

7.4 and a functional isoform lacking transmembrane domains 11 and 12. FEBS Lett. 581, 2599–2604.

Craig, P.M., Wood, C.M., McClelland, G.B., 2010. Water chemistry alters gene expression and physiological end points of chronic waterborne copper exposure in zebrafish, *Danio rerio*. Environ. Sci. Technol. 44, 2156–2162.

D'Souza, L., Devi, P., Shridhar, D.M., Naik, C.G., 2008. Use of Fourier Transform Infrared (FTIR) spectroscopy to study cadmium-induced changes in *Padina tetrastromatica* (Hauck). Anal. Chem. Insights 3, 135–143.

Davis, R., Mauer, L., 2010. Fourier transform infrared (FT-IR) spectroscopy: a rapid tool for detection and analysis of foodborne pathogenic bacteria. Res. Technol. Educ. Topics 1, 1582-1594

De Boeck, G., De Smet, H., Blust, R., 1995. The effect of sublethal levels of copper on oxygen consumption and ammonia excretion in the common carp, *Cyprinus carpio*. Aquat. Toxicol. 32, 127–141.

De Samber, B., Evens, R., De Schamphelaere, K., Silversmit, G., Masschaele, B., Schoonjans, T., Vekemans, B., Janssen, C.R., Van Hoorebeke, L., Szalóki, I., Vanhaecke, F., Falkenberg, G., Vincze, L., 2008. A combination of synchrotron and laboratory X-ray techniques for studying tissue-specific trace level metal distributions in *Daphnia magna*. J. Anal. At. Spectrom. 23, 829.

Derek H. Alsop, James C. McGeer, D.G.M. andChris M.W., 1999. Costs of chronic waterborne zinc exposure and the consequences of zinc acclimation on the gill/zinc interactions of rainbow trout in hard and soft water. Environ. Toxicol. Chem. 18, 1014–1025.

Dethloff, G.M., Schlenk, D., Hamm, J.T., Bailey, H.C., 1999. Alterations in physiological

parameters of rainbow trout (*Oncorhynchus mykiss*) with exposure to copper and copper/zinc mixtures. Ecotoxicol. Environ. Saf. 42, 253–264.

Dexter, D.T., Carayon, A., Vidailhet, M., Ruberg, M., Agid, F., Agid, Y., Lees, A.J., Wells, F.R., Jenner, P., Marsden, C.D., 1990. Decreased ferritin levels in brain in Parkinson's disease. J. Neurochem. 55, 16–20.

Di Toro, D.M., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C., 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. Environ. Toxicol. Chem. 20, 2383–2396.

Diaz, J., Ingall, E., Vogt, S., De Jonge, M.D., Paterson, D., Rau, C., Brandes, J.A., 2009. Characterization of phosphorus, calcium, iron, and other elements in organisms at sub-micron resolution using X-ray fluorescence spectromicroscopy. Limnol. Oceanogr. Methods 7, 42–51.

Dondero, F., Piacentini, L., Banni, M., Rebelo, M., Burlando, B., Viarengo, A., 2005. Quantitative PCR analysis of two molluscan metallothionein genes unveils differential expression and regulation. Gene 345, 259–270.

Driessnack, M.K., Matthews, A.L., Raine, J.C., Niyogi, S., 2016. Interactive effects of chronic waterborne copper and cadmium exposure on tissue-specific metal accumulation and reproduction in fathead minnow (*Pimephales promelas*). Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 179, 165–173.

Dumas, P., Miller, L., 2003. The use of synchrotron infrared microspectroscopy in biological and biomedical investigations. A Collect. Pap. Present. Shedding New Light Dis. Opt. Diagnostics New Millenn. (SPEC 2002) Reims, Fr. 23-27 June 2002 32, 3–21.

Endo, T., Kimura, O., Hatakeyama, M., Takada, M., Sakata, M., 1997. Effects of zinc and copper

on cadmium uptake by brush border membrane vesicles. Toxicol. Lett. 91, 111–120.

Ercal, N., Gurer-Orhan, H., Aykin-Burns, N., 2001. Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. Curr. Top. Med. Chem. 1, 529–539.

Evans, D.H., 1987. The fish gill: site of action and model for toxic effects of environmental pollutants. Environ. Health Perspect. 71, 47–58.

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85, 97–177.

Eyckmans, M., Celis, N., Horemans, N., Blust, R., De Boeck, G., 2011. Exposure to waterborne copper reveals differences in oxidative stress response in three freshwater fish species. Aquat. Toxicol. 103, 112–120.

Finlayson, B.J., Verrue, K.M., 1982. Toxicities of copper, zinc, and cadmium mixtures to juvenile chinook salmon. Trans. Am. Fish. Soc. 111, 645–650.

Foran, C., Peterson, B., Benson, W., 2002. Influence of parental and developmental cadmium exposure on endocrine and reproductive function in Japanese medaka (*Oryzias latipes*). Physiol. Part C Toxicol. & Pharm 133(3), 345-354

Fu, H., Lock, R.A.C., Bonga, S.E.W., 1989. Effect of cadmium on prolactin cell activity and plasma electrolytes in the freshwater teleost *Oreochromis mossambicus*. Aquat. Toxicol. 14, 295–306.

Fukuda, N., Hokura, A., Kitajima, N., Terada, Y., Saito, H., Abe, T., Nakai, I., 2008. Micro X-ray fluorescence imaging and micro X-ray absorption spectroscopy of cadmium hyper-accumulating plant, *Arabidopsis halleri*, using high-energy synchrotron radiation. J. Anal.

At. Spectrom. 23, 1068.

Gachot, B., Poujeol, P., 1992. Effects of cadmium and copper on zinc transport kinetics by isolated renal proximal cells. Biol. Trace Elem. Res. 35, 93–103.

Galvez, F., Wong, D., Wood, C.M., 2006. Cadmium and calcium uptake in isolated mitochondria-rich cell populations from the gills of the freshwater rainbow trout. Am. J. Physiol. Integr. Comp. Physiol. 291, R170-6.

Garrick, M.D., Singleton, S.T., Vargas, F., Kuo, H.C., Zhao, L., Knöpfel, M., Davidson, T., Costa, M., Paradkar, P., Roth, J.A., Garrick, L.M., 2006. DMT1: Which metals does it transport?, in: Biological Research. pp. 79–85.

Glynn, A.W., 1996. The concentration dependency of branchial intracellular cadmium distribution and influx in the zebrafish (*Brachydanio rerio*). Aquat. Toxicol. 35, 47–58.

Goto, D., Wallace, W.G., 2010. Metal intracellular partitioning as a detoxification mechanism for mummichogs (*Fundulus heteroclitus*) living in metal-polluted salt marshes. Mar. Environ. Res. 69, 163–171.

Gregory, D.S., Martin, A.C.R., Cheetham, J.C., Rees, A.R., 1993. The prediction and characterization of metal binding sites in proteins. Protein Eng. 6, 29–35.

Grosell, M., 2011. Copper, in: Wood Farrell, A.P., & Brauner, C.J., C.M. (Ed.), Fish Physiology: Homeostasis and Toxicology of Essential Metals. Academic Press, New York, pp. 53–135.

Grosell, M., McDonald, M.D., Wood, C.M., Walsh, P.J., 2004. Effects of prolonged copper exposure in the marine gulf toadfish (*Opsanus beta*) I. Hydromineral balance and plasma nitrogenous waste products. Aquat. Toxicol. 68, 249–262.

Grosell, M., Wood, C.M., 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. J. Exp. Biol. 205, 1179–1188.

Hamilton, J.G., Farrell, R.E., Chen, N., Feng, R., Reid, J., Peak, D., 2015. Characterizing Zinc Speciation in Soils from a Smelter-Affected Boreal Forest Ecosystem. J. Environ. Qual. 45, 684–692.

Hansen, J.A., Welsh, P.G., Lipton, J., Suedkamp, M.J., 2002. The effects of long-term cadmium exposure on the growth and survival of juvenile bull trout (*Salvelinus confluentus*). Aquat. Toxicol. 58, 165–174.

Harada, E., Hokura, A., Takada, S., Baba, K., Terada, Y., Nakai, I., Yazaki, K., 2010. Characterization of cadmium accumulation in willow as a woody metal accumulator using synchrotron radiation-based X-ray microanalyses. Plant Cell Physiol. 51, 848–853.

Has-Schön, E., Bogut, I., Strelec, I., 2006. Heavy metal profile in five fish species included in human diet, domiciled in the end flow of River Neretva (Croatia). Arch. Environ. Contam. Toxicol. 50, 545–551.

Hempe, J.M., Cousins, R.J., 1991. Cysteine-rich intestinal protein binds zinc during transmucosal zinc transport. Proc. Natl. Acad. Sci. U. S. A. 88, 9671–4.

Hogstrand, C., 2011. Zinc, in: Wood Farrell, A.P., & Brauner, C.J., C.M. (Ed.), Fish Physiology: Homoestasis and Toxicology of Essential Metals. Academic Press, New York, pp. 135–186.

Hogstrand, C., Reid, S., Wood, C., 1995. Ca^{2+} versus Zn^{2+} transport in the gills of freshwater rainbow trout and the cost of adaptation to waterborne Zn^{2+}. J. Exp. Biol. 198, 337–348.

Hogstrand, C., Verbost, P.M., Bonga, S.E., Wood, C.M., 1996. Mechanisms of zinc uptake in gills of freshwater rainbow trout: interplay with calcium transport. Am. J. Physiol. 270, R1141-7.

Holcombe, D.A.B.G.W., 1978. Toxic effects of zinc on fathead minnows Pimephales promelas in soft water. J. Fish Biol. 13.

Ide-Ektessabi, A., Kawakami, T., Watt, F., 2004. Distribution and chemical state analysis of iron

in the Parkinsonian substantia nigra using synchrotron radiation micro beams. Nucl. Instruments Methods Phys. Res. Sect. B Beam Interact. with Mater. Atoms 213, 590–594.

Inagaki, K., Mikuriya, N., Morita, S., Haraguchi, H., Nakahara, Y., Hattori, M., Kinosita, T., Saito, H., 2000. Speciation of protein-binding zinc and copper in human blood serum by chelating resin pre-treatment and inductively coupled plasma mass spectrometry. Analyst 125, 197–203.

Ishihara, A., Mugiya, Y., 1987. Ultrastructural evidence of calcium uptake by chloride cells in the gills of goldfish, Carassius auratus. J. Exp. Zool. 242, 121–129.

Jomova, K., Baros, S., Valko, M., 2012. Redox active metal-induced oxidative stress in biological systems. Transit. Met. Chem. 37, 127–134.

Jones, K.W., Bronson, S., Brink, P., Gordon, C., Mosher-Smith, K., Brown, M., Chaudhry, S., Rizzo, A., Sigismondi, R., Whitehurst, M., Lukaszewski, A., Kranz, D., Bland, K., Gordan, D., Lobel, J., Sullivan, J., Metzger, M., O'Shea, C., Harris, C., Arezzo, R., Kambhampati, M.S., 2009. Bivalve characterization using synchrotron micro X-ray fluorescence, in: Acta Physica Polonica A. pp. 477–481.

Kamunde, C., MacPhail, R., 2011. Subcellular interactions of dietary cadmium, copper and zinc in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol.

Komjarova, I., Blust, R., 2009. Multimetal interactions between Cd, Cu, Ni, Pb, and Zn uptake from water in the zebrafish, *Danio rerio*. Environ. Sci. Technol. 43, 7225–7229.

Komjarova, I., Blust, R., 2008. Multi-metal interactions between Cd, Cu, Ni, Pb and Zn in water flea, *Daphnia magna*, a stable isotope experiment. Aquat. Toxicol. 90, 138–144.

Komjarova, I., Bury, N.R., 2014. Evidence of Common Cadmium and Copper Uptake Routes in Zebra fish, *Danio rerio*. Enviromental Sci. Technol. 48, 12946–12951.

Korbas, M., MacDonald, T.C., Pickering, I.J., George, G.N., Krone, P.H., 2012. Chemical form matters: Differential accumulation of mercury following inorganic and organic mercury exposures in zebrafish larvae. ACS Chem. Biol. 7, 411–420.

Kwong, R.W.M., Andrés, J.A., Niyogi, S., 2010. Molecular evidence and physiological characterization of iron absorption in isolated enterocytes of rainbow trout (*Oncorhynchus mykiss*): Implications for dietary cadmium and lead absorption. Aquat. Toxicol. 99, 343–350.

Laurén, D.J., McDonald, D.G., 1985. Effects of copper on branchial ionoregulation in the rainbow trout, *Salmo gairdneri* (Richardson) - Modulation by water hardness and pH. J. Comp. Physiol. B 155, 635–644.

Lee, J., Prohaska, J.R., Thiele, D.J., 2001. Essential role for mammalian copper transporter Ctr1 in copper homeostasis and embryonic development. Proc. Natl. Acad. Sci. U. S. A. 98, 6842–7.

Liu, K.-Z., Bose, R., Mantsch, H.H., 2002. Infrared spectroscopic study of diabetic platelets. Vib. Spectrosc. 28, 131–136.

Lizardo-Daudt, H.M., Bains, O.S., Singh, C.R., Kennedy, C.J., 2008. Cadmium chloride-induced disruption of testicular steroidogenesis in rainbow trout, *Oncorhynchus mykiss*. Arch. Environ. Contam. Toxicol. 55, 103–110.

Lombi, E., Susini, J., 2009. Synchrotron-based techniques for plant and soil science: Opportunities, challenges and future perspectives. Plant Soil. 320(1), 1-35.

Lovell, M.A., Robertson, J.D., Teesdale, W.J., Campbell, J.L., Markesbery, W.R., 1998. Copper, iron and zinc in Alzheimer's disease senile plaques. J. Neurol. Sci. 158, 47–52.

Luo, L., Zhang, S., 2010. Applications of synchrotron-based X-ray techniques in environmental science. Sci. China Chem.

Luoma, S.N., Rainbow, P.S., 2008. Metal contamination in aquatic environments: science and lateral management. Cambridge University Press.

Lynch, N.R., Hoang, T.C., O'Brien, T.E., 2016. Acute toxicity of binary-metal mixtures of copper, zinc, and nickel to *Pimephales promelas*: Evidence of more-than-additive effect. Environ. Toxicol. Chem. 35, 446–457.

MacDonald, T.C., Malgorzata Korbas, A., Ashley James, cd K., Nicole Sylvain, abc J., Hackett, M.J., Nehzati, S., Krone, P.H., Graham George, bc N., Pickering abe, I.J., 2015. Metallomics Interaction of mercury and selenium in the larval stage zebrafish vertebrate model:Interaction of mercury and selenium in the larval stage zebrafish vertebrate model. Metallomics 7, 1199–1292.

Mallatt, J., 1985. Fish Gill Structural Changes Induced by Toxicants and Other Irritants: A Statistical Review. Can. J. Fish. Aquat. Sci. 42, 630–648.

Mantsch, H.H., Chapman, D., 1996. Infrared spectroscopy of biomolecules.

Marcelli, A., Cricenti, A., Kwiatek, W.M., Petibois, C., 2012. Biological applications of synchrotron radiation infrared spectromicroscopy. Biotechnol. Adv. 30, 1390–1404.

McGeer, J., Niyogi, S., Smith, S., 2011. Cadmium, in: Wood Farrell, A.P., & Brauner, C.J., C.M. (Ed.), Homeostasis and Toxicology of Non-Essential Metals. Volume 31B. Academic Press, Newyork, pp. 125–169.

McGeer, J.C., Szebedinszky, C., McDonald, D.G., Wood, C.M., 2000. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout. 1: Iono-regulatory disturbance and metabolic costs. Aquat. Toxicol. 50, 231–243.

Mebane, C.A., Dillon, F.S., Hennessy, D.P., 2012. Acute toxicity of cadmium, lead, zinc, and their mixtures to stream-resident fish and invertebrates. Environ. Toxicol. Chem. 31, 1334–1348.

Mebane, C.A., Hennessy, D.P., Dillon, F.S., 2008. Developing acute-to-chronic toxicity ratios for lead, cadmium, and zinc using rainbow trout, a mayfly, and a midge. Water. Air. Soil Pollut. 188, 41–66.

Minghetti, M., Leaver, M.J., Carpene, E., George, S.G., 2008. Copper transporter 1, metallothionein and glutathione reductase genes are differentially expressed in tissues of sea bream (*Sparus aurata*) after exposure to dietary or waterborne copper. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 147, 450–459.

Misra, S., Peak, D., Chen, N., Hamilton, C., Niyogi, S., 2012. Tissue-specific accumulation and speciation of selenium in rainbow trout (*Oncorhynchus mykiss*) exposed to elevated dietary selenomethionine. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 155, 560–565.

Naddy, R.B., Cohen, A.S., Stubblefield, W.A., 2015. The interactive toxicity of cadmium, copper, and zinc to Ceriodaphnia dubia and rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 34, 809–15.

Naito, W., Kamo, M., Tsushima, K., Iwasaki, Y., 2010. Exposure and risk assessment of zinc in Japanese surface waters. Sci. Total Environ. 408, 4271–4284.

Nieboer, E., Richardson, D.H.S., 1980. The replacement of the nondescript term “heavy metals” by a biologically and chemically significant classification of metal ions. Environ. Pollution. Ser. B, Chem. Phys. 1, 3–26.

Niyogi, S., Kamunde, C., Wood, C., 2006. Food selection, growth and physiology in relation to dietary sodium chloride content in rainbow trout (*Oncorhynchus mykiss*) under chronic waterborne Cu. Aquat. Toxicol. 77, 210-221

Niyogi, S., Kent, R., Wood, C.M., 2008. Effects of water chemistry variables on gill binding and acute toxicity of cadmium in rainbow trout (*Oncorhynchus mykiss*): A biotic ligand model

(BLM) approach. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 148, 305–314.

Niyogi, S., Nadella, S.R., Wood, C.M., 2015. Interactive effects of waterborne metals in binary mixtures on short-term gill-metal binding and ion uptake in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 165, 109–119.

Niyogi, S., Wood, C.M., 2004. Biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. Environ. Sci. Technol. 38, 6177–6192.

Niyogi, S., Wood, C.M., 2003. Effects of chronic waterborne and dietary metal exposures on gill metal-binding: implications for the biotic ligand model. Hum. Ecol. Risk Assess. 9, 813–846.

Nriagu, J.O., Pacyna, J.M., 1988. Quantitative assessment of worldwide contamination of air, water and soils by trace metals. Nature 333, 134–139.

Olsson, P., Hogstrand, C., 1987. Subcellular distribution and binding of cadmium to metallothionein in tissues of rainbow trout after exposure to ^{109}Cd in water. Environ. Toxicol. Chem. 6, 867–874.

OECD (2009). Guidance document for the diagnosis of endocrine-related histopathology of fish gonads.

Olsson, P.E., Kling, P., Hogstrand, C., 1998. Mechanisms of heavy metal accumulation and toxicity in fish, in: Metal Metabolism in Aquatic Environments. Springer, pp. 321–350.

Padovan, A., Munksgaard, N., Alvarez, B., McGuinness, K., Parry, D., Gibb, K., 2012. Trace metal concentrations in the tropical sponge *Spheciospongia vagabunda* at a sewage outfall: Synchrotron X-ray imaging reveals the micron-scale distribution of accumulated metals. Hydrobiologia 687, 275–288.

Palaniappan, P., Vijayasundaram, V., 2008. FTIR study of arsenic induced biochemical changes on the liver tissues of fresh water fingerlings, *Labeo rohita*. Rom J Biophys 18, 135–144.

Palaniappan, P.L.R.M., Nishanth, T., Renju, V.B., 2010. Bioconcentration of zinc and its effect on the biochemical constituents of the gill tissues of Labeo rohita: An FT-IR study. Infrared Phys. Technol. 53, 103–111.

Palaniappan, P.L.R.M., Pramod, K.S., 2010. FTIR study of the effect of $nTiO_2$ on the biochemical constituents of gill tissues of Zebrafish (*Danio rerio*). Food Chem. Toxicol. 1–7.

Palaniappan, P.R., Nishanth, T., Renju, V.B., 2010. Bioconcentration of zinc and its effect on the biochemical constituents of the gill tissues of *Labeo rohita*: An FT-IR study. Infrared Phys. Technol. 53, 103–111.

Palaniappan, P.R., Vijayasundaram, V., 2009. Effect of arsenic intoxication on proteins and lipids of *Labeo rohita* gill tissues: An FT-IR study. Toxicol. Environ. Chem. 91, 1125–1134.

Pan, T., Liao, B., Huang, C., Lin, L., 2005. Epithelial Ca^{2+} channel expression and Ca^{2+} uptake in developing zebrafish. Am. J. 289, 1202–1211.

Paquin, P.R., Gorsuch, J.W., Apte, S., Batley, G.E., Bowles, K.C., Campbell, P.G.C., Delos, C.G., Di, D.M., Dwyer, R.L., Galvez, F., Gensemer, R.W., Goss, G.G., Hogstrand, C., Janssen, C.R., Mcgeer, J.C., Naddy, R.B., Playle, R.C., Santore, R.C., Schneider, U., Stubblefield, W.A., Wood, C.M., Benjamin, K., 2002. The biotic ligand model : a historical overview 133, 3–35.

Payan, P., Mayer-Gostan, N., Pang, P.K.T., 1981. Site of calcium uptake in the fresh water trout gill. J. Exp. Zool. 216, 345–347.

Perry, S.F., Flik, G., 1988. Characterization of branchial transepithelial calcium fluxes in freshwater trout, *Salmo gairdneri*. Am. J. Physiol. 254, R491-8.

Perry, S.F., Laurent, P., 1993. Environmental effects on fish gill structure and function, in: Fish Ecophysiology. pp. 231–264.

Playle, R.C., Dixon, D.G., Burnison, K., 1993. Copper and cadmium binding to fish gills: modification by dissolved organic carbon and synthetic ligands. Can. J. Fish. Aquat. Sci. 50, 2667–2677.

Polette, L.A., Gardea-Torresdey, J.L., Chianelli, R.R., George, G.N., Pickering, I.J., Arenas, J., 2000. XAS and microscopy studies of the uptake and bio-transformation of copper in *Larrea tridentata* (creosote bush). Microchem. J. 65, 227–236.

Powell, S., 2000. The antioxidant properties of zinc. J. Nutr. 130, 1447S–54S.

Qiu, A., Hogstrand, C., 2004. Functional characterisation and genomic analysis of an epithelial calcium channel (ECaC) from pufferfish, *Fugu rubripes*. Gene 342, 113–123.

Qiu, A., Shayeghi, M., Hogstrand, C., 2005. Molecular cloning and functional characterization of a high-affinity zinc importer (DrZIP1) from zebrafish (*Danio rerio*). Biochem.J 388, 745–754.

Rainbow, P.S., 1997. Trace metal accumulation in marine invertebrates: Marine biology or marine chemistry? J. Mar. Biol. Assoc. United Kingdom 77, 195–210.

Reiley, M.C., 2007. Science, policy, and trends of metals risk assessment at EPA: how understanding metals bioavailability has changed metals risk assessment at US EPA. Aquat. Toxicol. 84, 292–298.

Richards, J.G., Curtis, P.J., Burnison, B.K., Playle, R.C., 2001. Effects of natural organic matter source on reducing metal toxicity to rainbow trout (*Oncorhynchus mykiss*) and on metal binding to their gills. Environ. Toxicol. Chem. 20, 1159–1166.

Roch, M., McCarter, J.A., 1984. Hepatic metallothionein production and resistance to heavy metals by rainbow trout (*Salmo gairdneri*) exposed to an artificial mixture of zinc, copper and cadmium. Comp. Biochem. Physiol. Part C, Comp. 77, 71–75.

Roesijadi, G., 1996. Metallothionein and its role in toxic metal regulation. Comp. Biochem. Physiol. Part C Pharmacol. Toxicol. Endocrinol. 113, 117–123.

Roeva, N.N., Sidorov, A. V, Yurovitskii, Y.G., 1999. Metallothioneins, proteins binding heavy metals in fish. Biol. Bull. Acad. Sci. C/C Izv. Akad. Nauk SERIIA Biol. 26, 617–622.

Rulíšek, L., Havlas, Z., 2000. Theoretical Studies of Metal Ion Selectivity. 1. DFT Calculations of Interaction Energies of Amino Acid Side Chains with Selected Transition Metal Ions (Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Hg^{2+}). J. Am. Chem. Soc. 122, 10428–10439.

Sahi, S. V., Israr, M., Srivastava, A.K., Gardea-Torresdey, J.L., Parsons, J.G., 2007. Accumulation, speciation and cellular localization of copper in *Sesbania drummondii*. Chemosphere 67, 2257–2266.

Salt, D.E., Prince, R.C., Pickering, I.J., 2002. Chemical speciation of accumulated metals in plants: Evidence from X-ray absorption spectroscopy. Microchem. J. 71, 255–259.

Sappal, R., Burka, J., Dawson, S., Kamunde, C., 2009. Bioaccumulation and subcellular partitioning of zinc in rainbow trout (*Oncorhynchus mykiss*): Cross-talk between waterborne and dietary uptake. Aquat. Toxicol. 91, 281–290.

Schamphelaere, K. De, 2004. Bioavailability and chronic toxicity of zinc to juvenile rainbow trout (*Oncorhynchus mykiss*): comparison with other fish species and development of a biotic ligand. Environ. Sci. 38, 165–174.

Settle, F.A., 1997. Infrared Spectroscopy. Handb. Instrum. Tech. Anal. Chem. 21, 252–276.

Sharma, N.C., Gardea-Torresdey, J.L., Parsons, J., Sahi, S. V, 2004. Chemical speciation and cellular deposition of lead in *Sesbania drummondii*. Environ. Toxicol. Chem. 23, 2068–2073.

Shay, N., Mangian, H., 2000. Zinc and health: current status and future directions. J Nutr 1493–1499.

Solomon, E.I., Lowery, M.D., 1993. Electronic structure contributions to function in bioinorganic chemistry. Science 259, 1575–1581.

Spry, D.J., Wood, C.M., 1988. Zinc influx across the isolated, perfused head preparation of the rainbow trout (*Salmo gairdneri*) in hard and soft water. Can. J. Fish. Aquat. Sci. 45, 2206–2215.

Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radic. Biol. Med. 18, 321–336.

Szczerbowska-Boruchowska, M., 2008. X-ray fluorescence spectrometry, an analytical tool in neurochemical research. X-Ray Spectrom. 37, 21–31.

Tan, Q.G., Wang, Y., Wang, W.X., 2015. Speciation of Cu and Zn in Two Colored Oyster Species Determined by X-ray Absorption Spectroscopy. Environ. Sci. Technol. 49, 6919–6925.

Taylor, L., McGeer, J., Wood, C., 2000. Physiological effects of chronic copper exposure to rainbow trout (*Oncorhynchus mykiss*) in hard and soft water: evaluation of chronic indicators. Environmental.

Taylor, P., 2008. Modelling Bioaccumulation and Toxicity. Risk Assess.

Thorn, K., Cerrato, R.M., Rivers, M.L., 1995. Elemental Distributions in Marine Bivalve Shells as Measured by Synchrotron X-Ray Fluorescence. Biol. Bull. 188, 57.

Toyran, N., Turan, B., Severcan, F., 2007. Selenium alters the lipid content and protein profile of rat heart: An FTIR microspectroscopic study. Arch. Biochem. Biophys. 458, 184–193.

Tuurala, H., Soivio, A., 1982. Structural and circulatory changes in the secondary lamellae of *Salmo gairdneri* gills after sublethal exposures to dehydroabietic Acid and Zinc. Aquat. Toxicol. 2, 21–29.

Twining, B.S., Baines, S.B., Fisher, N.S., Maser, J., Jacobsen, C., Tovar-sanchez, A., Saudo-

wilhelmy, S.A., Vogt, S., San, S.A., 2003. Quantifying Trace Elements in Individual Aquatic Protist Cells with a Synchrotron X-ray Fluorescence Microprobe Quantifying Trace Elements in Individual Aquatic Protist Cells with a Synchrotron X-ray Fluorescence Microprobe. Society 75, 3806–3816.

Twining, B.S., Baines, S.B., Vogt, S., De Jonge, M.D., 2008. Exploring ocean biogeochemistry by single-cell microprobe analysis of protist elemental composition, in: Journal of Eukaryotic Microbiology. pp. 151–162.

UNEP, 2010. Final Review of Scientific Information on Cadmium. Nairobi, Kenya.

USEPA, 2007. Aquatic Life Ambient Freshwater Qaulity Criteria - Copper. US Environmental Protection Agency, Washington, DC.

Valko, M., Morris, H., Cronin, M.T.D., 2005. Metals, toxicity and oxidative stress. Curr. Med. Chem. 12, 1161–1208.

Verbost, P.M., Van Rooij, J., Flik, G., Lock, R.A.C., Wendelaar Bonga, S.E., 1989. The movement of cadmium through freshwater trout branchial epithelium and its interference with calcium transport. J. Exp. Biol. 145, 185–197.

Viarengo, A., Nott, J.A., 1993. Mechanisms of heavy metal cation homeostasis in marine invertebrates. Comp. Biochem. Physiol. Part C Comp. Pharmacol. 104, 355–372.

Vijver, M.G., Elliott, E.G., Peijnenburg, W.J.G.M., de Snoo, G.R., 2011. Response predictions for organisms water-exposed to metal mixtures: A meta-analysis. Environ. Toxicol. Chem. 30, 1482–1487.

Wangsongsak, A., Utarnpongsa, S., Kruatrachue, M., Ponglikitmongkol, M., Pokethitiyook, P., Sumranwanich, T., 2007. Alterations of organ histopathology and metallolhionein mRNA expression in silver barb, *Puntius gonionotus*, during subchronic cadmium exposure. J.

Environ. Sci. 19, 1341–1348.

Weng, N., Wang, W.X., 2014. Variations of trace metals in two estuarine environments with contrasting pollution histories. Sci. Total Environ. 485–486, 604–614.

WHO/UNEP, 1989. GEMS - Global freshwater quality. Blackwell Reference, Oxford.

Wicklund-Glynn, A., 2001. The influence of zinc on apical uptake of cadmium in the gills and cadmium influx to the circulatory system in zebrafish (*Danio rerio*). Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 128, 165–172.

Wood, C.M., 2011. An Introduction to Metals in Fish Physiology and Toxicology: Basic Principles, in: Wood Farrell, A.P., & Brauner, C.J., C.M. (Ed.), Fish Physiology: Homeostasis and Toxicology of Essential Metals. Academic Press, New York, pp. 25–65.

Woodling, J., Brinkman, S., Albeke, S., 2002. Acute and chronic toxicity of zinc to the mottled sculpin *Cottus bairdi*. Environ. Toxicol. Chem. 21, 1922–6.

Yano, J., Yachandra, V.K., 2009. X-ray absorption spectroscopy. Photosynth. Res. 102, 241–254.

Yee, N., Benning, L.G., Phoenix, V.R., Ferris, F.G., 2004. Characterization of metal-cyanobacteria sorption reactions: a combined macroscopic and infrared spectroscopic investigation. Environ. Sci. Technol. 38, 775–782.

Zalups, R.K., Ahmad, S., 2003. Molecular handling of cadmium in transporting epithelia. Toxicol. Appl. Pharmacol. 186, 163–188.

Zelikoff, J.T., Bowser, D., Squibb, K.S., Frenkel, K., 1995. Immunotoxicity of low level cadmium exposure in fish: an alternative animal model for immunotoxicological studies. J. Toxicol. Environ. Heal. Part A Curr. Issues 45, 235–248.

Zhao, H., Wu, L., Chai, T., Zhang, Y., Tan, J., Ma, S., 2012. The effects of copper, manganese and zinc on plant growth and elemental accumulation in the manganese-hyperaccumulator

Phytolacca americana. J. Plant Physiol. 169, 1243–1252.