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The sublethal physiological effects of exposure to copper and silver mixtures on rainbow trout (Oncorhynchus mykiss)

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By

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Abstract

The mechanisms behind metal-metal interactions in freshwater environments are currently not well understood. Freshwater environments consist of many different types of metals, from those naturally present such as copper (Cu) and those that originate from anthropogenic sources like silver (Ag). Both Cu and Ag use apical sodium (Na^+) channels for uptake into the gills of freshwater fish. In the gills, the mechanisms of Cu^{2+} and Ag^+ toxicity appear to be similar to one another, which is by inhibiting Na^+ /potassium (K^+)- adenosine triphosphatase (NKA) and carbonic anhydrase (CA). Inhibition of NKA and CA results in ionoregulatory disturbances where branchial Na^+ and chloride (Cl^-) uptake is reduced and can result in mortality. The overall goal of this research was to build a better understanding of the interactions between Cu and Ag in the context of sub-chronic impacts of metal mixtures on the rainbow trout (*Onchorhynchus mykiss*). Juvenile rainbow trout were exposed for 10 and 14 days to Cu-only (10-day: 1.0 μM Cu^{2+}, 14-day: 0.35 μM Cu^{2+}), Ag-only (0.04 μM Ag^+) or a Cu^{2+} + Ag^+ mixture (10-day: 1.0 μM Cu^{2+} + 0.04 μM Ag^+; 14-day: 0.35 μM Cu^{2+} + 0.04 μM Ag^+). The effects of Cu-Ag interactions were assessed by measuring bioaccumulation in whole gill, liver and kidney samples, subcellular distribution in the gills and liver, and plasma Na^+ and Cl^- content. Mortalities were dose dependent and greatest in the mixture exposures. Significant accumulation of Cu and Ag in the gills and kidney were a result of a more than additive affect by metal interactions. Cu accumulation in the liver was also more than additive but no effect was observed on hepatic Ag. Subcellular distribution of Cu mainly occurred in metal sensitive fractions (MSF) while Ag accumulated mainly in biologically detoxified fractions (BDF). Fish exposed to the mixture for 10-days experienced more than additive disruption in plasma Na^+ but not in plasma Cl^-. During 14-days, mixture-exposed fish experienced a more than additive disruption on ion regulation where plasma Na^+ and Cl^- were significantly less than Cu^{2+} or Ag^+-only exposed fish. Overall,

the effects of metal interactions on bioaccumulation, physiological effects and mortalities were based on exposure concentration.

Authors Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

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Glossary

Cu: copper (in general)

Cu^{2+}: Ionic copper

Ag: Silver (in general)

Ag^{+}: Ionic silver

Na^{+}: Sodium ion

Cl^{-}: Chloride ion

NKA: sodium/ potassium-adenosine triphosphate

CA: Carbonic anhydrase

MRG: Metal rich granules

MT: Metallothionein

MTLP: Metallothionein like proteins

BDF: Biologically detoxified fractions

MSF: Metal sensitive fractions

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Chapter 1 Introduction

1. Background

1.1 Copper

Cu is a common element and is essential for important biological processes in aquatic organisms including cellular respiration, defense against free radical by-products, neurotransmitter functions and connective tissue biosynthesis (Harris and Gitlin, 1996). In pristine freshwater environments, Cu concentrations may range from 0.003 to 0.47 μM (USEPA, 2007). Environmental concentrations, however, can increase up wards of 1.6 μM or greater due to pollution by activities such as mining, smelting, and industrial production of Cu products (USEPA, 2007). At these elevated concentrations, Cu can become more harmful than beneficial for aquatic organisms as increasing concentrations will likely lead to toxicity and even mortality. Since Cu is an essential element and a potent toxicant, it is crucial for organisms to use homeostatic mechanisms such as the release of the metal-binding protein metallothionein (MT) and metallothionein like proteins (MLTP) to balance between deficient or excess amounts (Kamunde et al., 2002).

1.1.1 Copper Toxicity in Fish

The gills of fish are a multi-functional organ that is responsible for respiration, nitrogenous waste excretion, acid-base balance, osmoregulation, and the uptake of essential elements including Cu (Wilson and Laurent, 2002; Evans et al., 2005). The main toxic form of Cu is in the free ionic state of Cu^{2+}. Cu^{2+} is believed to be reduced to Cu^{+} (Grosell, 2012) and compete with sodium (Na^{+}) for uptake through the apical Na^{+} channels (Grosell and Wood, 2002). A metal reductase has not yet been demonstrated in fish for although a protein similar to duodenal cytochrome *b* (dcb) found in mammalian intestines has been hypothesized for reducing Cu^{2+} to Cu^{+} (Scheiber et al., 2010; Grosell, 2012). On the apical side of the gills, Cu may also enter

through Na^+/ proton (H^+)-exchanger. Although this method still remains a controversial means of uptake (Grosell, 2012; Harley and Glover, 2014). Once up taken in the gill cell, Cu^{2+} is thought to be transferred across the basolateral membrane and into the circulatory system via Na^+/potassium (K^+)- adenosine triphosphatase (NKA) (Figure 1.1) (Grosell, 2012; Harley and Glover, 2014). NKA functions in maintaining Cu^{2+} levels but when concentrations get too high, NKA then becomes a key target site for toxic effects. During acute exposure to elevated Cu^{2+} concentrations, osmoregulatory disturbances may occur through non-competitive inhibition of the NKA, leading to decreased Na^+ influx coupled with increased Na^+ efflux on the gills (Laurèn and McDonald, 1986, 1987a,b). A cascade of additional internal disturbances may also follow this inhibition including the swelling of red blood cells from the imbalance between Na^+ and chloride (Cl^-) levels, resulting in cardiovascular collapse (Grosell et al., 2002; Grosell, 2012).

During chronic exposure to sublethal Cu concentrations, osmoregulatory disturbances caused by Na^+ imbalance are observed but are typically short-lived (De Boeck et al., 2003). Fish are capable of handling this ion imbalance by increasing their tolerance during the acclimation period, a phenomenon that can be explained through a damage-repair model proposed by McDonald and Wood (1993). In this repair model, a "shock phase" will be initiated with the onset of ion loss and cell damage, which usually lasts for less than two days (McDonald and Wood, 1993). If the fish is able to survive the shock phase, then the "recovery phase" will follow while the fish continues to acclimate in the presence of the contaminant (McGeer et al., 2007). During the recovery phase, the fish will increase the mobilization of MT and MTLP to regulate essential free metal ions (ex. Cu^{2+} and Zn^{2+}) and remove non-essential metals (ex. Ag^+) (De Boeck et al., 2003; Leonard et al., 2014). An increase in NKA synthesis to restore Na^+ homeostasis is another mechanism that has been documented in Cu-exposed fish in attempts of increasing tolerance (Laurèn and McDonald, 1987a,b; McGeer et al., 2000b,c). Over time, if the

fish is be able to acclimate to these conditions well enough, it will either return to its pre-exposure physiological conditions or establish a new threshold limit (McGeer et al., 2000c).

1.2 Silver

Ag is a potent toxicant to aquatic organisms and has been researched since the early 1990's. Industrial applications such as mining, the manufacturing of jewelry and effluents from sewage treatment plants are a few contributing sources of Ag in the aquatic environment (Sathya et al., 2012). For the protection of aquatic life, the Canadian ambient water quality criterion for Ag is set at 2.32 nM (0.25 μg/L) (CCME, 2007; 2015). Ag concentrations in surface water vary depending upon nature of the environment within the watershed and input by anthropogenic sources. In Canada, total Ag concentrations sampled from thousands of surface waters ranged from below detection (<0.01 ug/L) to as high as 10.00 ug/L between 2008 and 2013 (CCME, 2015). Dissolved Ag concentrations (Ag molecules capable of bypassing a 0.45 μM filter) in natural freshwaters range between 5-50 pM and in highly contaminated sites, concentrations may be up to 1000 times greater (Wood, 2012). It is important to note that the potential for dissolved Ag toxicity in natural waters will typically be less than that of tests conducted in laboratory settings. This is due to the high tendency for Ag^{+} to form complexes with a number of organic and inorganic ligands such as dissolved organic carbons, chlorides, and thiosulphate ($S_2O_3^{2-}$), all of which are found in natural waters (Brauner and Wood, 2002; Morgan et al., 2004a,b; Bertram and Playle, 2005; Wood, 2012). Once a complex is formed with a ligand, Ag becomes biologically unavailable to bind to the toxic sites of an aquatic organism. However, protection against toxicity by these ligands has been shown to be effective during acute Ag exposure (McGeer et al., 2000a) but results from chronic exposures have shown to be less protective (Brauner and Wood, 2002a; Brauner et al., 2003; Detholff et al., 2007).

1.2.1 Silver Toxicity In Fish

During waterborne exposure, uptake of Ag^+ involves mimicking the ion Na^+ and enters the gills through an apical Na^+ channel that is driven by the electrical gradient of the enzyme H^+-ATPase (Bury and Wood, 1999) (Figure 1.1). Ag^+ exerts toxicity by directly targeting NKA and carbonic anhydrase (CA) in the gills of fish leading to ionoregulatory disturbances in Na^+ and Cl^- (Morgan et al., 2004a,b, 2005; Sathya et al., 2012) (Figure 1.1). Several studies in trout have shown that decreased Na^+ influx due to Ag are associated with the inhibitation of NKA (Hogstrand et al., 1998; Morgan et al., 2004a; Bury, 2005), while it has been postulated that inhibition of CA activity may be involved with the decrease in plasma Cl^-. CA is responsible for the hydration of carbon dioxide (CO_2) to produce H^+ and bicarbonate (HCO_3^-) in which are counter-ions for the exchange of extracellular Na^+ (with H^+) and Cl^- (with HCO_3^-) to maintain acid-base balance in gill ionocytes (Figure 1.1). By targeting CA, Ag^+ may then inhibit the production of the counter-ions for exchange with Na^+ and Cl^-, resulting in ionoregulatory disturbances (Webb and Wood, 1998; Brauner and Wood, 2002b; Evans et al., 2005; Wood, 2012). It has been shown that Ag^+ is capable of inhibiting CA in both *in vitro* (Soyut et al., 2008) and *in vivo* for freshwater fish (Morgan et al., 1997, 2004a), however the apparent mechanism is still not well understood.

Previous studies have documented the accumulation of Ag in fish liver to be associated with processes of detoxification rather than a site for toxicity (Galvez et al., 2002; Bury, 2005). In the liver, methods of detoxification in fish involve the induction of MT and MTLP to sequester and store Ag for potential elimination (Galvez et al., 2002; Mayer et al., 2003). Hogstrand et al. (1996) found the liver contained substantially greater MT than in the gills of trout after exposure to waterborne Ag^+, which further reflects the detoxification role of the liver for potential protection against Ag^+ toxicity (Galvez et al., 2002). Ag bound to MT and MTLP can possibly

lead to the displacement of pre-bound metals (those of lower binding affinity) and could result in additional toxicity by these freed metals (Hogstrand and Wood, 1998).

1.2.2 Interactions of Metal Mixtures

In contaminated aquatic ecosystems, organisms are typically exposed to a mixture of metals. Interactions between metals in a mixture may lead to additive, more than additive (synergistic) or less than additive (antagonistic) effects on toxicity (Playle, 2004). Two models that can be used to predict metal mixture effects are the 'concentration addition' and 'response (effect) addition' concepts. Concentration addition assumes that each metal contributes to the overall effect through a common site of action and that the overall effect is equal to the sum of effects by the individual metals (Altenburger et al., 2000; Clemow and Wilkie, 2015). The response addition model assumes that the combined effect in a mixture is based on the probability that each metal has a different mode of toxic action (Atlenburger et al., 2000; Rider, 2005; Clemow and Wilkie, 2015). If the total effect in a mixture is greater than the predicted sum of effects by the individual metals, then effect is more than additive (synergistic) (Newman and Unger, 2003; Altenburger et al., 2000). When the total effect is lower than the predicated sum of effect by the individual metals, then the effect is less than additive (antagonistic). For example, the concentration addition approach can be applied in predicting the effects of a mixture on plasma ion disruption based on the measured individual effect of each metal. If the total effect were more or less than additive, this would indicate that an interaction between the metals has occurred (Newman and Unger, 2003; Clemow and Wilkie, 2015).

Since many metals share similar mechanisms of toxicity it is likely that additive or more than additive effects will occur when present as a mixture, even at concentrations below environmental quality guidelines (Cooper et al., 2009; Le et al., 2013). As an example, two

metals that have been shown to interact with one another are Cu^{2+} and cadmium (Cd^{2+}), where the presence of Cu^{2+} enhanced the uptake of Cd in rainbow trout (McGeer et al., 2007) and may lead to additive effects on toxicity. From a mechanistic point of view, Cu^{2+} and Ag^{+} are two other metals that will very likely show more than additive interactions once they have bioaccumulated within an organism. Since both Cu^{2+} and Ag^{+} use apical Na^{+}-channels for uptake into the gills, competition may occur. During the uptake phase, however, Ag^{+} will bind more readily due to a higher binding affinity of log K'= 10.0 on the gills of fish compared to the affinity of Cu^{2+} which is log K'= 7.4 (Playle, 2004). It is possible that at lower concentrations, Ag^{+} may promote an increase of Na^{+} uptake pathways through homeostatic processes that can result in elevated Cu^{2+} uptake by Na^{+}-sensitive and Na^{+}-dependent pathways (Grosell, 2012). Once the metals have bioaccumulated, more than additive interactions are then possible since both Cu^{2+} and Ag^{+} target basolateral NKA and CA to induce toxicity. Since both Cu^{2+} and Ag^{+} affect ion regulation, measuring plasma Na^{+} and Cl^{-} is one method of determining the presence of an interaction. Other more than additive effects are also possible as Ag^{+} can disrupt the homeostasis of other non-essential metals such as Cd^{2+} and mercury (Hg^{2+}) that have been sequestered by MT, allowing these metals to be freed and become biologically available to cause toxicity (Mayer et al., 2003).

1.4 Subcellular Metal Distribution

Metal contaminants can be investigated at various levels of biological organization, ranging from processes that occur at the cellular level to impacts that affect an entire ecosystem (Eyckmans et al., 2012). The most common approach when exploring metal bioaccumulation in fish has been at the organ level (i.e whole tissues) (Kamunde and MacPhail, 2011). The issue with investigating at the organ level is that the results from metal mixture exposures are not always consistent with the data to that of single metals. They are not consistent because scenarios

involving metal-metal interactions (i.e, additive and more than or less then additive effects) can influence the severity of toxicity. Furthermore, elevated metal concentrations measured in tissues do not always result in adverse effects. The lack of an effect may imply that the organism possesses mechanisms that function to maintain internal metal concentrations and prevent toxicity (Kamunde, 2009). One such mechanism includes the release of the detoxifying compounds MT, MLTP and metal-rich granules (MRG) (Wallace and Luoma, 2003;Wallace et al., 2003; Rainbow, 2007; Kamunde, 2009; Wang, 2012), which can be explained through the 'spillover hypothesis'.

The 'spillover hypothesis' proposes that when metals accumulate to a level below the threshold that causes toxicity, then the organism has effectively been able to detoxify the metals through the mobilization of MT and MRG (Wang and Rainbow, 2006). If the organism is no longer able to remove the metals at a sufficient rate where the mechanism of detoxification cannot remove the metals as quickly as it is being accumulated, then the threshold is surpassed. Once surpassed, toxicity will occur due to excessive metal accumulation onto the toxic target sites (Kamunde, 2009). While whole tissue burden results can signify the presence of a metal interaction, it is believed that investigating toxicity at the cellular level through the processes of 'subcellular fractionation' will serve as a better method in determining metal toxicity rather than at the organ level (Wang, 2012).

The process of subcellular fractionation involves the isolation and quantification of accumulated metals in specific cellular compartments of a tissue. The protocol for subcellular fractionation involves whole tissues being centrifuged at rapid speeds upwards of 800 to 100,000 times gravity to obtain specific subcellular compartments (Wallace et al., 2003). These subcellular compartments are separated into 5 fractions 1) cellular debris (membrane), 2) organelle fractions (nucleus, mitochondria, microsomes and lysosomes), 3) heat denatured

proteins (HDP), 4) MRG, and 5) heat stable proteins (containing MT and MTLP) (HSP) (Figure 1.2) (Wang and Rainbow, 2006; Wang, 2012). These fractions can further be categorized into two different pools, biologically detoxified fraction (BDF: MRG, HSP) and metal sensitive fraction (MSF: organelles, HDP) (Eyckmans et al., 2012). Metals in BDF are considered to be detoxified by MT and MTLP and unavailable to induce toxicity while those in the MSF are biologically active and able to cause toxicity (Wallace et al., 2003). To assure that fractions have not raptured during subcellular fractionation, citrate synthase activity in the mitochondria and cytosol can be measured. High citrate synthase activity in the mitochondrial fraction and low activity in the cytosol would validate that fractions were intact during high fraction isolation via centrifugation. The use of subcellular fractionation will contribute in determining interactive effects between Ag and Cu during accumulation within each fraction and how it may relate to observed physiological effects.

1.6. Rainbow Trout

The rainbow trout (*Oncorhynchus mykiss*) are a species of salmonid that is native to western North America and are commonly found in waters all over Canada (Environment Canada, 1990). Today, the rainbow trout is used as the world's standard cool-water fish for freshwater pollution research in aquatic toxicology since background studies have proven that this species is sensitive to a wide array of aquatic contaminants (Environment Canada, 1990). Additionally, rainbow trout are an ideal specimen to be used for toxicity tests because they are inexpensive, widely available, easy to culture in the laboratory, and have the ability to provide easy lifetime bioassays (Bailey, et al., 1996; Environment Canada, 1990). Overall, the rainbow trout can serve as a potential indicator of the harmful effects caused by exposure to aquatic contaminants where the results can

implicated into decisions made for setting water quality criteria for human health or for the protection of the environment (Bailey et al., 1996).

1.7. Objectives

The characteristics of Cu^{2+} and Ag^{+} toxicity are similar and interactions between both metals are likely to occur when present as a mixture. The overall goal of this research project was to further the understanding of the sublethal and physiological effects of Cu^{2+} and Ag^{+} acting simultaneously as a mixture on the rainbow trout. The two primary objectives of this research were:

1. To understand how both Cu^{2+} and Ag^{+} would interact upon accumulation in the tissues of the rainbow trout as a whole, and how these metals are distributed in subcellular fractions from the gills, liver and kidney. This was achieved by comparing exposed trout to two individual metal exposures of Cu^{2+}-only or Ag^{+}-only and to a Cu^{2+} + Ag^{+} mixture exposure. Whole tissue accumulation in the gills and liver is documented in *Chapter 2- The effect of Cu + Ag mixture exposure on bioaccumulation and ion balance of rainbow trout (Oncorhynchus mykiss).* Bioaccumulation in whole tissues of the gills, liver and kidney as well as subcellular distribution in the gills and liver is documented in *Chapter 3- Assessing the effects of metal interactions during Cu + Ag mixture exposure on bioaccumulation and ion balance of rainbow trout (Oncorhynchus mykiss)*
2. To understand the physiological effects caused by Cu^{2+} and Ag^{+} separately, followed by Cu and Ag simultaneously by analyzing plasma ion concentration in exposed fish. This was achieved by measuring plasma Na^{+} and Cl^{-} in trout exposed to a single metal of Cu^{2+}-only or Ag^{+}-only and comparing the results to trout exposed to a Cu^{2+} + Ag^{+} mixture. Results on plasma ion composition can be found in *Chapter 2* and *Chapter 3*

The hypotheses that were tested include:

1) Cu and Ag will bioaccumulate in whole tissues and subcellular fractions such that:
    a. Ag will accumulate at the same rate in the gills, liver and kidney of both Ag^{+}-only and mixture exposed fish.
    b. Cu and Ag will significantly accumulate in the gills, liver, and kidney in a a time and concentration dependent manner.
    c. Ag will accumulate selectively in metal detoxified fractions due to detoxification and over exposure time, will accumulate in metal sensitive fractions. Cu will accumulate in all fractions since it is an essential metal.

    Rationale: Ag^{+} (log K'= 10) has as greater binding affinity at the gills of fish compared to Cu^{2+} (log K'= 7.4) (Playle, 2004). Cu is an essential metal and may bind to a number of proteins/enzymes for metabolic function, storage or detoxification (Kamunde and MacPhail, 2008).

2) Interactions between Cu^{2+} and Ag^{+} in the mixture exposure will significantly decrease plasma Na^{+} and Cl^{-}. Ag-only and Cu-only exposures will decrease plasma Na^{+} and Cl^{-}, but to a lesser extent.

    Rationale: Both Cu^{2+} and Ag^{+} have been shown to disrupt Na^{+} balance by inhibiting NKA and CA activity (Morgan et al., 2004ab; Goss et al., 2011; Zimmer et al., 2012).

1.8 Figures

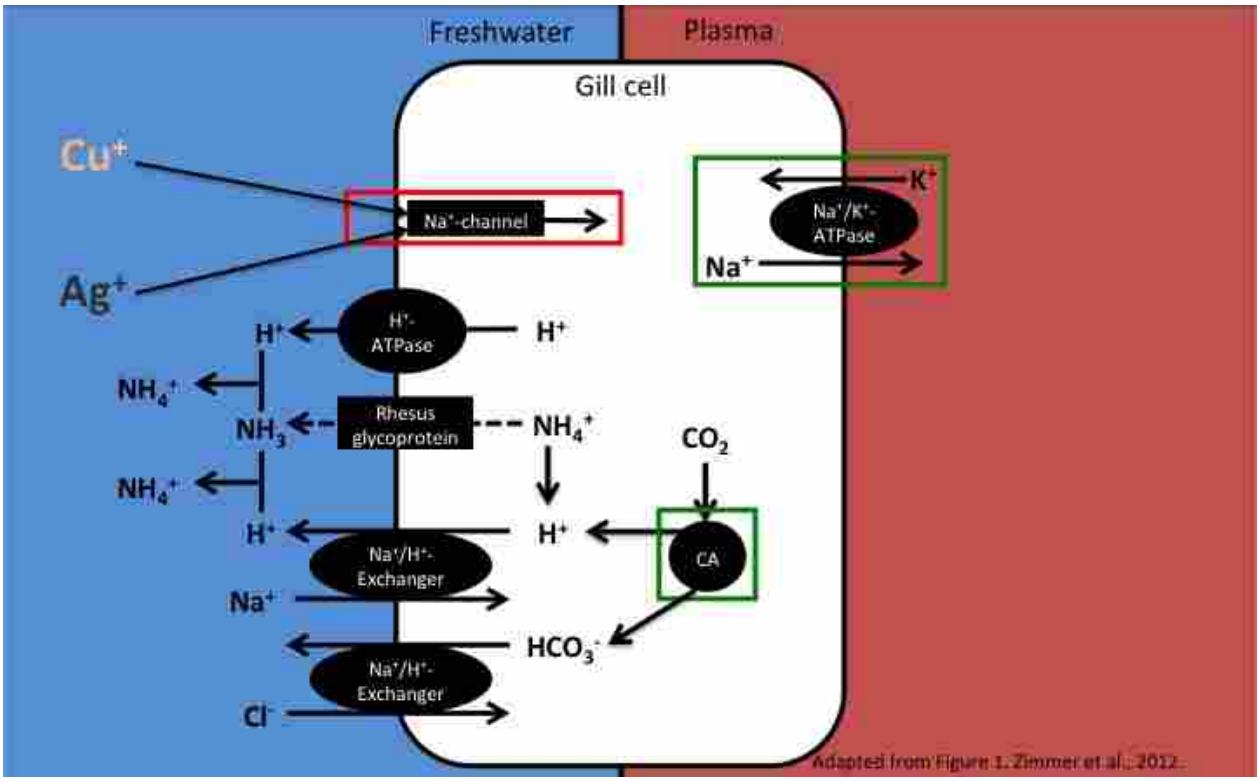

Figure 1.1. Copper and Silver Uptake Pathway. Copper (Cu^{2+}) and silver (Ag^{+}) both use sodium channels (Na^{+}) for uptake on the apical side of the gill cells. It is believed that Cu^{2+} is reduced to Cu^{+} by an apical metal reductase that has not yet been described in fish before uptake. Once accumulated within the cell, Cu^{2+} and Ag^{+} may cause toxicity by inhibiting basolateral sodium/potassium- adenosine triphosphate (NKA) and carbonic anhydrase (CA). A red box indicates the shared uptake channel of Cu^{2+} and Ag^{+}. Green boxes represent sites of toxic action by Cu^{2+} or Ag^{+}.

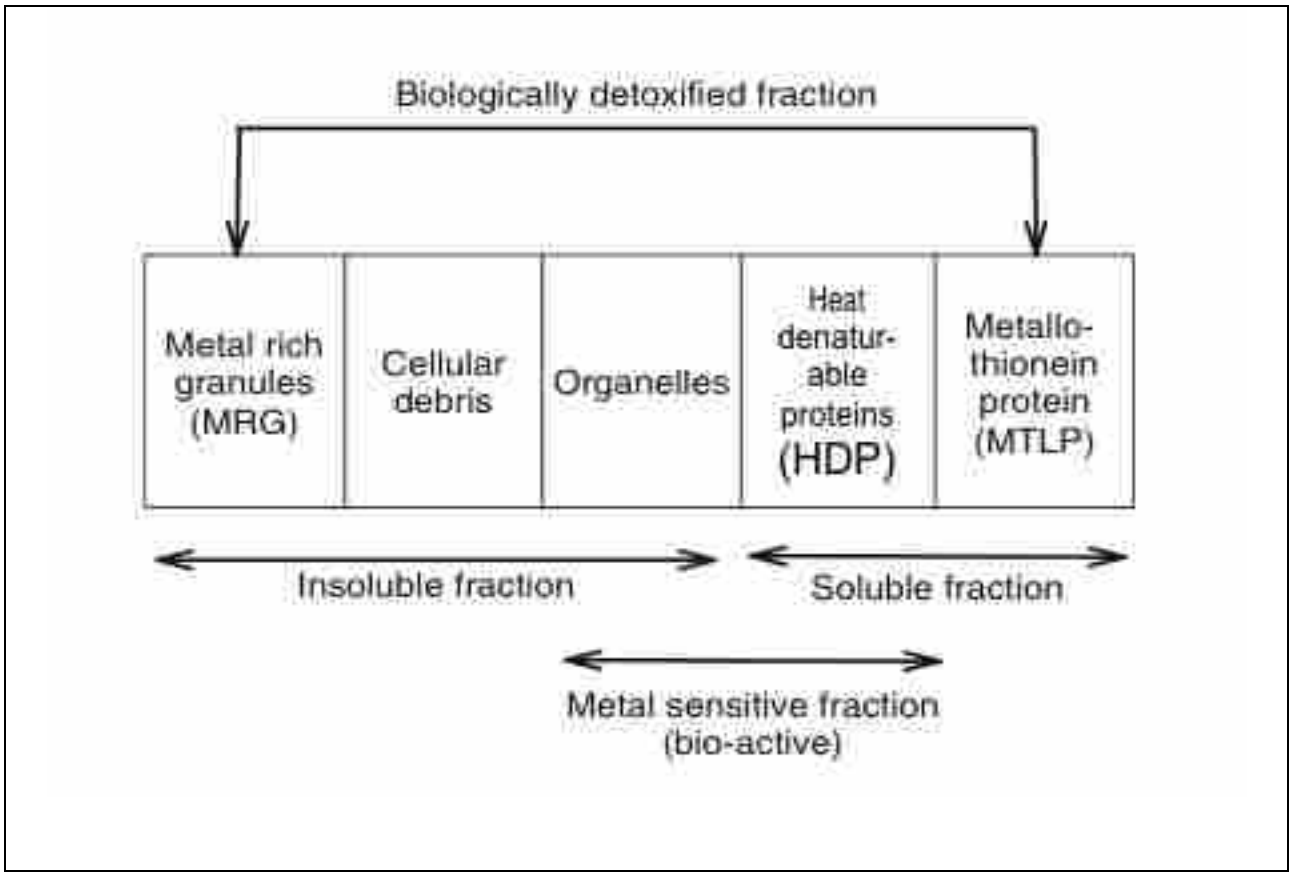

Figure 1.2. Subcellular Fraction Categories. Diagram displays the five (5) different categories of fraction during subcellular fractionation. The subcellular fractions of metals identified in aquatic organisms are; 1) cellular debris (membrane), 2) organelle fractions (nucleus, mitochondria, microsomes and lysosomes), 3) heat denatured proteins (HDP), 4) metal rich granules (MRG) and 5) heat stable proteins (HSP). Different fractions can be categorized into soluble (cytosol), insoluble, metal sensitive fraction (bio-active), and biologically detoxified fractions.

Adapted from Wang and Rainbow, 2006.

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Chapter 2

The effect of Cu + Ag mixture exposure on bioaccumulation and ion balance of rainbow trout (Oncorhynchus mykiss)

2.1 Abstract

Aquatic organisms are naturally exposed to a mixture of metals and the prediction of toxicity can be complicated by metal-metal interactions. The purpose of this study was to link bioaccumulation and physiological effects to interpret metal-metal interactions by exposing rainbow trout (*Oncorhynchus mykiss*) to a control (0.0 μM), 1.0 μM Cu^{2+}-only, 0.04 μM Ag^{+}-only and a mixture of 1.0 μM Cu^{2+} + 0.04 μM Ag^{+} for 10 days. Fish exposed to the Cu^{2+}-only and the mixture resulted in 33 % and 63 % mortality rate, respectively. A mortality rate of 8 % occurred in fish exposed to Ag^{+}-only while 4 % occurred in the controls. Results for fish exposed to the Cu^{2+} + Ag^{+} mixture were only conclusive till day 3 due to the high rate of mortalities. Gill accumulation of Ag was less in mixture-exposed fish than in Ag-only exposure fish on day 1 as a result of Cu^{2+} influence but may also be a result of the lethality of the metal concentration. Ag^{+} did not influence Cu accumulation in the gills of mixture-exposed fish, as there was no difference compared to Cu^{2+}-only exposed fish, indicating that mortalities were due to both Cu^{2+} and Ag^{+}. Liver content of Cu did not differ between mixture and Cu^{2+}-only exposure groups on day 1 but on day 3, a significant decline in accumulation perhaps related to the onset of mortality. Mixture exposed fish has significantly less Ag accumulation in the liver relative to Ag^{+}-only exposed fish between days 1 and 3 likely due to the high Cu^{2+} concentration in the mixture in addition to high background concentrations. Plasma Na^{+} was significantly decreased in fish exposed to the mixture in comparison to both Cu^{2+}-only and Ag^{+}-only exposed fish by day 3, implying an additive effect occurred due to a Cu^{2+} and Ag^{+} interaction. Plasma Cl^{-} was also significantly decreased in mixture-exposed fish and Cu^{2+}-only exposed fish relative to controls on day 1. Less than additive effect was observed on plasma Cl^{-} of mixture-exposed fish and was not predicted, most likely as a result of mortality.

2.2 Introduction

The prediction of metal mixture toxicity is an enduring and complex issue. Interactions between metals can cause deviations from the expected biological actions of each metal, leading to additive, more than additive (synergistic) or less than additive (antagonistic) effects during exposure. Additionally, adverse effects that are a result of interactions in mixtures can occur at concentrations that are below environmental quality guidelines for individual metals (Cooper et al., 2009). From a physiological standpoint, Cu^{2+} and Ag^{+} are two metals that have similar toxicity characteristics with one another. Both Cu^{2+} and Ag^{+} compete with Na^{+} for uptake through apical Na^{+} channels in gills of rainbow trout (Bury and Wood, 1999; Grosell and Wood, 2002). Once taken up at the gills, Cu^{2+} and Ag^{+} toxicity by inhibiting two key enzymes, NKA and carbonic anhydrase (CA) (Soyut et al., 2008; Zimmer et al., 2012), resulting in ionoregulatory disturbances where plasma Na^{+} and Cl^{-} are reduced (Lauren and Mcdonald, 1985; Morgan et al., 2004a).

The presence of elevated Cu in aquatic environments is primarily a result of industrial discharges from sources including mining, municipal sewage, usage of agricultural pesticides and fertilizers (Eisler, 1998). Naturally occurring concentrations of Cu in freshwater environments may range from 0.003 μM to 0.47 μM (USEPA, 2007). In contaminated freshwaters, Cu concentrations may reach greater than 1.58 μM (USEPA, 2007). Elevated concentrations of Cu can lead to adverse effects in aquatic biota. In waters with low concentrations of DOC and low pH (<6 pH), Cu in the ionic form of Cu^{2+} is the most predominant species found (Hong et al., 2010). As an essential metal, Cu^{2+} is generally regulated in liver of fish (Kamunde et al., 2002a; Kamunde et al., 2002b) with concentrations that are 25 to100-fold greater than other organs (Kjoss et al., 2005; Tellis et al., 2012).

Natural concentrations of Ag may range from 0.005 nM to 0.05 nM in pristine freshwaters (Wood, 2012). Ag concentrations, however, can increase 1000 times or more (Wood, 2012) with extensive discharges from the mining industry, photographic processing, effluents from treatment plants and medical waste into freshwaters (Sathya et al., 2012). Ag in the free ion form of Ag^+ is considered to be a toxic metal to aquatic organisms (Hogstrand et al., 1996; Mayer et al., 2003). Unlike with Cu, Ag speciation is not dependent on pH but the presence of DOC, chlorides or sulfides can lead to complexation, resulting in decreased toxicity (Lin et al., 2002; Wood, 2012). It is generally accepted that Ag^+ is readily taken up by the gills in fish from the water (Wood, 2012) and accumulates primarily in the liver (Galvez et al., 2002; Burry, 2005).

The objective of this study was to assess metal interactions by linking bioaccumulation with physiological effects in rainbow trout during a 10-day exposure to waterborne Cu^{2+} and Ag^+ mixture. Bioaccumulation was measured on a whole tissue level by examining metal content in the gills and liver. Physiological effects were characterized by survival and disturbances in ion regulation. It was predicted that when present as a mixture, Ag^+ would bind more readily in the gills relative to Cu^{2+} because of Ag^+'s greater binding affinity of log K'= 10, opposed to Cu^{2+}'s affinity of log K'= 7.4 (Playle, 2004). Therefore there would be less Cu accumulation when it is present with Ag^+ compared to a Cu^{2+}-only exposure. It was also predicted that fish exposed to the Cu^{2+} and Ag^+ mixture would experience greater ion loss compared to either Ag^+-only, Cu^{2+}-only and the control since both metals target NKA and CA to cause toxicity.

2.3 Materials and Methods

2.3.1 Fish Culturing

Juvenile rainbow trout (*Oncorhynchus mykiss*) were purchased from Rainbow Springs Hatchery, Thamesford Ontario and maintained in 200L polyethylene holding tanks. Holding

tanks were supplied with continuously flowing (1.6 L/min) of mixed reversed osmosis water and dechlorinated city (Waterloo, Ontario) water to achieve a conductivity of 270 ± 30 uS, pH of 5-9 and temperature of 15 ± 3 degrees Celsius. Rainbow trout were fed on daily basis with commercial fish food (Skretting, Moore Clarke Canada, Vancouver British Columbia) at 2 % body weight. Culturing of fish and experimentation was conducted in accordance with Canadian Council on Animal Care, using protocols reviewed by the Wilfrid Laurier University Animal Care Committee.

2.3.2 Ten Day Exposure

Juvenile rainbow trout 30-50 g in wet weight were exposed to one of three metals treatments, either of 1.0 μM Cu^{2+}-only, 0.04 μM Ag^{+}-only, or a mixture of 1.0 μM Cu^{2+} plus 0.04 μM Ag^{+}. An unexposed (control) group was also included. These concentrations were based on a range finder to determine which nominal concentrations produced gill accumulation saturation over 6 hours. All exposures were done with a single replicate. Trout were randomly selected from the holding tanks and placed into 30 L polyethylene exposure tanks with n=12 for each treatment and controls. Exposure tanks were supplied with a continuous flow of mixed reverse osmosis, dechlorinated city (Waterloo, Ontario) water with a conductivity of 268 ± 20 uS. pH of 7.7 ± 0.1 and temperature of 14.5 ± 0.5^{o}C. Fish were acclimated for a minimum of two weeks prior to experimentation (Figure 2.1). For exposure, stock solutions (1.0 μM Cu^{2+}-only, 0.04 μM Ag^{+}-only or 1.0 μM Cu^{2+} + 0.04 μM Ag^{+}) were made from concentrated $CuSO_4$ and /or$AgNO_3$ solutions at 1000 times the desired concentration. Exposures were done as flow through by continuously metering (QG6 pump, Fluid Metering Inc., Oyster Bay, New York) stock solutions into the mixing head tanks at a desired rate (1mL/min) to achieve the desired exposure concentrations. The metering of exposure solutions into the head tanks was monitored at least

twice daily. Daily water measurements for temperature and conductivity were also conducted using a portable meter (YSI, Yellow Springs, Ohio).

2.3.3 Sample Collection and Storage

Unfiltered and filtered (0.45 μM) water samples (20 mL) were collected on days 0, 1, 3, 5 and 10. Water samples were acidified to 1.0% with 16 N HNO_3 (Trace metals grade, Fisher Scientific, Nepean, Ontario) and measured for Cu^{2+} and Ag^{+} by graphite furnace atomic absorption spectrophotometry (AAS: PinAAcle 900, Perkin Elmer, Woodbridge, Ontario). Sampling of tissues (gills and liver) and plasma was conducted on days 0, 1, 3, 5 and 10. Fish were not fed 24 hours prior to sampling to ensure that the gut was cleared of ingesta (Nadella et al., 2007). Sampled fish were first anesthetized using a dose of tricaine methanesulfonate (MS222: 0.3 g/L^{-1}) buffered 1:1 with $NaHCO_3$. Fish were then euthanized with a blow to the head, blotted dry with paper towels and then weighed (g). Blood samples were collected via caudal puncture (0.5-1.0 mL) using a 21-gauge needle and syringe, placed into microcentrifuge tubes containing 10.0 μL of heparinized Courtland saline (Lithium salt, Sigma Aldrich Canada, Oakville, Ontario) and centrifuged at 13,000 RPM for 3 minutes to separate blood and plasma. Plasma was isolated, placed in separate microcentrifuged tubes and stored immediately at -40 ^{o}C. Gills samples were removed, rinsed vigorously in deionized water for 15 seconds and blotted dry and placed in micro centrifuged tubes. Liver samples were also collected. All tissue samples were placed stored at -40 ^{o}C prior to analysis.

2.3.4 Metal and Plasma Ion Analysis

Tissue samples were weighed and 1N HNO_3 (5:1 volume to weight) was added to digest the tissue. Tissue samples were digested at 80 ^{o}C for 3 hours and then vortexed for 15 seconds then centrifuged for 2 minutes at 10,000-x *g* and then allowed to settle before analysis (Janes and

Playle, 1995; 2000). Digested samples were diluted using deionized water acidified to 1.0% using concentrated (16 N) HNO_3 and analyzed for Cu^{2+} and/or Ag^{+} content by graphite furnace AAS (PinAAcle 900, Perkin Elmer, Woodbridge, Ontario). Two certified reference materials, TM 15.2 and TM 23.5 (National Resource Council of Canada) were used to verify sample concentrations. Plasma samples (20 µL per sample) were analyzed for Cl^{-} content using a chloride analyzer (Chloride Analyzer 926, Cole-Parmer Canada Inc., Montreal, Quebec). Remaining plasma samples (10µL per sample) were diluted 1000 times with deionized water acidified to 1.0% with 16 N HNO_3 and analyzed for Na^{+} content by flame AAS (PinAAcle 900, Perkin Elmer, Woodbridge, Ontario).

2.3.5 Calculations and Statistical Analysis

Cu^{2+} and Ag^{+} concentrations were adjusted for tissue weight as well as dilution factors and presented as nmol Cu or Ag g^{-1} of tissue wet weight. Plasma Na^{+} and Cl^{-} are presented as mmol L^{-1}. Tissue accumulation results are presented as the mean with ± 1 standard error of the mean (SEM). Sigma Plot (Version 11.0- Systat Software Inc., San Jose) was used to conduct statistical analysis. Collected data were compared through one-way analysis of variance (ANOVA), using a significant difference of $P < 0.05$ as the limit of significance. When there was a significant difference amongst the treatment groups, a Tukey HSD post hoc test was performed to determine which treatment groups were different.

Additive effects on plasma ion balance was predicted and tested using the following equation based on the approach outlined by Norwood et al., 2003 and adapted from Niyogi et al., 2015:

$$\text{Simple Additivity (\% effect)} = [(a + b) - f] \times 100$$

where *a* and *b* represent measured % difference/effect of plasma ion content by metal 1 and 2 relative to the controls, and *f* is the interaction factor conceived by multiplying *a* and *b*. Additive effects on % mortality was predicted in a similar manner as above, where *a* and *b* represented the measured % mortality by metal 1 and 2 and *f* is the interaction factor calculated by multiplying *a* and *b.* Interactive effects on bioaccumulation were determined by comparing the % differences/effect in tissue concentration between treatment groups and the controls. All measured interactions were validated by testing for statistical significance.

2.4 Results

2.4.1 Water Concentrations and Survival

In this study, total and dissolved concentrations in the exposure water were measured for Cu^{2+} (Table 2.1) and Ag^{+} (Table 2.2) over 10 days. There was no significant difference in measured samples and therefore all values are presented as total concentration. Fish were exposed to a nominal concentration of 1.0 µM Cu-only, 0.04 µM Ag^{+}-only, and a mixture of 1.0 µM Cu^{2+} with 0.04 µM Ag^{+} for 10 days (Table 2.1; Table 2.2). Mortalities occurred during the span of the study with a majority on days 3 through 10 with a 33 % (8) in Cu^{2+}-only exposure and 63 % (15) in the mixture-exposure (Table 2.4). Over 10 days, there was 8 % (2) mortality in the Ag^{+}-only exposure and 4 % (1) mortality in the controls (Figure 2.4).

2.4.2 Bioaccumulation: Tissue Accumulation

In the gills, Ag accumulation was approximately 2.5-fold less in mixture-exposed fish relative to Ag^{+}-only exposed fish on day 1 but was 1.5-fold greater by day 3 (Figure 2.3). There was no difference in Cu accumulation in the gills of mixture and Cu^{2+}-only exposed fish by day 3 (Figure 2.3). Ag accumulation in the liver of Ag-only and mixture exposed fish were 190 % and

169 % greater than in the controls, respectively. Ag accumulation in the liver was significantly lower (P= 0.005) in the mixture-exposed fish compared to Ag^{+}-only exposed fish on days 1 and 3 (Figure 2.4). Cu accumulation in the liver was similar between the mixture and Cu^{2+}-only exposure on day 1. By day 3, Cu accumulation in the liver of mixture-exposed fish was less than those exposed to Cu^{2+}-only and controls (Figure 2.4).

2.4.3 Ion Regulation

Plasma Na^{+} and Cl^{-} concentrations were measured for each day of exposure. Fish exposed to the mixture had a significant reduction in plasma Na^{+} relative to the Cu^{2+}-only (P= <0.001) and Ag^{+}-only (P= <0.001) exposed fish on day 3. Between both single metal exposed fish, plasma Na^{+} was further decreased in the Ag^{+}-only exposure compared to Cu^{2+}-only exposure on days 3 and 10 (Figure 2.5). Plasma Cl^{-} content in mixture and Cu^{2+}-only exposure fish were similar to each other on day 1 and in both case, were significantly lower (P= 0.004) than Ag-only exposed fish (Figure 2.5). On day 3, plasma Cl^{-} was similar between mixture-exposed fish and the Cu^{2+}-only (P= 0.634) and Ag^{+}-only (P= 0.754) exposed fish were not significantly different.
Refer to Appendix A for *Chapter 2* data.

2.5. Discussion

The use of 1.0 μM of Cu^{2+} in the single metal and mixture exposure was more lethal than anticipated. The Cu^{2+}-only exposure resulted in 33% mortality by day 10 while in the mixture exposure; there was 63% mortality by day 3 of the study. Ag accumulation was greater in the gills of trout exposed to Ag^{+}-only relative to those exposed to the mixture on day 1. This was unexpected as both metals are taken up by apical Na^{+} channels in the gills of trout and Cu^{2+} would not be expected to out compete Ag^{+} based on binding affinity (Niyogi and Wood, 2004). Ag accumulation in mixture exposed fish was predicted to be similar or more than in those

exposed to Ag only due to a higher binding affinity. However, since the Cu^{2+} concentration in this test was 25-fold greater than the Ag^{+} concentration (1.0 μM Cu^{2+} vs 0.04 μM Ag^{+}), it is possible that competition for gill binding sites occurred in favour of Cu^{2+}, leading to less Ag^{+} uptake in mixture-exposed fish. It is perhaps likely that with an increase concentration difference between Cu^{2+} and Ag^{+} in a mixture, greater competition could occur where Ag^{+} uptake is inhibited, as seen in a study by Bury and Hogstrand (2002). Their study found that Atlantic salmon fry exposed to a mixture containing 100-fold excess of Cu^{2+} at (1.0 μM Cu^{2+} and 0.04 μM Ag^{+}) resulted in inhibition of Ag^{+} influx at the gills. In another study, Balisterieri and Mebane (2014) also considered the use of increasing concentrations of cadmium (Cd^{2+}), Cu^{2+}, lead (Pb^{2+}), and zinc (Zn^{2+}) in evaluating the metal mixture toxicity. Their results showed that as metal concentrations increased for Cu^{2+}, Pd and Zn, they all bound more successfully in the gills of trout even though Cd^{2+} has a higher binding affinity (Balisterieri and Mebane, 2014). Balisterieri and Mebane (2014) showed that Zn^{2+}, an essential metal with a lower binding affinity, could potentially outcompete Cd^{2+}, a non-essential, higher binding affinity metal. It is possible that when Cu^{2+} or Zn^{2+} exposure concentrations are elevated, homeostatic mechanisms involved in regulating essential metals may in some way minimize the uptake of other metals (Ribeyre et al., 1995). In this study, because mortalities occurred in both the Cu-only and mixture exposure it is apparent that trout were not able to regulate Cu^{2+} and the greater difference in concentration lead to less Ag accumulation on day 1. It is important to note that the difference in Ag accumulation only occurred on day 1 of exposure. By day 3, Ag accumulation in the gills was similar between Ag^{+}-only and mixture exposed trout (Figure 2.3). It was predicted using the simply additivity concept (adapted from Niyogi et al., 2015) that the combined effect of Cu^{2+} and Ag^{+} would lead to a 39 % mortality rate in mixture-exposed fish over 10 days; however, measured mortality rate was 63 % (Table 2.4). Since the measured % mortality rate was greater than predicted, the Cu^{2+} +

Ag^+ exposure indicates that a more than additive effect occurred. The mortalities associated with the mixture exposure (Figure 2.2) would appear to be unrelated to Ag bioaccumulation as tissue concentrations in Ag^+-only exposed fish were similar (gills) or greater (liver) compared with mixture-exposed fish.

In previous studies, it has been demonstrated that the liver of fish is the main site for accumulation of several metals including Ag (Hogstand and Wood, 1998; Galvez et al., 2002; Bury, 2005) and Cu (De Beock et al., 2004; Kamunde and MacPhail, 2008; Eyckmans et al., 2012). Specifically in rainbow trout, Cu^{2+} has been shown to be present at high concentrations in the liver under controlled conditions of low Cu (Eyckmans et al., 2012). High Cu^{2+} concentrations in the liver could possibly provide a means of internal regulation when in excess (Eyckmans et al., 2012). As such, it was common for control fish in this study to have relatively high Cu content in the liver as observed. Fish exposed to 1.0 μM of Cu^{2+}-only had Cu liver accumulations there were more than double that of controls but this was not significant (Table 2.4). Similarly, De Beock et al., (2004) exposed rainbow trout to 1.0 μM for 4 days and found that Cu^{2+} exposed fish and controls showed no significant differences in Cu liver content. Based on the work of Grosell and colleagues (Grosell et al., 2001) hepatic Cu concentrations during exposure are held in check via enhanced biliary elimination and this seems likely to have occurred in this study.

Fish exposed to Ag^+-only had significant accumulation in the liver relative to the controls on each sampling day. Mixture exposed fish had significantly lower Ag liver accumulation than Ag^+-only exposed fish with 7.4-fold less on day 1 and 2.4-fold less on day 3. It is possible that with a high background concentration of Cu in the liver in addition to the 1.0 μM of Cu^{2+} in the mixture exposure, this could have influenced Ag accumulation. However, since the mixture-exposed fish all died within the first 3 days, the process of death may have limited Ag

accumulation in the liver. This may also have occurred with Cu accumulation in the liver of mixture-exposed fish since concentrations were significantly less than those exposed to Cu^{2+}-only on day 3 (Figure 2.4). Thus, predictions that Ag would accumulate similarly in both Ag^{+}-only and mixture exposed fish was not supported in for liver accumulation.

Cu^{2+} and Ag^{+} are among a group of metals that have been shown to cause ionoregulatory disturbances in fish. Several studies have demonstrated that the mode of toxicity for both Cu^{2+} and Ag^{+} involves inhibiting NKA and possibly CA to cause disturbances with ion homeostasis (Lauren and Mcdonald, 1985; Paquin et al., 2002; Morgan et al 2004a). In previous studies, a similar disturbance in internal Na^{+} and Cl^{-} was observed in rainbow trout exposed to Cu^{2+} (Lauren and McDonald, 1985; Wilson and Taylor 1993) and Ag^{+} (Galvez and Wood, 2002). In this study, fish exposed Cu^{2+} or Ag^{+} alone had a slight disturbance in plasma Na^{+} compared to controls during the span of 10 days. Mixture exposed fish on the other hand, exhibit significant plasma Na^{+} loss by day 3 compared to the single metal exposed fish, as hypothesized. It was also hypothesized that plasma Cl^{-} disruption would also be the greatest in mixture-exposed fish but was not supported since concentrations were similar were between treatment groups by day 3. It was predicted using the simple additivity model that the combined effect of Cu^{2+} and Ag^{+} in the mixture would result in a 15 and 42 % decrease in plasma Na^{+} and plasma Cl^{-}, respectively (Table 2.3). Predicted effects on plasma ions, however, did not follow simple addition in mixture-exposed fish since measured % differences on plasma Na^{+} and Cl^{-} were 92 % and 22 %, respectively (Table 2.3). This suggests that the effect of the mixture exposure was more than additive on plasma Na^{+} disruption but less than additive on plasma Cl^{-} on day 3 (Table 2.3). Based on these results, a precise conclusion cannot be drawn since statistical analyses were not conducted between % predicted and % measured values. It is clear that an interaction occurred between Cu^{2+} and Ag^{+}, producing a more than additive effect on plasma Na^{+} disruption and on

mortality. This then would have been related to significantly less Cu and Ag accumulation in mixture-exposed fish. The more than additive disruption on plasma Na^+ perhaps involved a joint action of Cu^{2+} and Ag^+ that increased binding to NKA and CA where the number (or activity) of NKA and CA might have been up-regulated to maintain Na^+ homeostasis (Tellis et al., 2012). Greater plasma Na^+ loss would have then caused increased blood viscosity and pressure that lead to a series of other events and ended in mortality via cardiovascular collapse (Hogstrand and Wood, 1998).

In this study, responses to single-metal exposures of either Cu^{2+} or Ag^+ were used to assess mixture effects. Notable differences in tissue metal accumulation and disturbances in plasma ion were observed between mixture and single metal exposures. It was apparent that the addition of 0.04 μM Ag^+ to an exposure of 1.0 μM Cu^{2+} resulted in greater toxicity even though Ag^+ on its own did not induce mortality. Tissue accumulation of metals in mixture-exposed fish indicated that exposure to Cu^{2+} concentration was associated with reduced uptake of Ag^+ in the liver. Predictions on Ag accumulation in fish exposed to Ag^+-only and the mixture and were supported where Ag concentrations in the gills were similar in both treatments. However, predictions on Ag accumulation were not supported for the liver since tissue concentrations were greater in Ag^+-only exposed fish compared to those exposed to the mixture. Plasma Na^+ disruption was the greatest in mixture-exposed fish and indicated that disruption on ion balance was more than additive. A less than additive effect was observed on plasma Cl^- loss where concentrations were similar in all treatment groups by day 3. Thus, hypothesis on plasma Cl^- loss were not supported. Overall, findings in this experiment were impacted by events related to mortality. This was indicated in mixture-exposed fish where significantly less plasma Na^+ and hepatic Cu and Ag were observed compared to single-metal exposed fish.

2.6. Tables and Figures

Table 2.1. Means (± SEM (n)) for measured dissolved Cu concentrations in water samples from control, Cu-only and mixture exposures over 10-days. Values are represented in μmol/L (μM). Samples were taken directly from the head tank and exposure (in replicate) tanks.

	Water in Head Tank	**Water Exposure Tank**
Exposure	**Dissolved (μM)**	**Dissolved (μM)**
Control (0 μM)	0.03 ± 0.01 (4)	0.03 ± 0.01 (8)
Cu^{2+}-only (0.35 μM)	0.45 ± 0.23 (4)	0.40 ± 0.14 (8)
Mixture (0.35 μM Cu^{2+} + 0.04 μM Ag^{+})	0.52 ± 0.26 (4)	0.43 ± 0.15 (8)

Table 2.2. Means (± SEM (n)) for measured dissolved Ag concentrations in water samples from control, Ag^{+}-only and mixture exposures over 10 days. Values are represented as nmol/L (nM). Samples were taken directly from the head tank and exposure (in replicate) tanks.

Exposure	**Water in Head Tank Dissolved (nM)**	**Water Exposure Tank Dissolved (nM)**
Control (0 μM)	0.00 ± 0.00 (4)	0.00 ± 0.00 (8)
Ag^{+}-only (0.04 μM)	40.15 ± 20.07 (4)	37.53 ± 13.27 (8)
Mixture (0.35 μM Cu^{2+} + 0.04 μM Ag^{+})	50.99 ± 05.50 (4)	43.89 ± 15.52 (8)

Table 2.3. Measured and predicted effects on plasma Na^+ and Cl^- balance in rainbow trout exposed to Cu^{2+}-only, Ag^+-only or mixture exposure on day 3. Predicted effects were estimated based on principles of simple additivity (see Calculation, Methods section for details). Values are represented as % difference of plasma Na^+ or Cl^- content relative to the controls.

Exposure	**Measured % difference of plasma Na^+**	**Predicted % difference of plasma Na^+**	**Measured % difference of plasma Cl^-**	**Predicted % difference of plasma Cl^-**
Cu^{2+}-only (1.0 μM)	0	-	31	-
Ag^+-only (0.04 μM)	15	-	16	-
Mixture (1.0 μM Cu^{2+} + 0.04 μM Ag^+)	92	15	22	42

Table 2.4. Measured and predicted % mortality rate in rainbow trout exposed to Cu^{2+}-only, Ag^+-only or mixture on day 3. Predicted effects were estimated based on principles of simple additivity (see Calculation, Methods section for details). Values are represented as % mortality.

Exposure	**Measured % mortality rate**	**Predicted % mortality rate**
Controls (0 μM)	4	
Cu^{2+}-only (1.0 μM)	33	-
Ag^+-only (0.04 μM)	8	-
Mixture (1.0 μM Cu^{2+} + 0.04 μM Ag^+)	63	39

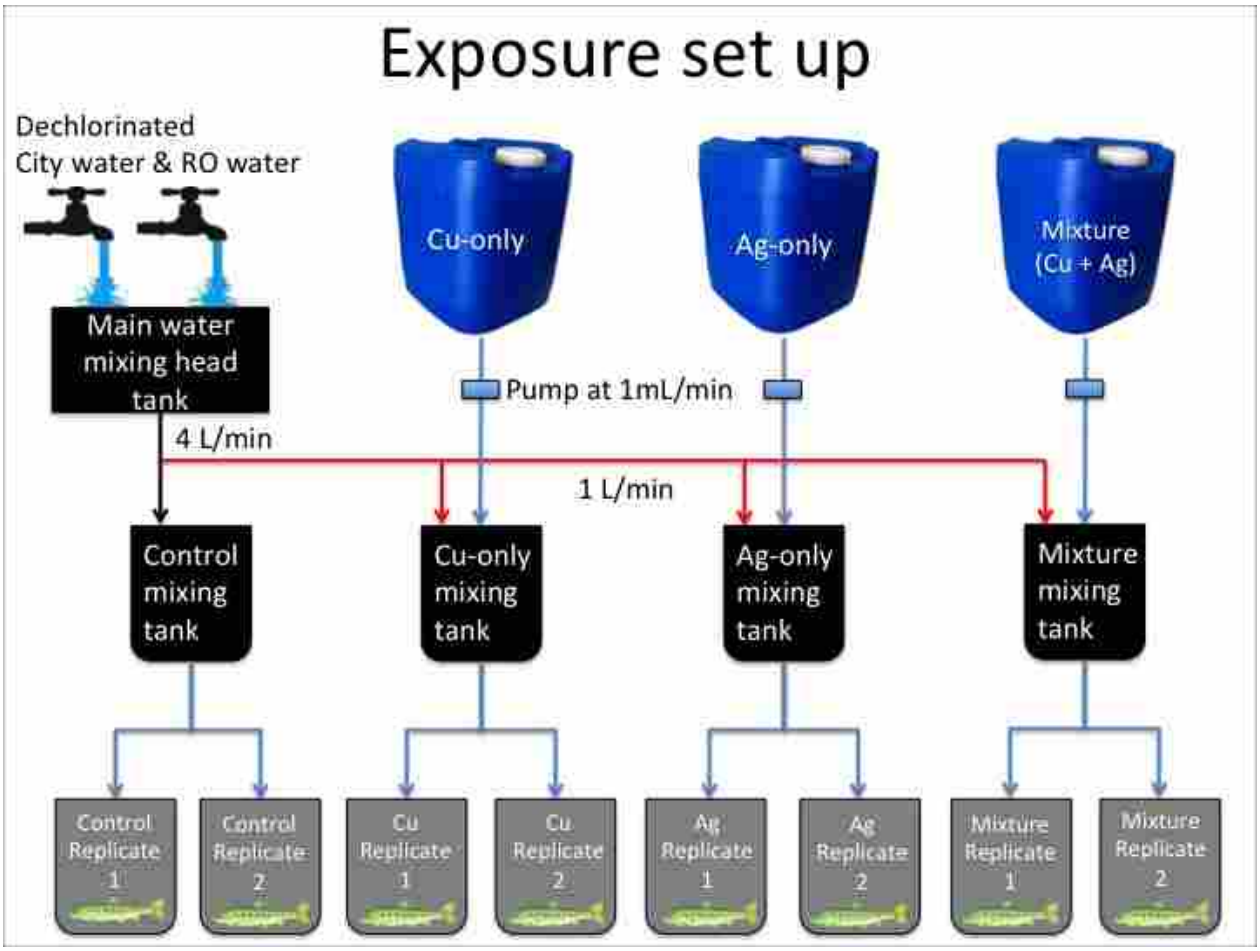

Figure 2.1. Experimental Set Up. Exposure system is comprised of one main head tank that is used to mix dechlorinated city water and reversed osmosis (RO) water, feeding into four other separate treatment mixing tanks at 1L/min each. These four treatment mixing tanks include a control; copper (Cu) only, silver (Ag) only and Cu + Ag mixture. A pump will feed a stock solution at 1000 x the desired concentration at 1mL/min for each concentration (except for the control). Each treatment head tank will feed into single-replicate exposure tanks.

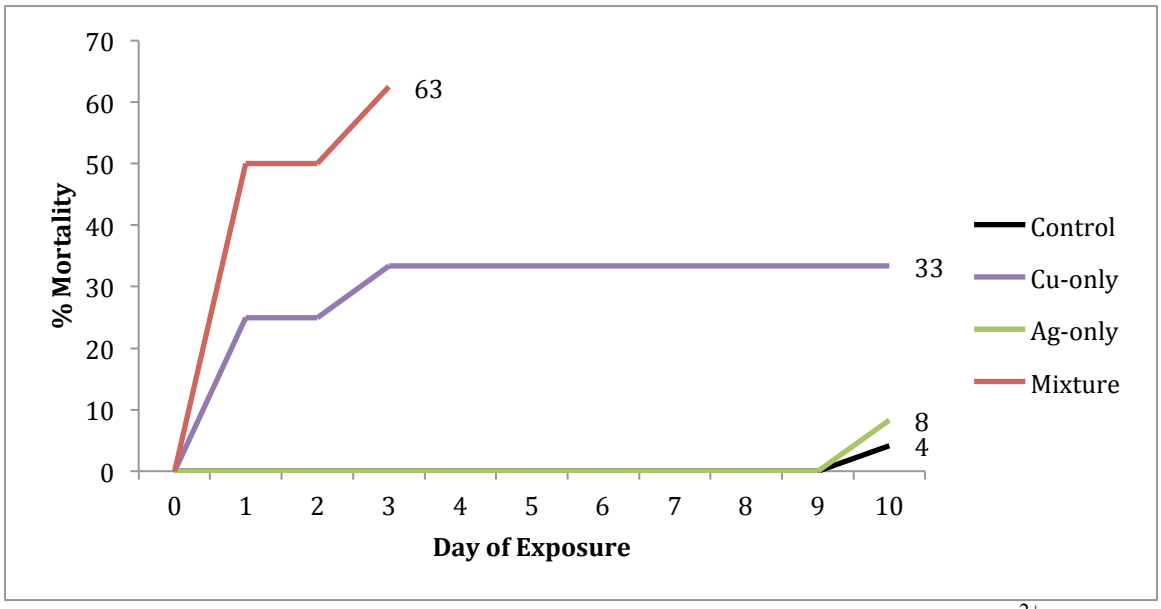

Figure 2.2. Percent (%) mortality of rainbow trout exposed to either 1.0 µM Cu^{2+}-only (purple), 0.04 µM Ag^{+}-only (green), or 1.0 µM Cu^{2+} + 0.04 µM Ag^{+} mixture (red) for 10 days. A control group was also included (black). Values are represented as % mortality.

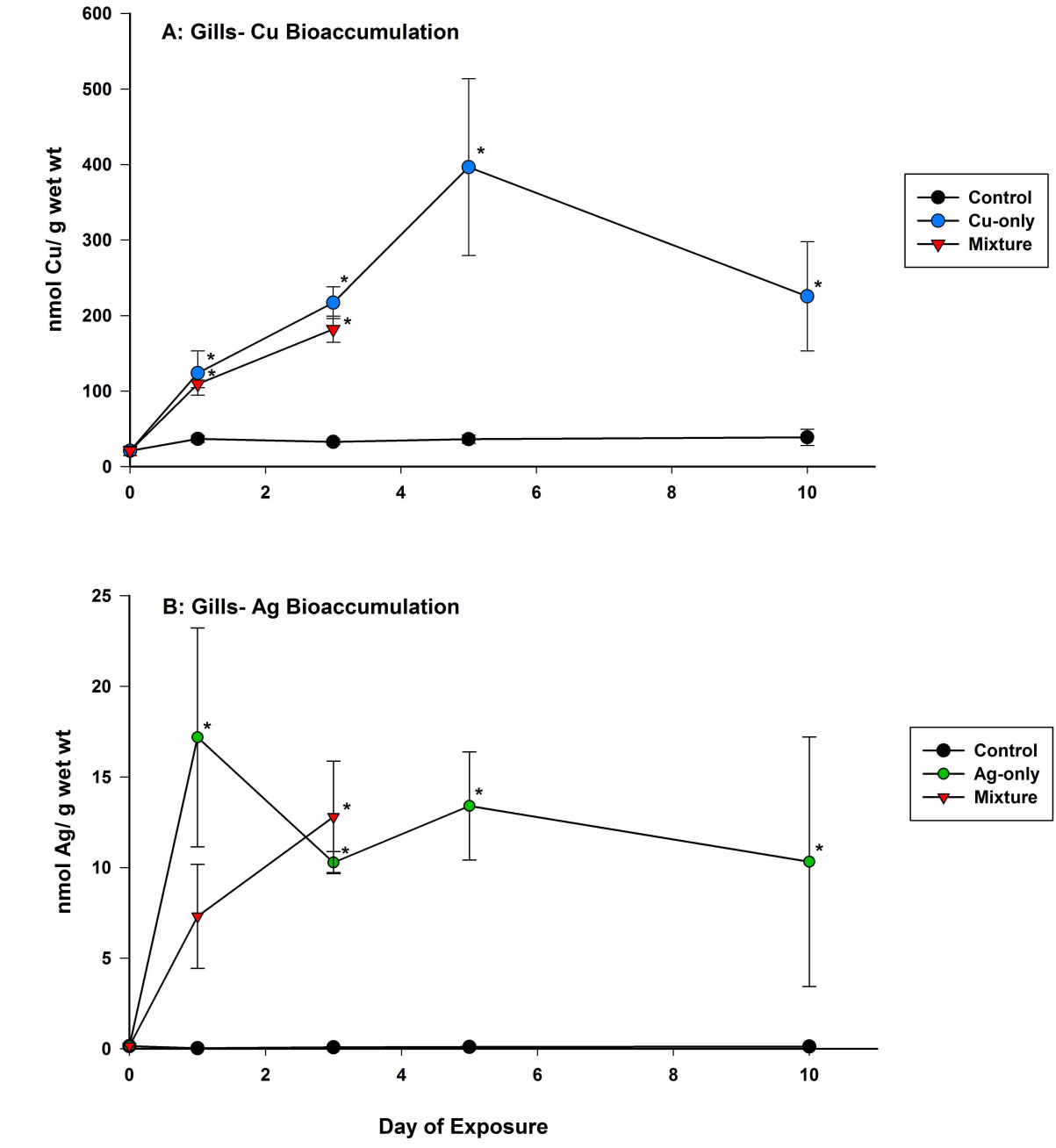
A: Gills- Cu Bioaccumulation
nmol Cu/ g wet wt
600
500
400
300
200
100
0
0
2
4
6
8
10
Control
Cu-only
Mixture
B: Gills- Ag Bioaccumulation
nmol Ag/ g wet wt
25
20
15
10
5
0
0
2
4
6
8
10
Day of Exposure
Control
Ag-only
Mixture

Figure 2.3. Gill accumulation of Cu (A) and Ag (B) in rainbow trout exposed to either control, 1.0 μM Cu^{2+}-only, 0.04 μM Ag^{+}-only or 1.0 μM Cu^{2+} + 0.04 μM Ag^{+} mixture for 10 days. Values are represented as means (nM /g tissue wet weight) ± 1 SEM. * represents a significant difference between exposed fish and controls on that sampling day while a † represents a significant difference between mixture-exposed fish and Ag^{+}-only (A) or Cu^{2+}-only (B) exposed fish (ANOVA $P < 0.05$).

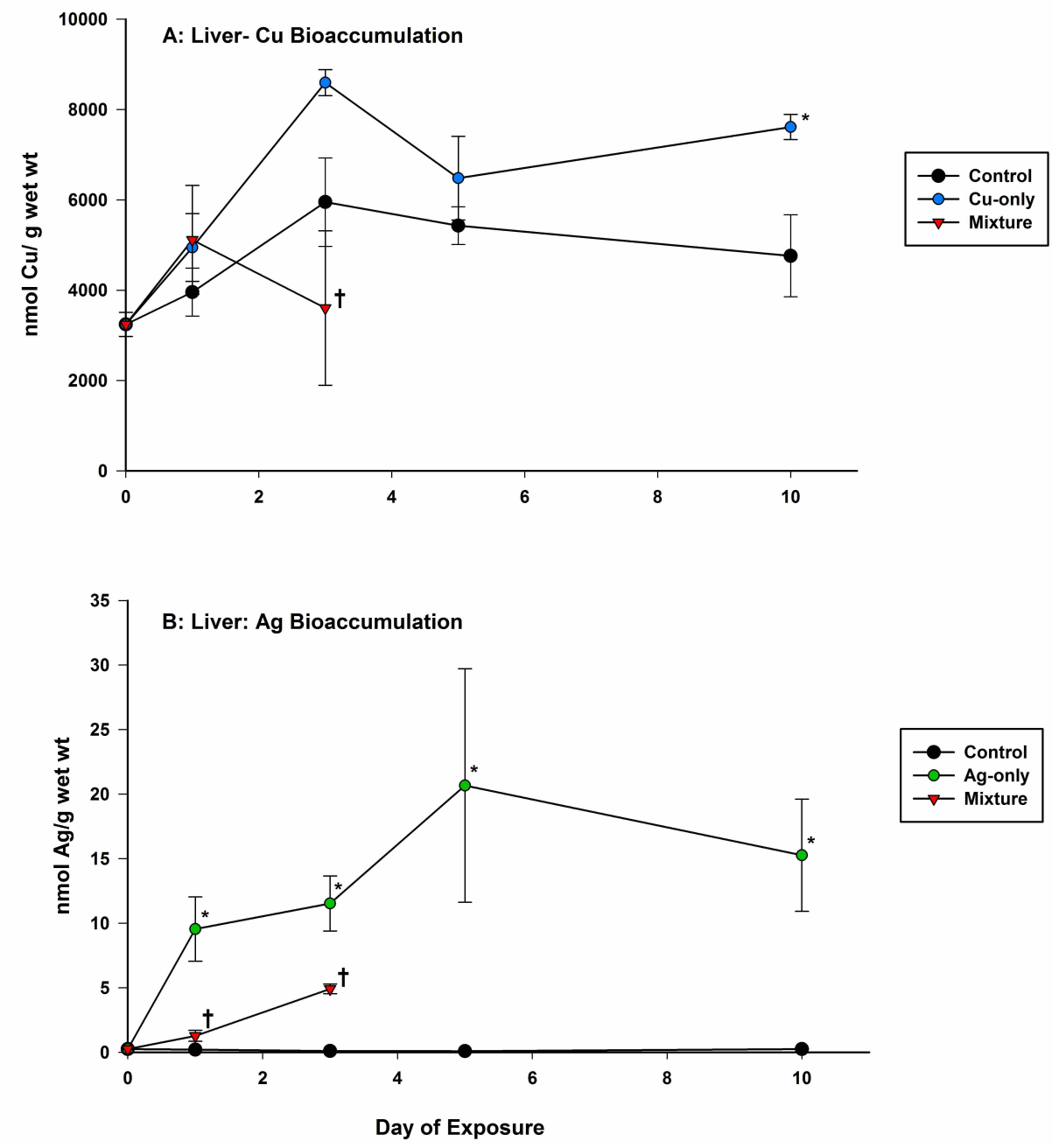
A: Liver- Cu Bioaccumulation
nmol Cu/ g wet wt
10000
8000
6000
4000
2000
0
0
2
4
6
8
10
*
†
Control
Cu-only
Mixture
B: Liver: Ag Bioaccumulation
nmol Ag/g wet wt
35
30
25
20
15
10
5
0
0
2
4
6
8
10
*
*
*
*
†
†
Control
Ag-only
Mixture
Day of Exposure

Figure 2.4. Liver accumulation of Cu (A) and Ag (B) in rainbow trout exposed to either control, 1.0 μM Cu^{2+}-only, 0.04 μM Ag^{+}-only or 1.0 μM Cu^{2+} + 0.04 μM Ag+ mixture for 10 days. Values are represented as means (nM /g tissue wet weight) ± 1 SEM. * represents a significant difference between exposed fish and controls on that sampling day while a † represents a significant difference between mixture-exposed fish and Ag^{+}-only (A) or Cu^{2+}-only (B) exposed fish (ANOVA $P < 0.05$).

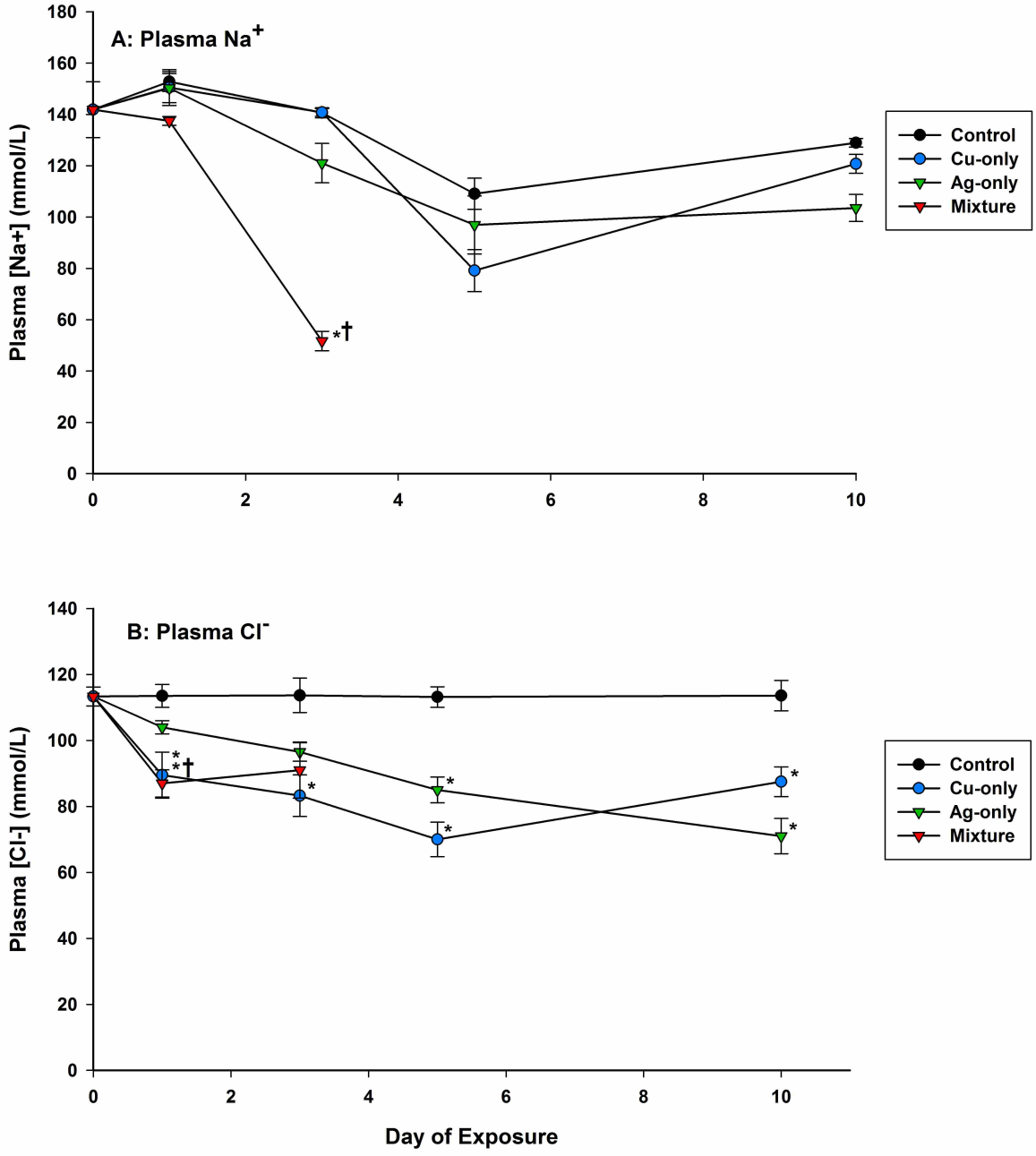
A: Plasma Na+
Plasma [Na+] (mmol/L)
180
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10
*†
Control
Cu-only
Ag-only
Mixture
B: Plasma Cl-
Plasma [Cl-] (mmol/L)
140
120
100
80
60
40
20
0
0
2
4
6
8
10
Day of Exposure
*
*†
Control
Cu-only
Ag-only
Mixture

Figure 2.5. Plasma Na^+ (A) and Cl^- (B) levels in rainbow trout exposed to either control, 1.0 μM Cu^{2+}-only, 0.04 μM Ag^+-only or 1.0 μM Cu^{2+} + 0.04 μM Ag^+ mixture for 10 days. Values are represented as means (Plasma [ion] mmol/L) ± 1 SEM. * represents a significant difference between exposed fish and controls on that sampling day while a † represents a significant difference between mixture-exposed fish and Ag^+-only (A) or Cu^{2+}-only (B) exposed fish (ANOVA $P < 0.05$).

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Chapter 3

Assessing the effects of metal interactions during Cu + Ag mixture exposure on bioaccumulation and ion balance of rainbow trout (Oncorhynchus mykiss)

3.1 Abstract

Cu-Ag metal interactions were studied in rainbow trout (*Oncorhynchus mykiss*) at the whole tissue (gills, liver, kidney) and subcellular level (gills, liver) in correlation to plasma ion concentrations (Na^+, Cl^-). Trout were exposed to 0.35 μM Cu^{2+}-only or 0.04 μM Ag^+-only as or to a binary mixture of 0.35 μM Cu^{2+} + 0.04 μM Ag^+ for 14 days. Sets of unexposed trout were included as controls. A mortality rate of 0 % occurred in fish exposed to 0.35 μM Cu^{2+}-only, 16 % fish exposed to 0.04 μM Ag^+-only and 31 % in fish exposed to the 0.35 μM Cu^{2+} + 0.04 μM Ag^+ mixture. Significant additional accumulation of Cu and Ag were observed in the tissues of mixture-exposed fish relative to the accumulation that occurred in fish exposed to Cu^{2+}-only or Ag^+-only. Subcellular distribution of Cu in the gills and liver occurred mainly in metal sensitive fractions (MSF: mitochondria, microsomes and lysosomes, heat-denatured proteins (HDP)) with fish exposed to either Cu^{2+}-only or Cu^{2+} + Ag^+ mixture. Ag partitioned primarily in biologically detoxified metal fractions (BDF: metal rich granules (MRG), heat-stable proteins (HSP)). A significant decrease in plasma ion was observed in fish exposed to the Cu^{2+} + Ag^+ mixture relative to single-metal exposures as plasma Na^+ was reduced by 36 % and plasma Cl^- by 40 % on day 14. Increase accumulation of Cu^{2+} + Ag^+ in the gills and kidney of mixture exposed fish was indicative of a synergistic effect produced by a Cu and Ag interaction. Cu and Ag interactions were also apparent in causing the greater decrease of plasma ions in mixture-exposed fish.

3.2 Introduction

Aquatic organisms are frequently exposed to a mixture of metals in contaminated environments. Some of these metals (e.g. Cu) have essential biological roles and are often regulated by the organism when internal concentrations are tolerable based on external concentrations (Komjarova and Blust, 2009). Other metals (e.g. Ag) in the mixture have no known biological importance and can be toxic at low concentrations (Wood, 2012). Metals that have a common uptake route into the gills of fish have been shown to interact with one resulting in competitive uptake inhibition (e.g lead: Pd^{2+} + cadmium: Cd^{2+}) (Komjarova and Blust, 2009) or enhanced uptake of a metal (e.g Cu^{2+} + Ag^{+}) (Niyogi et al., 2015). For instance, Cu^{2+} and Ag^{+} are two metals that been documented to be up taken via apical Na^{+} channels into the gills (Bury and Wood, 1999; Gorsell and Wood, 2002). Once in the gills, both Cu^{2+} and Ag^{+} have both been shown to have a similar mode of toxic action, which is the inhibition of NKA (Galvez et al., 2002; Grosell et al., 2003; Morgan et al., 2015) and carbonic anhydrase (CA: Morgan et al., 2004; Goss et al., 2011; Zimmer et al., 2012) to cause ionoregulatory disturbance. Realistically, Ag contamination in aquatic systems rarely occurs in isolation from other metals and information regarding potential interactive effects are lacking (Wood, 2012). Further knowledge about the interactions between Ag^{+} and Cu^{2+} during waterborne exposure to aquatic organisms could provide a better understanding of metal mixture toxicity.

Several studies have shown that Cu and Ag accumulate in a variety of tissues within fish during waterborne exposure. The gill for instance, is the primary uptake route and site of toxicity where it has been shown to accumulate significant amounts of Cu (Grosell et al., 2007; Harley and Glover, 2014) and Ag (Jane and Playle, 1995; Galvez et al., 2002). The liver has also been shown to accumulate significant amounts of Cu (De Beock et al., 2004; Kamunde and MacPhail,

2008; Eyckmans et al., 2012) and Ag (Galvez et al., 2002; Bury, 2005) and has been demonstrated to act as the main storage site for both metals during exposure. The biological significance of metal accumulation in tissues as a whole, however, may not provide a precise prediction of toxicity (Vijver et al., 2006; Kamunde and MacPhail, 2008) since not all portions contribute to toxicity. Mechanisms involved with internal handling function to regulate excess essential metals and detoxifying those that are harmful, making them biologically unavailable to induce toxicity. Detoxification takes place mainly in the liver with the synthesis of small cysteine-rich proteins known as metallothionein (MT) and metallothionein-like proteins (MTLP), which binds onto metals and provides short-term storage (Wang and Rainbow, 2006; Wood, 2012). Another means of detoxification involves the synthesis of metal-rich granules (MRG) to handle permanent storage of metals (Wood, 2012; Leonard et al., 2014). The subcellular fractionation technique is a widely used approach to quantify concentrations of sequestered metal from MT, MTLP and MRG (Vijver, 2004; Kamunde and MacPhail, 2008, Li et al., 2015). Therefore, since the gills are associated with sites of toxicity and the liver with detoxification, it would be informative to determine how Cu and Ag are distributed at the subcellular level in these tissues.

Within a cell, there are six subcellular fractions in which metals may accumulate in: i) cellular debris (membranes), ii) mitochondria, iii) microsomes and lysosomes, iv) heat denaturable proteins (HDP), v) NaOH resistant granules, and vi) heat stable proteins (HSP) (Wallace and Luoma, 2003; Giguere et al., 2006; Wang and Rainbow, 2006). These fractions can further be grouped in two general fractions, metal sensitive fraction (MSF) which includes mitochondria plus HDP, and biologically detoxified fraction (BDF) in which is comprised of NaOH resistant granules as well as HSP (Wallace and Luoma, 2003; Giguere et al., 2006; Wang and Rainbow, 2006; Kamunde and MacPhail, 2008). MSF are considered to be the toxic action

sites where as BDF contain metals that have been sequestered (i.e. detoxified) and unavailable to cause toxicity (Kraemer et al., 2005; Wang, 2012). The "spillover hypothesis" is the current working hypothesis for subcellular metal distribution studies. The hypothesis states that an organism's capacity to detoxify metals is limited to a certain threshold and once this threshold is surpass, the metals spill into other MSF to cause toxicity (Wallace et al., 2003; Kamunde, 2009).

The objective of this study was to further the understanding of the sublethal effects of a Cu and Ag mixture (0.35 μM Cu^{2+} and 0.04 μM Ag^{+}) in rainbow trout (*Oncorhynchus mykiss*) by linking whole tissue and subcellular accumulation to plasma ion content during a 14-day waterborne exposure. Bioaccumulation was investigated on a whole tissue level (gills, liver, kidney), as well as at the subcellular level (gills and liver). Studying the subcellular distribution of Cu and Ag in the gills and liver will provide insight into metal interactions at a subcellular level.

3.3 Methods

3.3.1 Fish Culturing

Juvenile rainbow trout (*Oncorhynchus mykiss*) were purchased from Rainbow Springs Hatchery, Thamesford Ontario and maintained in 200 L polyethylene holding tanks. Holding tanks were supplied with continuously flowing (1.6 L/min) of mixed reversed osmosis water and dechlorinated city (Waterloo, Ontario) water to achieve a conductivity of 270 ± 30 uS, pH of 5-9 and temperature of 15 ± 3 degrees Celsius. Rainbow trout were fed on a daily basis with commercial fish food (Skretting, Moore Clarke Canada, Vancouver British Columbia) at 2.0% body weight. Culturing of fish and experimentation was conducted in accordance with Canadian Council on Animal Care, and reviewed and set by the Wilfrid Laurier University Animal Care Committee.

3.3.2 Exposure Setup

Juvenile rainbow trout 60-100 g in wet weight were exposed to three separate metal treatments: 0.35 μM Cu-only, 0.04 μM Ag-only or a mixture of 0.35 μM Cu plus 0.04 μM Ag. An unexposed (control) group was also included. All exposures were done with a single replicate. Trout were randomly selected from the holding tanks and placed into 30 L polyethylene exposure tanks. Exposure tanks were supplied with a continuous flow of water (as in 3.3.1) with temperatures of 12.0 ± 1.0 ^{o}C and pH of 7.7 ± 0.1. Fish were acclimated for a minimum of two weeks prior to experimentation. For exposure to 0.35 μM Cu^{2+}-only, 0.04 μM Ag^{+}-only or 0.35 μM Cu^{2+} + 0.04 μM Ag^{+}, $CuSO_4 \bullet 5H_20$ and $AgNO_3$ (Sigma-Aldrich, St. Louis) were used to make stock solutions at 1000 times the desired concentration. All exposures were done as flow through by continuously metering (QG6 pump, Fluid Metering Inc., Oyster Bay, New York) stock solutions into mixing head tanks at a desired rate of 1mL/min, which then flowed at a rate of 1 L/min to achieve the target exposure concentrations in the tanks. Daily water measurements for temperature and conductivity were also conducted using a portable meter (YSI, Yellow Springs, Ohio).

3.3.3 Sample Collection and Storage

Unfiltered and filtered (0.45 μM) water samples (20 mL) were collected on days 0, 1, 3, 7 and 14. Water samples were acidified to 1.0% with 16 N HNO_3 (Trace metals grade, Fisher Scientific, Nepean, Ontario) and measured for Cu and Ag by graphite furnace atomic absorption spectrophotometry (AAS: PinAAcle 900, Perkin Elmer, Woodbridge, Ontario). Sampling of tissues (gills, liver and kidney) and plasma was conducted on days 0, 1, 3, 7 and 14. Fish were not fed 24 hours prior to sampling. Sampled fish were first anesthetized using a dose of tricaine methanesulfonate (MS222: 0.3 g/L^{-1}) buffered 1:1 with $NaHCO_3$. Fish were then euthanized with

a blow to the head, blotted dry with paper towels and then weighed (g). Blood samples were collected via caudal puncture (0.5-1.0 mL) using a 21 gauge needle and syringe, placed into micro centrifuge tubes containing 10.0 µL of heparinized Courtland saline (Lithium salt, Sigma Aldrich Canada, Oakville, Ontario) and centrifuged at 13,000 RPM for 3 minutes to separate blood and plasma. Plasma was isolated, placed in separate microcentrifuge tubes and stored immediately at -40 ºC. From each fish, gill samples were removed, rinsed vigorously in deionized water for 15 seconds and blotted dry and placed in microcentrifuge tubes. Liver and kidney samples were also collected from each fish. A portion of each tissue sampled (100-200 mg) was removed and placed into a microcentrifuge tube then snap-frozen in liquid nitrogen to be stored at -80 ºC prior to analysis for subcellular distribution. The remaining tissue samples were placed into another micro centrifuge tube and stored at -40 ºC prior to digestion and analysis for whole tissue metal burden.

3.3.4 Subcellular Distribution of Cu and Ag in tissues

Assessment of the subcellular distribution of Cu and Ag in gills and liver samples was done using a differential centrifugation technique (fractionation) outlined by Giguere et al. (2006) and Wallace et al. (2003). Tissue samples (100-200 mg) were weighed (CP224S, Sartorius, Elk Grove, IL) and diluted 3:1 wet weight with a buffer solution (20mM TRIS-base with 2mM of 2-mercaptoethanol and 0.2 mM phenylmethanesulfonyl fluoride, at pH 8.6). Tissues were kept on ice and homogenized at 20,000 RPM (Omini THQ, Omni Internatioanl, Marietta, GA). The first step of fractionation was to centrifuge the homogenized samples at 800 x *g* (IEC-CL31R Multispeed; Thermo Electron Corp., Milford, MA) for 15 minutes at 4 ^{o}C with the resulting supernatant being extracted and saved for further fractionation. The resulting pellet was suspended in 1mL of 1N NaOH and heated treated at 80 ºC for one hour and then centrifuged at

5000 x *g* for 10 minutes at 20 ºC, resulting in the MRG fraction as the pellet and cellular debris fraction as the supernatant.

From the first fractionation, the supernatant was centrifuged at 10 000 x *g* for 30 minutes at 4 ºC to produce the mitochondrial fraction as the pellet and the third supernatant (cytosol). The third supernatant was then centrifuged at 100 000 x *g* (Optima MAX; Beckman Instruments, Mississauga, ON, Canada) for 1 hour at 4 ºC, producing a fourth supernatant (proteins) and a pellet containing the microsomes and lysosomes fraction. In the last fractionation step, the fourth supernatant was heat treated at 80 ºC for 10 minutes, cooled for an hour at 4 ºC and then centrifuged at 50 000 x *g* for 10 minutes at room temperature, resulting in the HDP fraction as the supernatant and the HSP fraction as the pellet. Refer to Figure 3.1 for subcellular fractionation protocol.

3.3.4 Metal and Plasma Ion analyses

Pellets from the subcellular fractionation process and whole tissues were weighed and 1N HNO_3 (5:1 volume to weight) was added to digest the samples (Trace metal grade, Fisher Scientific, Nepean, ON). Supernatants from the subcellular fractionation process were also suspended and 1N HNO_3 (3:1 volume to weight) was added to digest the samples. Following the addition of acid, all tissue samples (whole and fractions) were digested by heat (80 ºC for 3 hours), followed by 15 seconds of vortex then centrifuged for 2 minutes at 10,000-x g and allowed to settle before analysis (Janes and Playle, 1995; 2000). Digested samples were diluted using deionized water acidified to 1.0% from concentrated (16 N) HNO_3 and analyzed for Cu and/or Ag content by graphite furnace AAS (PinAAcle 900, Perkin Elmer, Woodbridge, Ontario). Two certified reference materials, TM 15.2 and TM 23.5 (National Resource Council of Canada) were used to verify sample concentrations. Plasma (10 µL from a total of 20 µL per

sample) was analyzed for Cl^- content using a chloride analyzer (Chloride Analyzer 926, Cole-Parmer Canada Inc., Montreal, Quebec). Remaining plasma samples (10µL per sample) were diluted 1000 times with deionized water acidified to 1.0 % with 16 N HNO_3. Diluted plasma samples were analyzed for Na^+ content using flame AAS (PinAAcle 900, Perkin Elmer, Woodbridge, Ontario).

3.3.5 Protein and Citrate Synthase Analysis

Protein concentration the mitochondria and cytosol fraction of gill and liver homogenate were analyzed by the Bradford (1976) method, using a bovine serum albumin standard (23200, Thermoscientific, Rockford, Illinois). Citrate synthase activity was analyzed in the mitochondrial fraction along side the cytosol to check for fraction cross-contamination of ruptured organelles during the centrifugation process using a commercial kit (701040, Cayman Chemical, Ann Arbor, Michigan). Measurements for both protein and citrate synthase assays were done using a microplate spectrophotometer (Spectramax, Molecular Devices, Sunnyvale, California).

3.3.6 Calculations and Statistical Analyses

Cu and Ag concentrations in whole tissues and subcellular fractions were adjusted for tissue weight in addition to dilution factor and expressed as nmol Cu or Ag per g^{-1} of tissue wet weight. Plasma Na^+ and Cl^- concentrations are presented as mmol L^{-1}. Citrate synthase activities of measured fractions were adjusted for protein content and represented as nmol/min/mg protein. Sigma Plot (version 11.0- Systat Software, San Jose) was used to conduct statistical analysis. All data was subjected to one-way analysis of variance (ANOVA), followed by Tukey HSD post hoc test when possible. A significant difference of $P < 0.05$ was used as the limit of significance during analysis. All results are presented as the mean with ± 1 standard error of the mean (SEM).

Additive effects on plasma ion disruption were predicted and tested by using the following equation based on the approach outlined by Norwood et al., 2003 and adapted from Niyogi et al., 2015:

$$\text{Simple Additivity (\% effect)} = [(a + b) - f] \times 100$$

where *a* and *b* represent measured % difference of plasma ion content by metal 1 and 2 relative to the controls, and *f* is the interaction factor conceived by multiplying *a* and *b*. Additive effects on % mortality was predicted in a similar manner as above, where *a* and *b* represented the measured % mortality by metal 1 and 2 and *f* is the interaction factor calculated by multiplying *a* and *b.* Interactive effects on tissue bioaccumulation were determined by comparing % differences between treatment groups and the controls. All measured interactions were validated by statistical significance.

3.4 Results

3.4.1 Survival

There were no significant differences among paired total and dissolved metal concentrations in all treatment groups. Therefore, all analysis will be reported as total concentration (Table 3.1 and Table 3.2). Fish exposed to 0.04 μM Ag^{+}-only experience 16 % mortality while those in the 0.35 μM Cu^{2+} + 0.04 μM Ag^{+} mixture exposure had a 31 % mortality (Figure 3.2). No mortalities occurred in the 0.35 μM Cu^{2+}-only exposure or in the unexposed controls (Figure 3.2). Based on simple additivity model, the predicted mortality rate in mixture-expose fish was 16 % over 14 days.

3.4.2 Bioaccumulation: Whole tissues

Over the span of 14 days, Cu accumulations in the gills were 56 % greater in Cu^{2+}-only exposed fish and 98 % greater in mixture-exposed fish relative to the controls (Table 3.4). On all sampling days (1, 3, 7 and 14), Cu accumulation in the gills was significantly higher in mixture-exposed fish than in Cu^{2+}-only exposed fish (P= <0.001-0.004), roughly 1.9 times more by day 14 (Figure 3.3). Ag accumulation in the gills of mixture-exposed fish increased but more gradually for the rest of the exposure. Over 14 days, Ag^{+}-only exposed fish had a 175 % increase in accumulation while mixture-exposed had a 194 % increase of Ag in the gill compared to controls. On days 3, 7 and 14, Ag in the gills were significantly higher in mixture-exposed fish than in Ag-only exposed fish (P= <0.001-0.048), where tissue burden ranged from 3.9 to 5.7 times higher (Figure 3.3). In the liver, mixture-exposed fish accumulated 29 % more Cu than the controls while Cu concentrations were similar between Cu-only exposed fish and the controls with a 4 % difference over 14 days (Table 3.4). Fish exposed to the Cu^{2+} + Ag^{+} mixture accumulated 1.9 - 2.8 times more Cu in the liver compared to Cu^{2+}-only exposed fish on days 7 and 14 (Figure 3.4). Ag accumulation in the liver of mixture-exposed fish was significantly lower than in Ag^{+}-only exposed fish by day 14 (P= 0.007), about 2.1 times less but remained significantly higher relative to the controls (Figure 3.4). Compared to the controls, fish exposed to the mixture resulted in 175 % greater Ag accumulation in the liver while fish in the Ag^{+}-only exposure resulted 184 %. Similar to the gills, Cu and Ag also accumulated in the kidney of mixture-exposed fish with significant increases compared to the controls, 30 % and 184 % more respectively (Figure 3.5). In the case of fish exposed to Cu^{2+}-only, increases in renal Cu burden were delayed compared to the other tissues, occurring only at day 7 (Figure 3.5).

3.4.3 Bioaccumulation: Subcellular distribution

Citrate synthase activity was measured to determine whether cross contamination had occurred during the centrifugation process (Figure 3.6). Citrate synthase activity was significantly (P= <0.001) greater in the mitochondrial fraction than in the cytosol of the gill homogenate, approximately 17 times greater. In the liver homogenate, citrate synthase activity in the mitochondrial fraction was 49 times higher than in the cytosol, indicating that fractions were intact during subcellular fractionation.

The subcellular distribution of Cu in the gills resided mainly in the MSF of both Cu^{2+}-only and mixture exposed fish (Figure 3.7). In Cu^{2+}-only exposed fish, the mitochondria fraction accounted for 36-58 % of the total Cu in all (five) fractions; mitochondria fraction in mixture-exposed fish accounted for 50-52 % of total Cu in all fractions (Figure 3.7). Compared to Cu^{2+}-only exposed fish, significant accumulation occurred on day 14 in the MRG (P= <0.001), HSP (P= <0.001) and HDP (P=0.006) fractions from fish exposed to the mixture. Subcellular distribution of Ag in the gills occurred in all fractions with no significant differences between Ag^{+}-only and mixture-exposed fish. The majority of Ag accumulated in the BDF where MRG accounted for 63-87% of total Ag in all fractions while the HSP accounted for 2-3% in both Ag^{+}-only and mixture-exposed fish (Figure 3.8). In unexposed controls, Ag was present in all fractions of the gill with the majority residing in the MRG (Figure 3.8). For both Ag^{+}-only and mixture-exposed fish, Ag accumulation in the MSF at day 14 was the greatest in the mitochondria fraction, followed by microsomes and lysosomes, and then HDP (Figure 3.8).

In unexposed controls, Cu was distributed in all fractions of the liver and concentrations were considerably high compared to gills. Similar to distribution patterns in the gills, Cu accumulated mainly in the MSF and the mitochondria fraction (27-52 %; 30-55 %) made up the majority of all the fractions, followed by microsomes and lysosomes (14-60 %; 9-24 %) and then

HDP (1-14 %; 2-7%) for fish in both Cu^{2+}-only and the mixture exposures, respectively (Figure 3.9). Cu in the MRG was lower in both Cu^{2+}-only and mixture exposed fish compared to the controls and with no changes in accumulated between day 3, 7 and 14. In mixture exposed fish, Cu concentrations in HDP decreased over time while HSP increased (Figure 3.9). Ag accumulation in the liver of controls was found in all fractions. The distribution of Ag mainly occurred in BDF where MRG accounted for 43-79 % and HSP accounted for 3-8 % of total Ag in all fractions of Ag^{+}-only exposed fish. In mixture-exposed fish, MRG accounted for 62-83% and HSP accounted for 1-4% of total Ag in all fractions (Figure 3.10).

3.4.4 Ion regulation

Fish exposed to Cu^{2+}-only did not experience any significant ions disturbance where plasma Na^{+} concentrations was similar to those of the controls while plasma Cl^{-} was 3 % less than the controls over 14 days (Table 3.4). By day 3 both plasma Na^{+} and Cl^{-} were significantly reduced in Ag-only exposed fish and concentrations continued to fall with a 12 % decrease in plasma Na^{+} and a 14 % decrease in plasma Cl^{-} relative to controls over 14 days (Figure 3.11). Plasma ions in fish exposed to the mixture was significantly less than in the controls by day 1 and continued to decreased. It was predicted using the simple additivity model that mixture-exposed fish would experience a 12 % decrease in plasma Na^{+} and a 16 % decrease in plasma Cl^{-}. From a physiological perspective, by day 14 mixture exposed fish had a reduction of 36 % in plasma Na^{+} and 40 % in plasma Cl^{-} compared to the controls (Figure 3.11). Plasma Na^{+} was significantly less in mixture-exposed fish when compared to both Cu^{2+} ($P = <0.001$) and Ag^{+}-only ($P = 0.004$) exposure fish on day 3. Plasma Cl^{-} was significantly reduced in mixture-exposure fish on days 1, 3 and 7 compared to both Cu^{2+} and Ag^{+}-only exposed fish. On day 14, both plasma ions were

significantly less in mixture-exposed fish compared to fish from the Cu^{2+}-only (< 0.001) exposure but not the Ag^{+}-only (P =0.296) exposure (Figure 3.11).

Refer to Appendix B for *Chapter 3* data.

3.5 Discussion

The purpose of this study was to assess metal interactions by linking tissue bioaccumulation to physiological effects in rainbow trout (*Oncorhymchus mykiss*) during a 14-day exposure to a Cu^{2+} + Ag^{+} mixture. To develop an understanding of these effects, single-metal exposures (Cu^{2+}-only and Ag^{+}-only) were included along with the mixture exposure. Cu^{2+} concentrations in this experiment were reduced from 1.0 μM to 0.35 μM in this experiment to minimize mortalities. Fish exposed to either Ag^{+}-only or the mixture experienced a continuous decrease in both plasma Na^{+} and Cl^{-}. No significant effect was observed in fish exposed to Cu^{2+}-only, as levels were similar to those in the controls. Over time, the decrease in plasma ions also reflected the onset of mortalities since fish exposed to the Cu^{2+} + Ag^{+} mixture had a 31 % mortality rate whereas those exposed to the Ag^{+}-only had a 16 % mortality rate (Figure 3.2). Most of the mortalities occurred by day 3 in the mixture exposure while mortalities in the Ag^{+}-only exposure occurred during the last three days of the test. This early event of mortalities for mixture-exposed fish was associated with greater accumulation of both Cu^{2+} and Ag^{+} in the gills (Figure 3.3) but not in the liver (Figure 3.4) or kidney (Figure 3.5) when compared to the Cu^{2+}-only or Ag^{+}-only exposed fish. This elevated accumulation in mixture-exposed fish was also associated with greater plasma ion loss on days 1 and 3 (Figure 3.11). Interestingly there was no increase in the MSF on day 3 in the gills of Cu^{2+}-only (Figure 3.7) or Ag^{+}-only (Figure 3.8) exposed fish, but there was in the liver for fish from the Cu^{2+}-only exposure. The HDP fraction in the liver of mixture-exposed fish showed an increase of Cu compared to Cu^{2+}-only exposed fish

on day 3 and perhaps related interactions with Ag (Figure 3.9). Over the 14 days of exposure, gills and kidney of the fish exposed to the mixture accumulated more Cu and Ag compared to those from the Cu^{2+}-only or Ag^{+}-only exposure. In the liver, Cu accumulation was the greatest in mixture-exposed fish while Ag was the greatest in those exposed to Ag^{+}-only. In the subcellular fractions of the gills and liver, the majority of Ag accumulation occurred in the BDF in both Ag^{+}-only and mixture exposed fish while most of the Cu accumulated in the MSF in Cu^{2+}-only and mixture exposed fish. All tissue burdens in this study (whole tissue and subcellular samples) were measured on a wet weight basis rather than dry weight. This allowed for direct comparisons with other studies/published values that have also measured on a wet weight basis involving Cu^{2+} and Ag^{+}.

In this study, mixture effects on bioaccumulation were determined by comparing the % increase in tissue concentration between exposed fish and the controls over 14 days. Compared to the controls, the highest % accumulation of Cu and Ag was observed in mixture-exposed fish over 14 days, which suggests that accumulation was more than additive effect in the gills. This was supported statistically as Cu and Ag accumulation were significantly greater in mixture-exposed fish compared to Cu^{2+} or Ag^{+}-only exposed fish by day 14 (Figure 3.3). In the kidneys, the % differences in Cu^{2+} and Ag^{+} concentrations were also greater in mixture-exposed fish and suggested that a more than additive effect in accumulation occurred. Cu and Ag accumulation in the kidneys of mixture-exposed were also significantly greater than fish exposed to either single-metal, which supports that mixture effects were more than additive (Figure 3.5). In the liver, mixture-exposed fish accumulated significantly more Cu than Cu^{2+}-only exposed fish, indicating a more than additive but this was not observed in hepatic Ag accumulation (Figure 3.4). Ag accumulation in the liver was generally similar between Ag^{+}-only and mixture-exposed fish (Figure 3.4) although there were some significant differences, particularly on day 14. This would

suggest that the presence of background Cu and Cu^{2+} from the mixture exposure had either no interactive effect or an antagonistic effect on hepatic Ag accumulation. In this experiment, it was observed that as Cu and Ag accumulation in mixture-exposed fish increased, plasma ions decreased and mortalities occurred. A possible explanation for this linkage may include that the more than additive accumulation of Cu and Ag in the gills of mixture-exposed fish was perhaps related to Cu^{2+} enhancing Ag accumulation and vice versa. Greater accumulation in the gills would then lead to an increased number of Cu^{2-} and Ag^{+} binding to NKA and CA, resulting in a greater rate of plasma ion loss due to increased inhibitation of NKA and CA and accelerating the rate of the sequence of events involved in mortality by circulatory collapse (Hogstrand and Wood, 1998). While it was clear toxicity occurred in these exposed fish, it is unknown what portions of accumulated Cu and Ag influenced ion disruption since metals can are both sequestered and inactive (detoxified) or, are biologically active to bind to sensitive sites to cause toxicity. Although whole tissue concentrations can provide general but limited knowledge regarding metal-metal interactions and can lead to possible misconceptions on their toxicological effects. Since the gills and liver showed the greatest differences in Cu and Ag accumulation between mixture-exposed fish and the single-metal exposed fish in this study, we chose to examine the subcellular distribution in these tissues.

Interactive effects such as additivity, more than or less than additivity between metals in a mixture have been shown to influence accumulation and toxicity in aquatic organisms although little is known concerning the mechanisms associated with metal-metal interactions at the cellular level (Wallace and Luoma, 2003; Li et al., 2015). Examining the subcellular distribution of Cu and Ag in the gills and liver of rainbow trout over 14 days of exposure could provide a better link between metal accumulation and ion disturbance. Several studies identify microsomes and lysosomes as part of the MSF (Wallace et al., 2003; Eyckmans et al., 2012; Leonard et al. 2014;

Li et al., 2015), which can be appropriate for microsomes since they are involved in the synthesis and transport of proteins, making it a potential site of toxicity when metals are bound (Eyckmans et al., 2012). On the other hand, lysosomes are involved in metal storage for future elimination (Leonard et al., 2014, Li et al., 2015) and could therefore be considered as in the BDF. However, lysosomes have been shown to become "leaky" after metal exposure releasing hydrolytic enzymes into the cell (Viarengo et al., 1987), and for the purposes of this study, microsomes and lysosomes were categorized as part of MSF.

In the gills of Cu^{2+}-only exposed fish, Cu accumulation occurred mainly in MSF (Figure 3.7) without lethality. Cu^{2+}-only exposed fish tolerated 0.35 μM of Cu^{2+}, meaning that within the organelles, Cu perhaps accumulated mainly in the lysosomes. Cu accumulation the microsomes and lysosomes fraction of mixture-exposed fish was less than in Cu^{2+}-only exposed fish on day 3,7 and 14. Since Ag accumulation in the microsomes and lysosomes in the gills of fish exposed to Ag^{+}-only and the mixture were similar on day 3, 7 and 14 (Figure 3.8), it is possible that higher binding affinity Ag replaced Cu resulting in less Cu accumulation in the organelles of mixture exposed fish relative to those exposed to Cu^{2+}-only. Mixture-exposed fish also experienced significant Cu accumulation in the fractions MRG, HSP and HDP on day 14 when compared to Cu^{2+}-only exposed fish. It is difficult to assume that this increase of Cu in HDP was spilt over from MRG and HSP given that Cu^{2+} is an essential metal for biological functions and unselectively accumulates in all fractions (Kamunde and MacPhail, 2008), making it difficult to address the threshold capacity in these fractions. Additionally, since most of the mortalities occurred by day 3 in the mixture exposure, it is likely that Cu did not spill over as concentrations in BDF and MSF were similar between Cu^{2+}-only and mixture exposed fish during the early days of experiment (Figures 3.7). Ag accumulation in the gills occurred mainly in the BDF where MRG accounted for 63-87 % and 81-85% of total Ag in all the fractions within Ag^{+}-only and

mixture-exposed fish, respectively (Figure 3.8). Although a majority of Ag in both Ag^{+}-only and mixture-exposed fish accumulated predominately in BDF of the gills, mortalities were still occurring during the test. Like with Cu, it is difficult to presume that Ag accumulation into MSF is a result of spill over from BDF since background levels in this study showed Ag partitioning in all fractions and it is unclear whether a threshold exists in these fractions.

In correlation to whole tissue measurements, high background Cu content was observed in all fractions of the liver and again, further suggests a role in normal Cu-metabolism (Kamunde and MacPhail, 2008). The majority of Cu accumulation in the liver in both Cu^{2+}-only and mixture-exposed fish resided within the MSF between days 3, 7 and 14 (Figure 3.9). In comparison to high background concentrations in the liver and Cu accumulation in the fractions of exposed fish, two observations were made. First, Cu accumulation in the MRG fraction was nearly unchanged between days 3, 7 and 14 in fish from the Cu^{2+}-only and mixture exposures and was less than that of unexposed controls. It was observed that MRG Cu content was less in mixture-exposed fish than in Cu^{2+}-only exposed fish on days 3,7 and 14 as well. In a study conducted by Kamunde and MacPhail (2008), less hepatic Cu was found in the MRG of trout exposed to Cu alone (0.63 μM) than in the controls on days 7, 14 and 21 of their test. Kamunde and MacPhail (2008) suggested that less Cu in the MRG was likely a result of detoxification to limit further uptake and sequester Cu into an inactive form (Kamunde and MacPhail, 2008). Detoxification in fish from the mixture exposure might have been more directed towards sequestering Ag than Cu, which would explain less Cu accumulation in MRG than in fish exposed to Cu-only (Figure 3.9). In the HSP fraction, there was more Cu accumulation (even at background) than in the HDP fraction, which could further indicate a regulatory role for sequestering and removing excess Cu by MT and MTLP. Second, the absence of mortalities and disturbances on ion balances observed in the Cu^{2+}-only exposure suggests that fish were able to

tolerate above-background Cu concentrations in the MSF, but mixture exposed fish were not as tolerant. Therefore it seems possible that effects in the mixture exposure were related to Ag^{+}. It is difficult to determine the possibility of metal-metal interactions occurring within fractions of the liver due to high background concentrations but a few interpretations can be made. In a study conducted by Kamunde and MacPhail (2011) it was found that fish exposed to a Cu^{2+} + Cd^{2+} mixture accumulated lower Cu in the HDP fraction of the liver than those exposed to Cu^{2+}-only, while Cd^{2+} accumulation was enhanced. Kamunde and MacPhail (2011) suggested that Cd replaced Cu for binding sites in HDP, which lead to increased Cu accumulation in the HSP fraction of mixture-exposed fish. The suggestion of a non-essential metal with higher binding affinity to replace an essential metal with lower binding affinity also seemed possible to occur in this study. Since Ag accumulation in the HSP fraction was similar between Ag^{+}-only and mixture-exposed fish on days 3, 7 and 14 (Figure 3.10), it is likely that Ag bound onto MT and MTLP and displaced pre-bound (background) Cu, thus allowing freed Cu to accumulate into the MSF and increase toxicity. Additional displacement of Cu for Ag could occur in the HDP in which might explain why Cu in HDP faction decreased over time while increasing in HSP of mixture-exposed fish to be relocated into other fractions of the liver. Predicting metal-metal interactions in the cellular fractions of the liver can be complex due to undefined mechanisms of detoxification in addition to high background Cu^{2+} concentrations. The cellular debris contains tissue fragments, cell membranes and other cellular components of unknown functions (Wallace et al., 2003). Additionally, the cellular debris fraction in both the gills and liver accounted for less than 1-2% of the total Cu or Ag in all fractions, and therefore was not considered for interpretation in this study. There is also the potential for breakage or leakage of soluble constituents between fractions during centrifugation (De Duve, 1975; Kamunde and MacPhail, 2008; Eyckmans et al., 2012; Leonard et al., 2014). However, measured citrate synthase activity

in the mitochondrial fraction and cytosol from gill and liver homogenate showed a low indication of possible cross contamination (Table 3.6 and Figure 3.4).

The mechanism of toxicity by either Cu^{2+} or Ag^{+} in fish is by inhibitation of gill NKA and possibly CA resulting in the disruption of internal Na^{+} and Cl^{-} balance (Galvez and Wood, 2002; Grosell et al., 2003; Morgan et al., 2005). In this study, it was apparent that fish tolerated exposure to 0.35 μM of Cu^{2+} with no indication of ion disruption (Figure 3.11). On the other hand, fish exposed to 0.04 μM of Ag^{+}-only experienced a reduction in both plasma Na^{+} and Cl^{-} (Figure 3.9). It was predicted by simple additivity that the combined effect of Cu^{2+} and Ag^{+} would result in an additive disruption on plasma ions disruption but this did not occur. The measured Cu^{2+} and Ag^{+} effects on plasma Na^{+} (35 %) and Cl^{-} (16%) were greater than the predicted values (20 % in Na^{+}, 24 % in Cl^{-}) by simple additivity (Table 3.4). This indicated that the Cu^{2+} + Ag^{+} effect produced a more than additive disruption on plasma ions over 14 days, and supports the hypothesis that the greatest ion disruption would occur in mixture-exposed fish. Cu^{2+} and Ag^{+} are Na^{+} antagonists and believe to compete for uptake through apical Na^{+}-channel (Niyogi et al., 2015) but no competitive effect was observed in this study where less than additive effects would have occurred during uptake; rather it was apparent that both metals interacted and produced a more than additive effect towards ion disturbance. A similar outcome was observed in a study conducted by Niyogi et al. (2015) where rainbow trout exposed to a mixture containing 1.30 μM of Cu^{2+} and 0.09 μM of Ag^{+} experienced an additive inhibition in Na^{+} uptake when compared to treatments containing Cu^{2+} or Ag^{+} alone. Niyogi et al. also found that exposure to a mixture containing Ca^{2+}-antagonists Zn^{2+} and Cd^{2+}, produced an additive inhibition on Ca^{2+} influx in trout while a mixture containing Cd^{2+} and Cu^{2+} did not have any interactive effects on either Ca^{2+} or Na^{+} uptake (2015). In past studies, plasma Na^{+} and Cl^{-} loss in rainbow trout occurs during both Ag^{+} (Morgan et al., 1997, 2004; Galvez and Wood, 2002) and Cu^{2+} exposure (Lauren

and McDonald, 1985ab; Wilson and Taylor, 1993). Inhibition of NKA has mainly been associated with plasma Na^+ loss while inhibition of CA has been linked to plasma Cl^- loss (Grosell, 2012). Currently, only one *in vivo* study has demonstrated that exposure to Cu^{2+} can inhibit CA activity in freshwater fish (guppy, *Poecilia vivipara*) (Zimmer et al., 2012). Other studies have shown exposure to Ag^+ to inhibit CA activity (Morgan et al., 2004; Goss et al., 2011) and decrease Cl^- influx in the gills of rainbow trout (Morgan et al., 2004). It is possible that the mechanisms associated with the metal-metal interactions in this (Cu^{2+} + Ag^+) and in Noyogi et al.'s (2015) (Cu^{2+} + Ag^+ and Zn^{2+} + Cd^{2+}) study may involve targeting specific sites within a cell, where a binary mixture of metals with same mode of toxic action were shown to have additive and more than additive effects on ion disruption. In comparison to data in the 10-day mixture exposure (*Chapter 2*), the Cu^{2+} concentration used in the mixture exposure was much higher (1.0 μM v.s 0.35 μM in the 14-day experiment) and produced a more than additive effect on plasma Na^+ loss but a less than additive effect on plasma Cl^- loss. It would then seem that in both experiments, ion disruption in mixture-exposed fish was dependent on exposure concentrations.

The objective of this study was to better understanding of the sublethal effects of a Cu^{2+} + Ag^+ mixture in rainbow trout (*Oncorhynchus mykiss*) by linking tissue bioaccumulation to observed effects during a 14-day waterborne exposure. Our results suggest that a metal-metal interaction occurred between Cu^{2+} and Ag^+, producing a more than additive effect of increased accumulation of both metals in the gills. This was contrary the hypothesis where Ag accumulation was predicted to occur similarly in both Ag^+-only and mixture-exposed fish. The increase in Cu and Ag gill accumulation were followed by further interactions leading to an additive effect on reducing plasma Na^+ and Cl^-. Subcellular distribution in the gills indicated that Cu^{2+} did not influence the binding of Ag^+ in mixture-exposed fish, as results were similar in fish

exposed to Ag^{+}-only, thus the initial predictions were supported. The significant increase of Cu within the BDF and HDP from the gills of mixture-exposed fish showed that an interaction did occur between Cu^{2+} and Ag^{+} to produce a more than additive effect. The subcellular distribution of Cu and Ag in the liver showcased signs of detoxification, however, since mortalities were occurring in mixture-exposed fish, it is unclear how efficient this process was. In the controls, Cu and Ag accumulation occurred in all fractions of gills and liver, which supports the hypothesis for the subcellular distribution of Cu. However, this did not support the prediction that Ag would accumulate in the BDF first then in the MSF. Subcellular distribution of Ag in the liver was also complicated by the high background Cu concentrations, making observations and predictions towards interactive effects between Cu^{2+} and Ag^{+} challenging. This study demonstrates Ag^{+} can bind more readily than Cu^{2+} in the gills, at a whole tissue and subcellular level. Also, this study shows that Cu^{2+} and Ag^{+} do interact with one another rather than compete and exposure to both metals as mixture can increase the level of toxicity in rainbow trout as indicated by plasma ion levels.

3.6 Figures

Table 3.1. Means (± SEM (n)) for measured dissolved Cu concentrations in water samples from control, Cu^{2+}-only and mixture exposures over 14 days. Values are represented in µmol/ L (µM). Samples were taken directly from the head tank and exposure (in replicate) tanks.

	Water in Head Tank	**Water Exposure Tank**
Exposure	**Dissolved (µM)**	**Dissolved (µM)**
Control (0 µM)	0.03 ± 0.01 (4)	0.03 ± 0.01 (8)
Cu^{2+}-only (0.35 µM)	0.45 ± 0.23 (4)	0.40 ± 0.14 (8)
Mixture (0.35 µM Cu^{2+} + 0.04 µM Ag^{+})	0.52 ± 0.26 (4)	0.43 ± 0.15 (8)

Table 3.2. Means (± SEM (n)) for measured dissolved Ag concentrations in water samples from control, Ag^{+}-only and mixture exposures over 14 days. Values are represented in nmol/L (nM). Samples were taken directly from the head tank and exposure (in replicate) tanks.

Exposure	**Water in Head Tank Dissolved (nM)**	**Water Exposure Tank Dissolved (nM)**
Control (0 µM)	0.00 ± 0.00 (4)	0.00 ± 0.00 (8)
Ag^{+}-only (0.04 µM)	40.15 ± 20.07 (4)	37.53 ± 13.27 (8)
Mixture (0.35 µM Cu^{2+} + 0.04 µM Ag^{+})	50.99 ± 05.50 (4)	43.89 ± 15.52 (8)

Table 3.3. Measured and predicted effects on plasma Na^+ and Cl^- balance in rainbow trout exposed to Cu^{2+}-only, Ag^+-only or mixture exposure over 14 days. Predicted effects were estimated based on principles of simple additivity (see Calculation, Methods section for details). Values are represented as % difference in plasma Na^+ or Cl^- content relative to the controls.

Exposure	**Measured % difference of plasma Na^+**	**Predicted % difference of plasma Na^+**	**Measured % difference of plasma Cl^-**	**Predicted % difference of plasma Cl^-**
Cu^{2+}-only (0.35 μM)	0	-	3	-
Ag^+-only (0.04 μM)	12	-	14	-
Mixture (1.0 μM Cu^{2+} + 0.04 μM Ag^+)	19	12	23	16

Table 3.4. Measured and predicted % mortality rate in rainbow trout exposed to Cu^{2+}-only, Ag^+-only or mixture over 14-days. Predicted effects were estimated based on principles of simple additivity (see Calculation, Methods section for details). Values are represented as % mortality.

Exposure	**Observed % mortality rate**	**Predicted % mortality rate**
Cu^{2+}-only (0.35 μM)	0	-
Ag^+-only (0.04 μM)	16	-
Mixture (1.0 μM Cu^{2+} + 0.04 μM Ag^+)	31	16

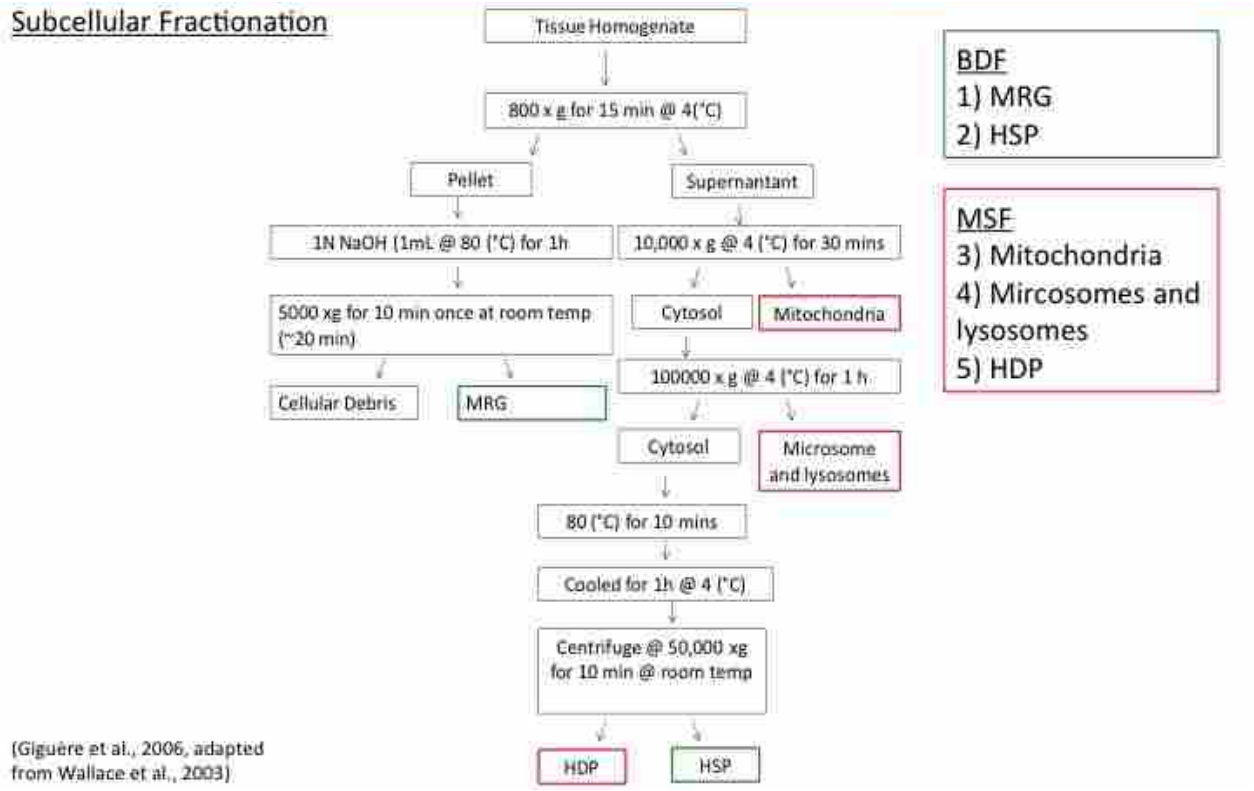

Figure 3.1. Subcellular fractionation protocol. Adapted from Giguere et al, 2006. Schematic for subcellular fractionation by differential centrifugation protocol including spin speed, temperature and duration for isolating various fractions. Biologically detoxified fractions (BDF: green box) comprise of metal rich granules (MRG) and heat stable proteins (HSP). Metal sensitive fractions (MSF: read boxes) include mitochondria, heat stable proteins (HSP), microsomes and lysosomes.

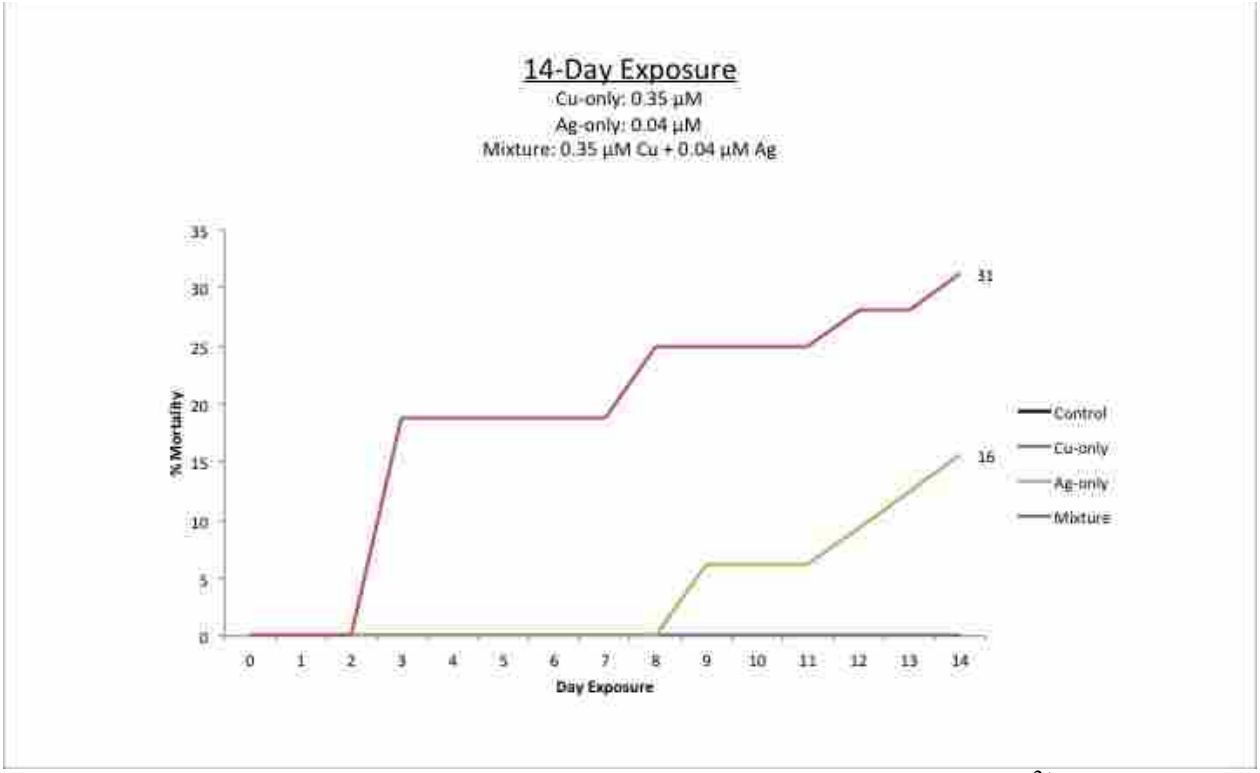

Figure 3.2. Percent (%) mortality of rainbow trout exposed to either 0.35 µM Cu^{2+}-only (purple), 0.04 µM Ag^{+}-only (green), or 1.0 µM Cu^{2+} + 0.04 µM Ag^{+} mixture (red) for 14 days. A control group was also included (black). Values are represented as % mortality.

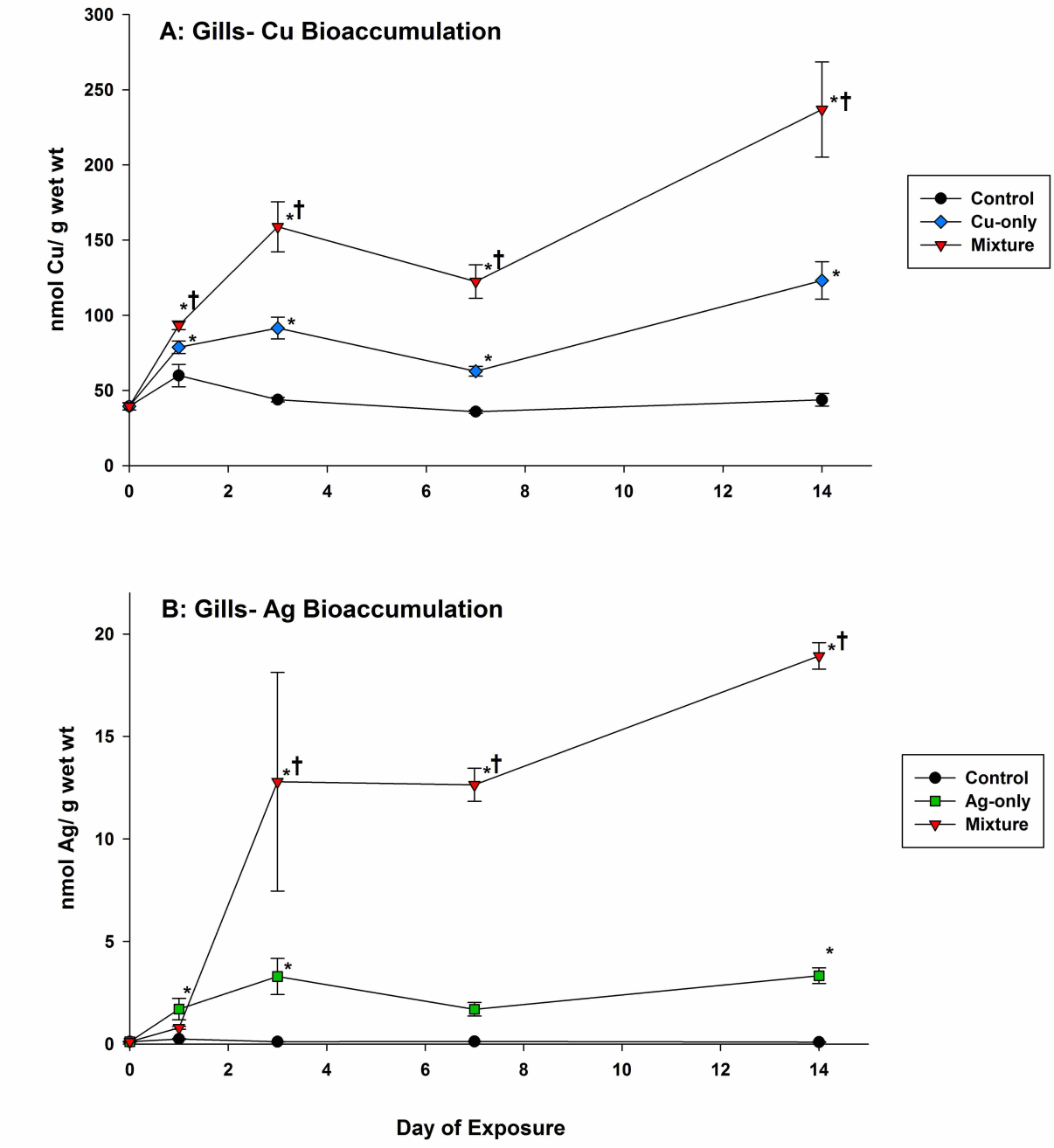
A: Gills- Cu Bioaccumulation
nmol Cu/ g wet wt
300
250
200
150
100
50
0
0
2
4
6
8
10
12
14
Control
Cu-only
Mixture
B: Gills- Ag Bioaccumulation
nmol Ag/ g wet wt
20
15
10
5
0
Control
Ag-only
Mixture
Day of Exposure

Figure 3.3. Gill accumulation of Cu (A) and Ag (B) in rainbow trout exposed to either control, 0.35 μM Cu^{2+}-only, 0.04 μM Ag^{+}-only or 0.35 μM Cu^{2+} + 0.04 μM Ag^{+} mixture for 14 days. Values are represented as means (nM /g tissue wet weight) ± 1 SEM). * represents a significant difference between exposed fish and controls on that sampling day while † represents a significant difference between mixture-exposed fish and Ag^{+}-only (A) and Cu^{2+}-only (B) exposed fish (ANOVA $P < 0.05$).

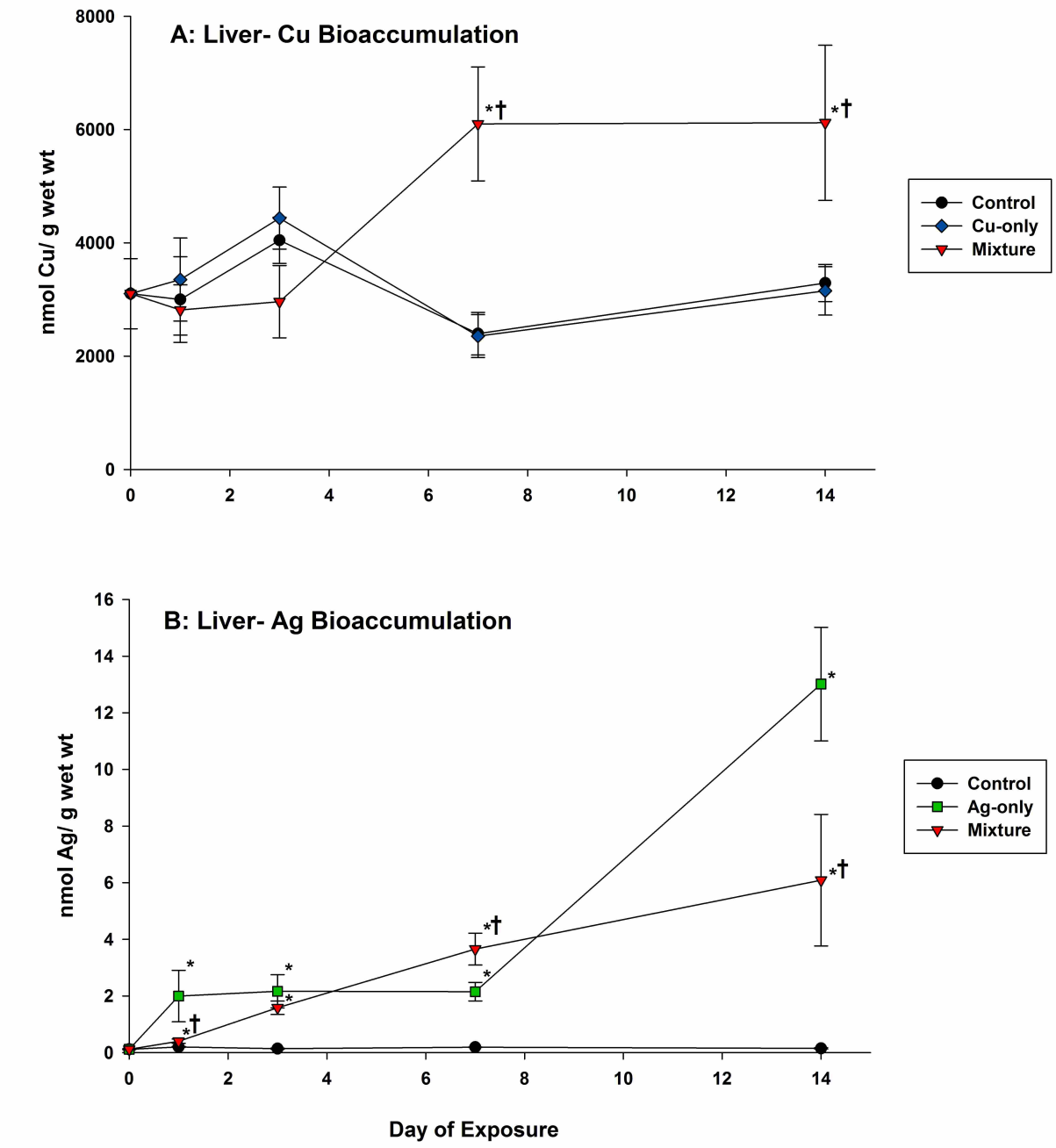
A: Liver- Cu Bioaccumulation
nmol Cu/ g wet wt
8000
6000
4000
2000
0
0
2
4
6
8
10
12
14
*†
*†
Control
Cu-only
Mixture
B: Liver- Ag Bioaccumulation
nmol Ag/ g wet wt
16
14
12
10
8
6
4
2
0
*
*
*
*
*†
*†
*
Control
Ag-only
Mixture
Day of Exposure

Figure 3.4. Liver Gill accumulation of Cu (A) and Ag (B) in rainbow trout exposed to either control, 0.35 μM Cu^{2+}-only, 0.04 μM Ag^{+}-only or 0.35 μM Cu^{2+} + 0.04 μM Ag^{+} mixture for 14 days. Values are represented as means (nM /g tissue wet weight) ± 1 SEM). * represents a significant difference between exposed fish and controls, relative to that sampling day while † represents a significant difference between mixture-exposed fish and Ag^{+}-only (A) and Cu^{2+}-only (B) exposed fish (ANOVA $P < 0.05$).

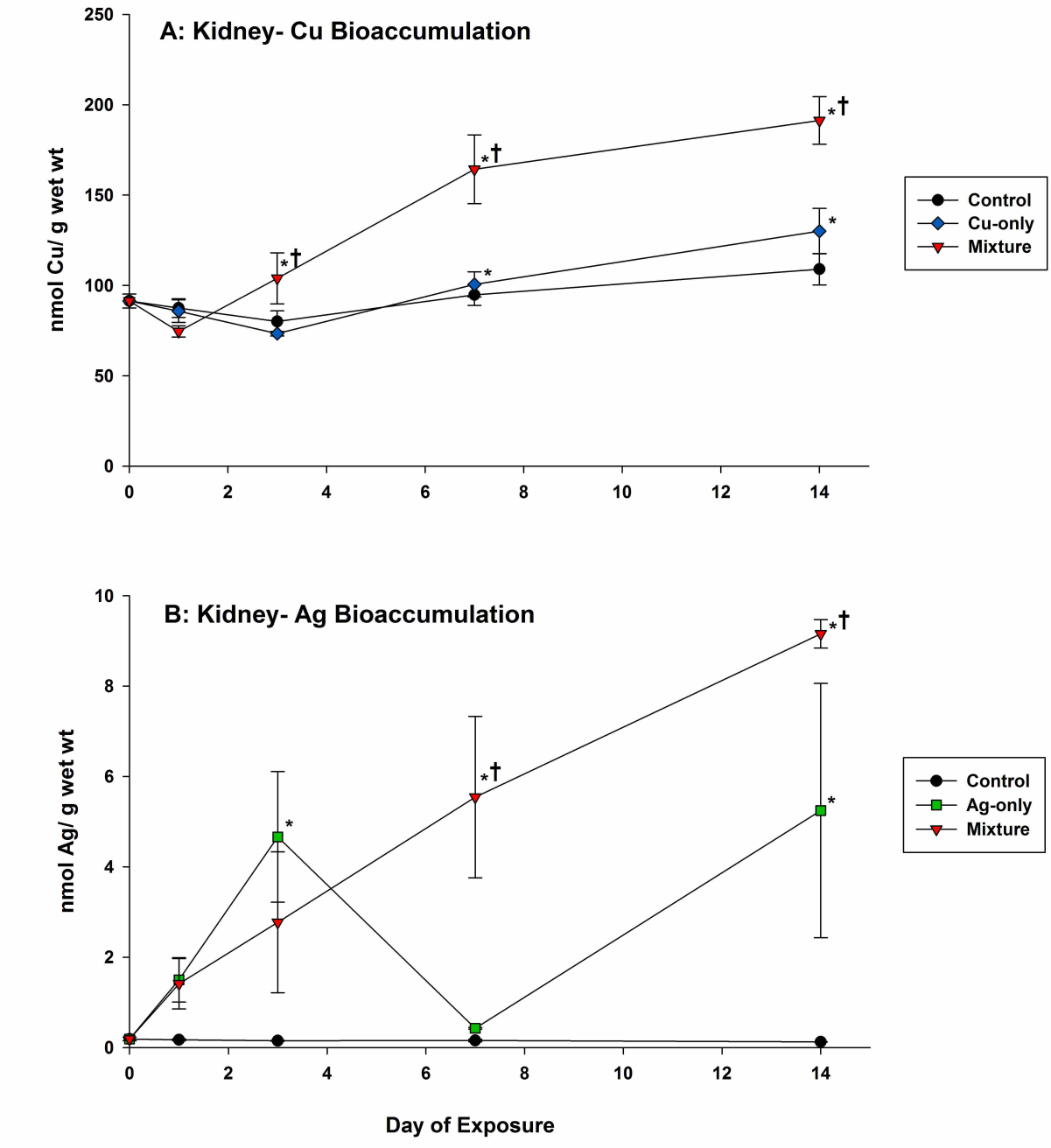
A: Kidney- Cu Bioaccumulation
nmol Cu/ g wet wt
250
200
150
100
50
0
0
2
4
6
8
10
12
14
Control
Cu-only
Mixture
B: Kidney- Ag Bioaccumulation
nmol Ag/ g wet wt
10
8
6
4
2
0
Control
Ag-only
Mixture
Day of Exposure

Figure 3.5. Kidney Gill accumulation of Cu (A) and Ag (B) in rainbow trout exposed to either control, 0.35 μM Cu^{2+}-only, 0.04 μM Ag^{+}-only or 0.35 μM Cu^{2+} + 0.04 μM Ag^{+} mixture for 14 days. Values are represented as means (nM /g tissue wet weight) ± 1 SEM. * represents a significant difference between exposed fish and controls on that sampling day while † represents a significant difference between mixture-exposed fish and Ag-only (A) and Cu-only (B) exposed fish (ANOVA $P < 0.05$).

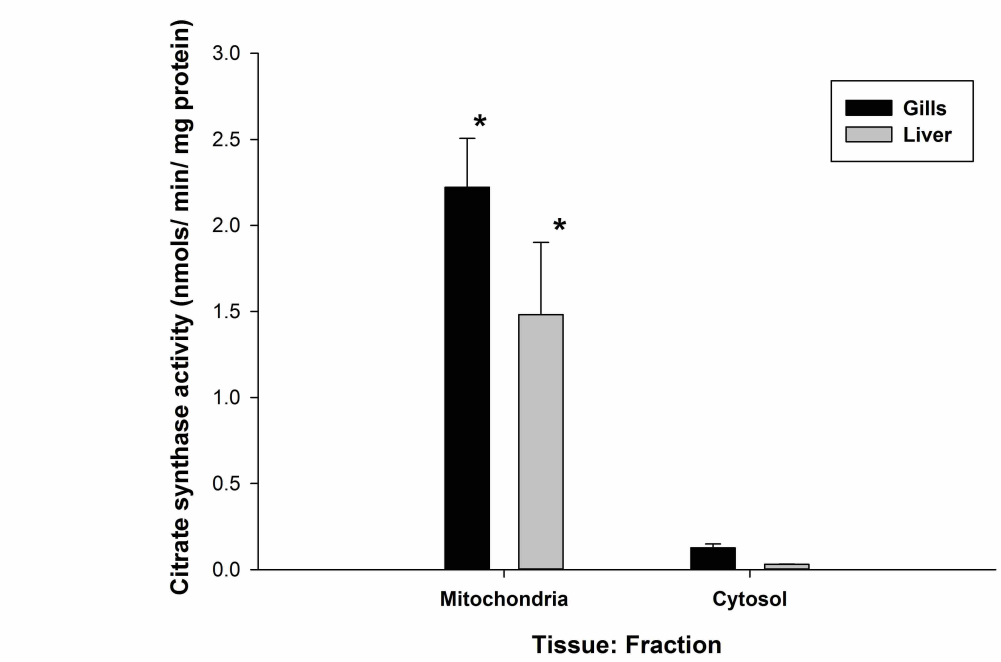

Figure 3.6. Citrate synthase activity measured in mitochondrial fraction and cytosol of rainbow trout gills and liver homogenate. Values are represented as mean (nM/ min/ mg protein) ± 1 SEM (6). * represents a significant difference between mitochondrial fraction and cytosol (ANOVA $P < 0.05$).

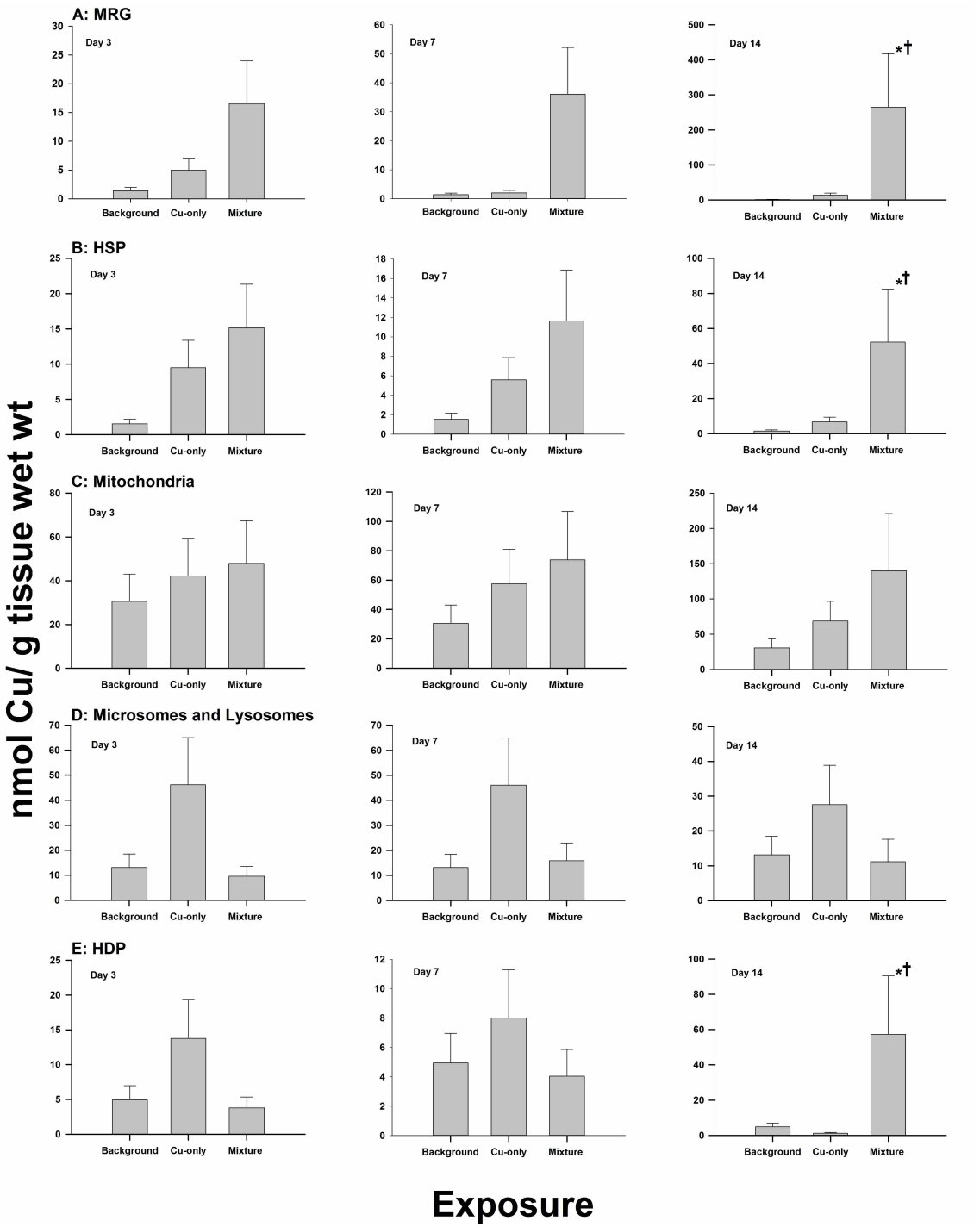
A: MRG
B: HSP
C: Mitochondria
D: Microsomes and Lysosomes
E: HDP
Day 3
Day 7
Day 14
*†
Background
Cu-only
Mixture
nmol Cu/ g tissue wet wt
Exposure

Figure 3.7. Gill subcellular distribution of Cu in MRG (A), HSP (B), mitochondria (C), microsomes and lysosomes (D) and HDP (E) fractions in controls (background) and rainbow trout exposed to either 0.35 μM Cu^{2+}-only or 0.35 μM Cu^{2+} + 0.04 μM Ag^{+} mixture for 14 days. Values are represented as means (nmol Cu/ g tissue wet weight) ± 1 SEM. * represents a significant difference between mixture-exposed fish and controls on that sampling day while † represents a significant difference between mixture-exposed fish and Cu-only exposed fish (ANOVA $P < 0.05$).

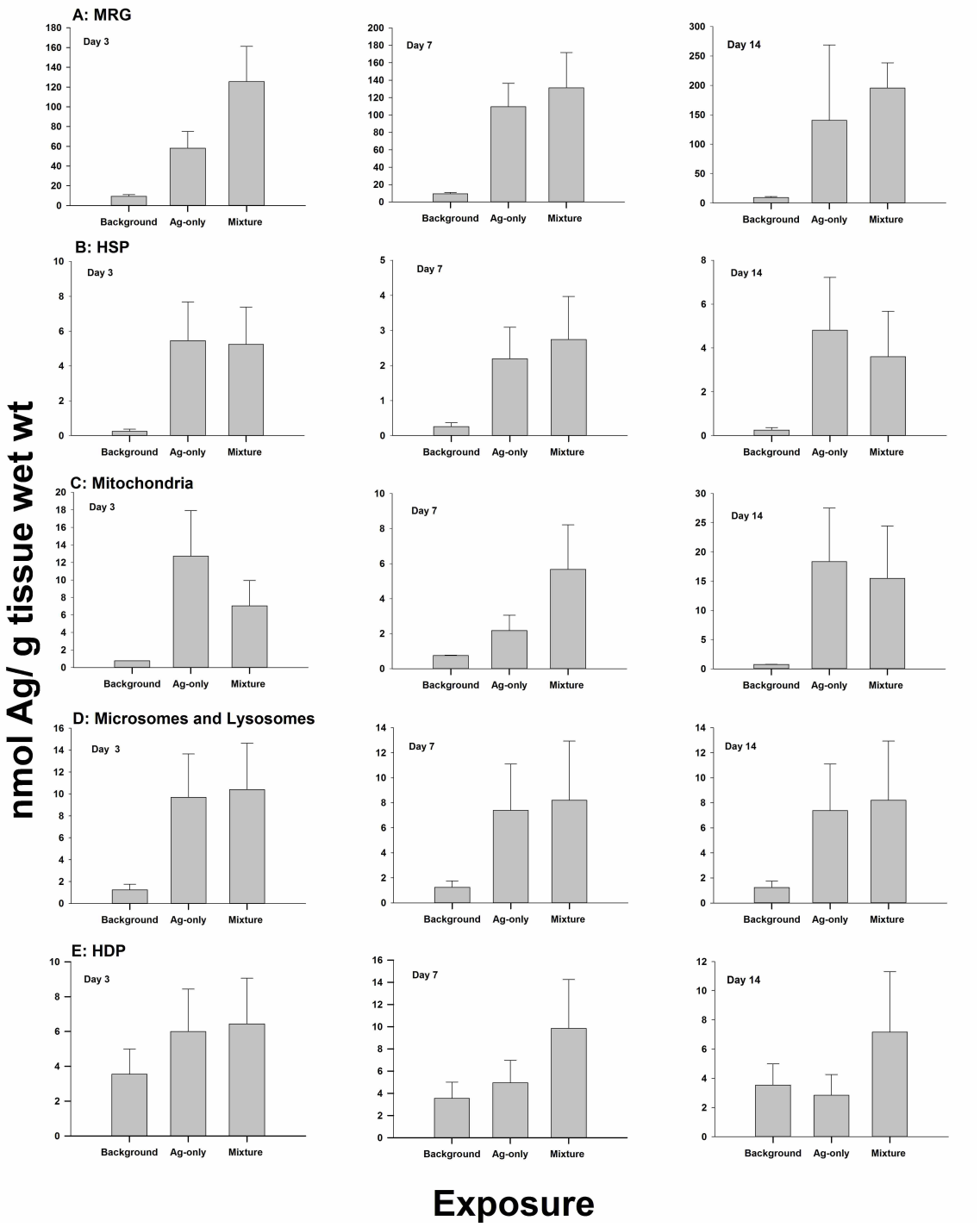
A: MRG
B: HSP
C: Mitochondria
D: Microsomes and Lysosomes
E: HDP
Day 3
Day 7
Day 14
Background
Ag-only
Mixture
nmol Ag/ g tissue wet wt
Exposure

Figure 3.8. Gill subcellular distribution of Ag in MRG (A), HSP (B), mitochondria (C), microsomes and lysosomes (D) and HDP (E) fractions in controls (background) and rainbow trout exposed either to 0.04 μM Ag^{+}-only or 0.35 μM Cu^{2+} + 0.04 μM Ag^{+} mixture for 14 days. Values are represented as means (nmol Ag/ g tissue wet weight) ± 1 SEM.

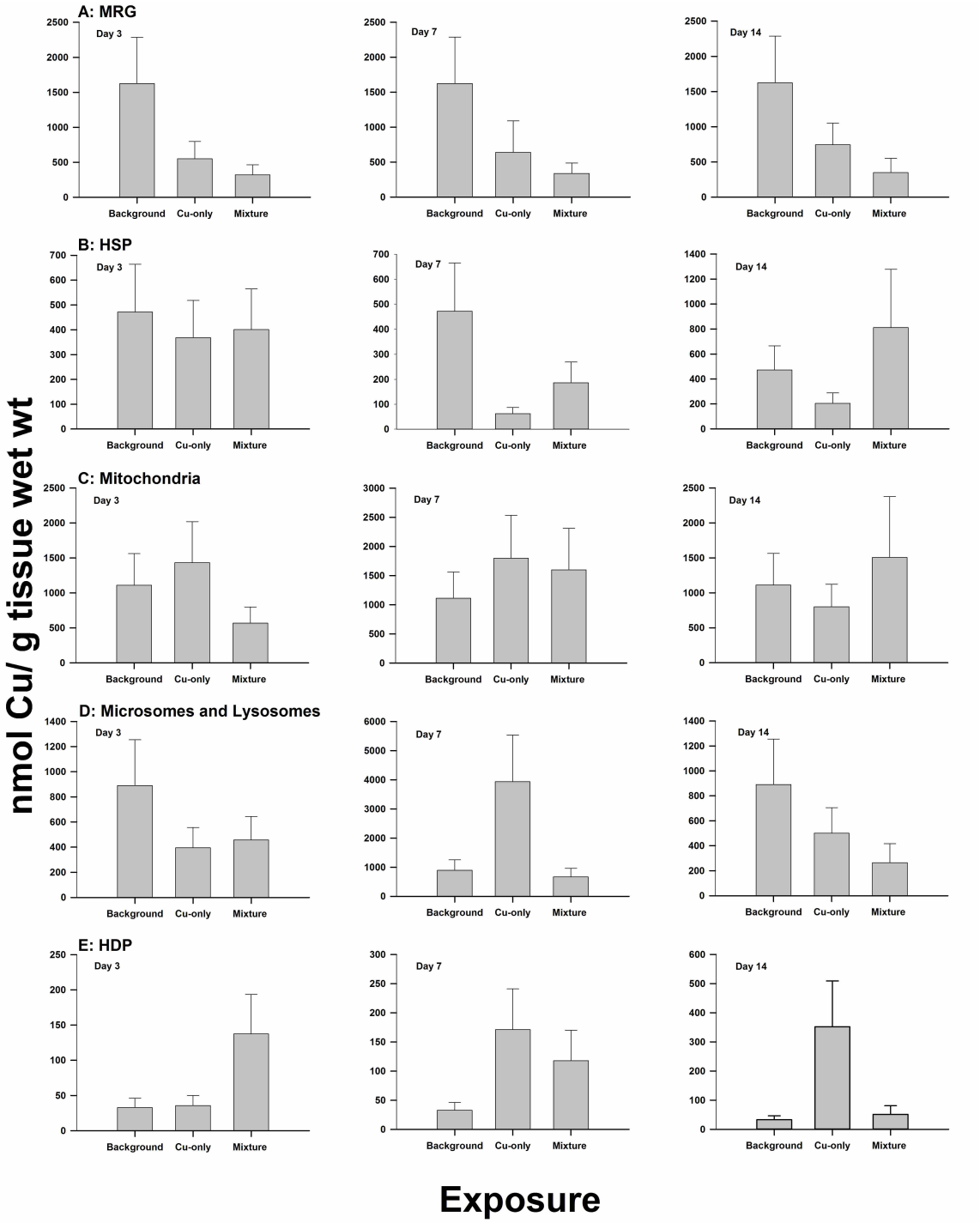

Figure 3.9. Liver subcellular distribution of Cu in MRG (A), HSP (B), mitochondria (C), microsomes and lysosomes (D) and HDP (E) fractions in controls (background) and rainbow trout exposed to either 0.35 μM Cu^{2+}-only or 0.35 μM Cu^{2+} + 0.04 μM Ag^{+} mixture for 14 days. Values are represented as means (nmol Cu/ g tissue wet weight) ± 1 SEM.

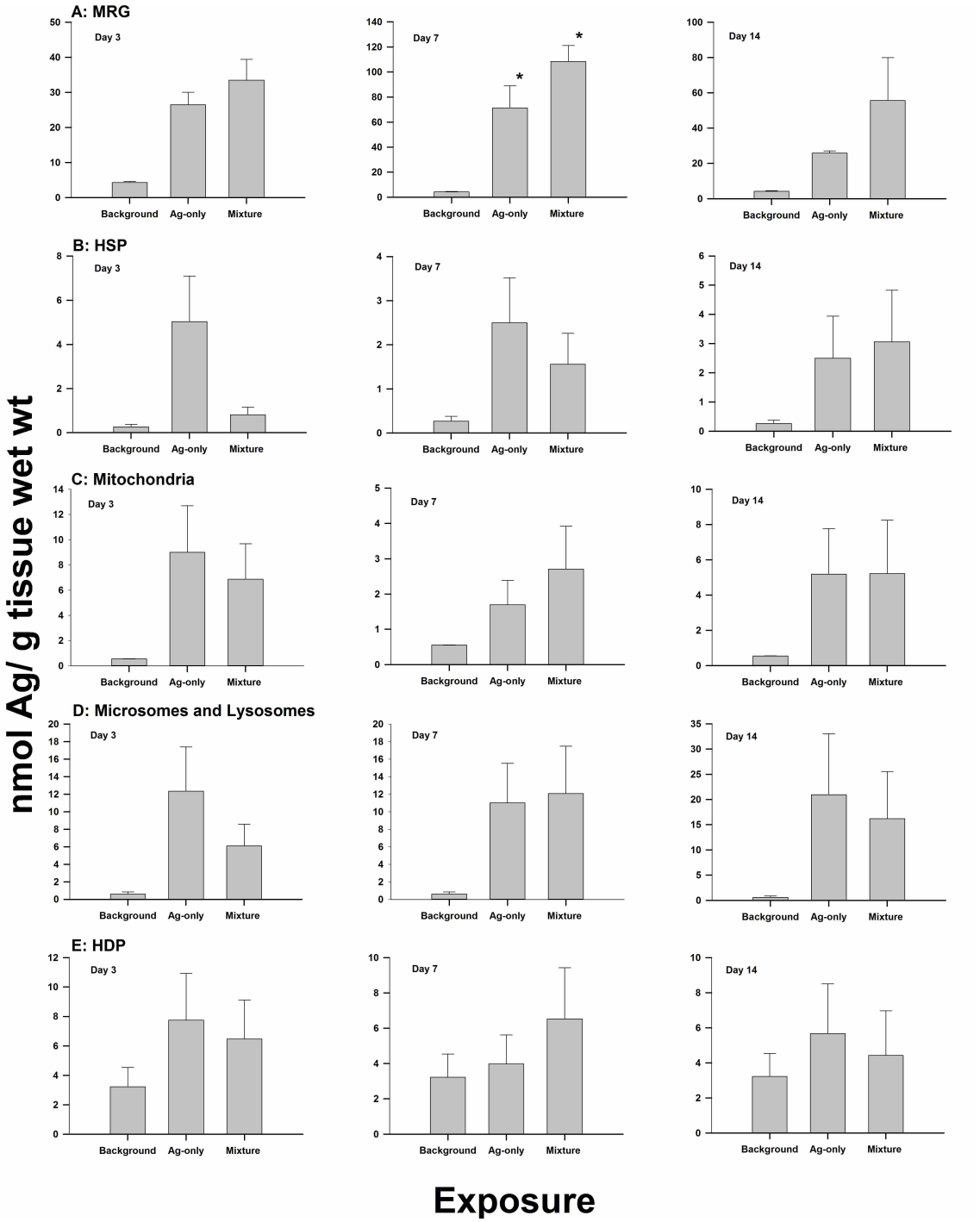
A: MRG
Day 3
Day 7
Day 14
B: HSP
Day 3
Day 7
Day 14
C: Mitochondria
Day 3
Day 7
Day 14
D: Microsomes and Lysosomes
Day 3
Day 7
Day 14
E: HDP
Day 3
Day 7
Day 14
Background
Ag-only
Mixture
nmol Ag/ g tissue wet wt
Exposure

Figure 3.10. Liver subcellular distribution of Ag in MRG (A), HSP (B), mitochondria (C), microsomes and lysosomes (D) and HDP (E) fractions in controls (background) and rainbow trout exposed to either 0.04 μM Ag^{+}-only or 0.35 μM Cu^{2+} + 0.04 μM Ag^{+} mixture for 14 days. Values are represented as means (nmol Ag/ g tissue wet weight) ± 1 SEM. * represents a significant difference between mixture-exposed fish and controls on that sampling day (ANOVA $P < 0.05$).

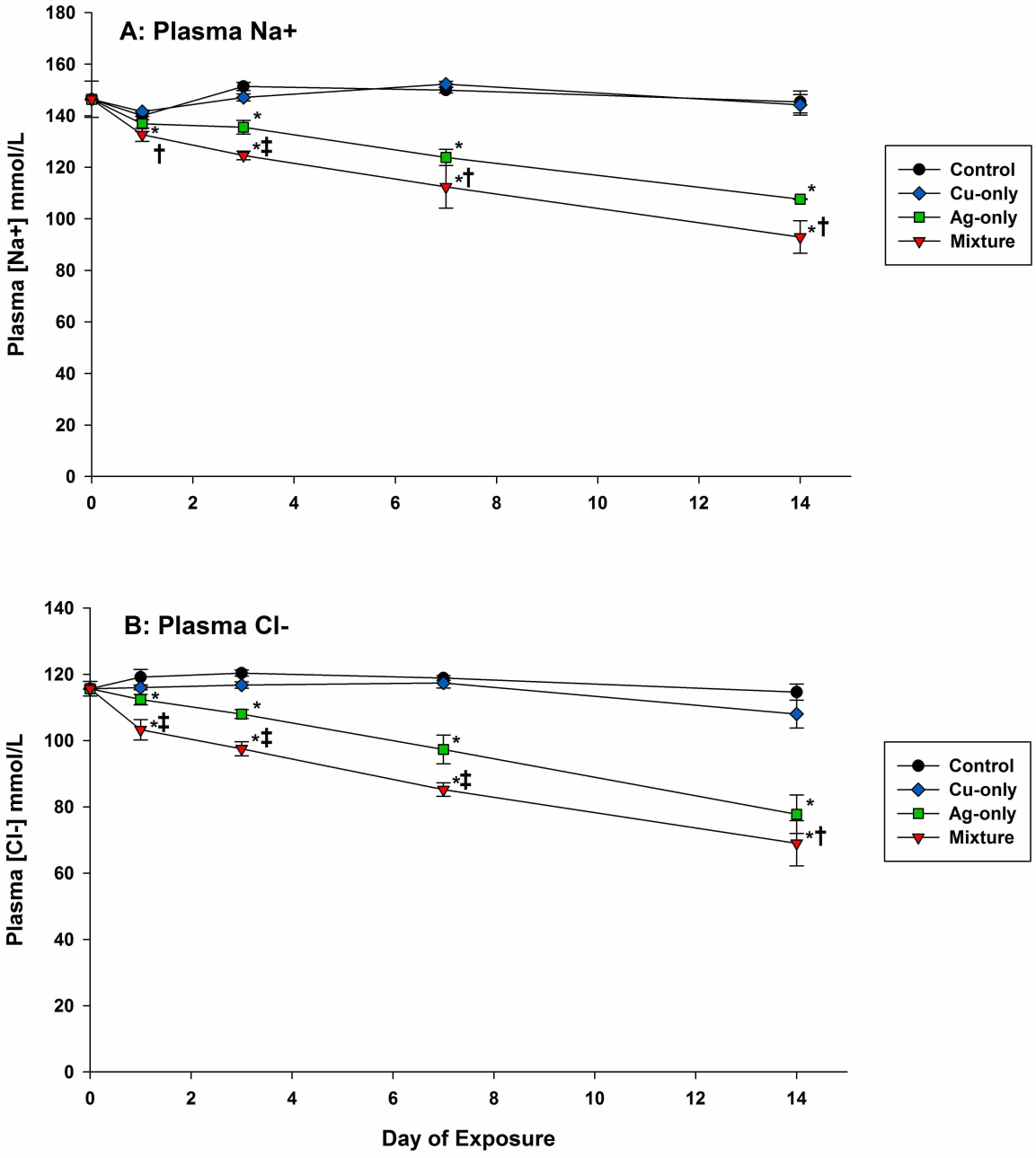
A: Plasma Na+
Plasma [Na+] mmol/L
180
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Control
Cu-only
Ag-only
Mixture
B: Plasma Cl-
Plasma [Cl-] mmol/L
140
120
100
80
60
40
20
0
Day of Exposure

Figure 3.11. Plasma Na^+ (A) and Cl^- (B) levels in rainbow trout exposed to either control, 1.0 μM Cu^{2+}-only, 0.04 μM Ag^+-only or 1.0 μM Cu^{2+} + 0.04 μM Ag^+ mixture for 14 days. Values are represented as means (Plasma [ion] mmol/L) ± 1 SEM. * represents a significant difference between exposed fish and controls on that sampling day while † represents a significant difference between mixture and Cu-only exposed fish. ‡ represents a significant difference between mixture-exposed fish and both single metal exposed fish (ANOVA $P < 0.05$).

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Chapter 4

An Integrative Interpreation of Cu and Ag Mixture Exposure on Rainbow Trout (Oncorhynchus mykiss).

4.1 Integration

It is well known that aquatic environments contain various mixtures of metals, from those that occur naturally due to the decay of vegetation, to those arising from anthropogenic activities. These mixtures may include metals such as Cu, which have essential biological roles in aquatic organisms (Grosell, 2012) and non-essential metals that have no dietary needs, such as Ag (Wood, 2012). Toxicity involving exposure to waterborne metals commonly involves the mimicry of essential ions including uptake by organisms through ion-specific channels. Studies involving Cu^{2+} or Ag^{+} exposure to rainbow trout have shown that both metals share a common means of uptake through apical Na^{+}- channels of the gills and both also share the same mode of toxic action by inhibiting NKA activity and possibly CA leading to ionoregulatory disturbances (Goss et al., 2011, Zimmer et al., 2012). Rainbow trout are generally used as model organisms because of their sensitivity to contaminate exposure (Environment Canada, 1990). For this reason, rainbow trout was used in this study to test the existence of Cu and Ag interactions and to link tissue accumulation at the whole and subcellular level with disturbances on ion regulation during exposure to a mixture.

In this study, it was hypothesized that Ag would accumulate at the same rate in the gills, liver and kidney of both Ag^{+}-only and mixture-exposed fish. It was also hypothesized that Ag would accumulate selectively in BDF due to detoxification first then accumulate in MSF and Cu would accumulate in all fractions of the cell because it is an essential nutrient. In the 10-day mixture exposure, similar Ag accumulation in the gills of Ag^{+}-only and mixture-exposed fish supported the initial thoughts on Ag binding. Subcellular distribution of Ag results from the 14-day mixture exposure also supported this prediction since accumulation was similar in all tissue (gills and liver) fractions between Ag^{+}-only and mixture-exposed fish. However, it was observed that Ag accumulated in all fractions of gills and liver of the controls and did not support the

prediction that Ag would be detoxified first before accumulating in the MSF. Gill and kidney accumulation of Ag was significantly greater in mixture-exposed fish compared to fish in the Ag^{+}-only exposure over 14 days, and did not support the hypothesis on Ag binding.

In both experiments, Ag accumulation in the liver was lower in mixture-exposed fish compared to those exposed to Ag^{+}-only and also did not support the hypothesis of Ag binding. Disruption of plasma ion balance was hypothesized to be greatest in fish exposed to a Cu^{2+} + Ag^{+} mixture while exposure to Cu^{2+} or Ag^{+} alone would result in less plasma ion disruption. This hypothesis was supported in in the 14-day mixture experiment where fish exposed to the mixture resulted in a more than additive disruption on both plasma Na^{+} and C^{l-} loss. In the 10-day mixture exposure, plasma Na^{+} loss was more than additive in mixture-exposed fish while plasma Cl^{-} loss was less then additive, which did not support the plasma ion disruption hypothesis.

In both the 10-day (*Chapter 2*) and 14-day experiment (*Chapter 3*), interactions between Cu^{2+} and Ag^{+} occurred and exacerbated metal bioaccumulation and disturbances to plasma ion balance, as well as leading to greater mortality. During the 10-day mixture exposure, bioaccumulation of Cu and Ag in the gills of mixture-exposed fish did not differ from those treated with Cu^{2+} or Ag^{+} alone (Figure 2.3). This indicated that Cu^{2+} did not influence Ag accumulation and vice versa on day 3. The mixture concentration of 1.0 μM Cu^{2+} + 0.04 μM Ag^{+} used in the 10-day experiment was more lethal than anticipated and resulted in a 64 % mortality rate where all mixture-exposed dying by day 3 (Table 2.2). High % mortality in the mixture-exposure ultimately impacted the findings in this experiment. The lack of significant differences in either Ag and Cu accumulation in the gills also weakened the connection between bioaccumulation and ion disruption. On the other hand, results from the 14-day mixture exposure strengthened the linkage between tissue bioaccumulation, ion disruption and mortalities. This was best seen in gill accumulation at a whole tissue level. In the 14-day mixture exposure, effects

on gill accumulation of Cu and Ag were more than additive (significantly greater) in mixture-exposed fish. Plasma Na^+ and Cl^- were significantly less in the mixture-exposed fish where a more than additive effect was observed ion disruption. A more than additive effect was also observed in the % mortality rates of mixture-exposed fish (31 %) compared to fish exposed to Cu^{2+} (0 %) or Ag^+ (15 %) alone (Table 3.4). In both experiments, interactions between Cu^{2+} and Ag^+ occurred, but not to the same degree and this were dependent on to exposure concentrations.

While it is obvious that a Cu and Ag interactions occur during simultaneous exposure of these metals, the mechanism(s) involved are still unclear. A proposed model involving Cu and Ag interactions in the gills of fish may support the findings in both the 10-day and 14-day mixture exposure experiments (Figure 4.1). It is proposed that Cu^{2+} and Ag^+ are Na^+- antagonists and compete with one another for uptake by apical Na^+-channels. It is believed that Cu^{2+} is reduced to Cu^+ by an apical metal reductase before it is taken up into the cell by the Na^+-channels (Grosell, 2012). Once in the gill cell, Cu^{2+} and Ag^+ will cause toxicity by inhibiting NKA, causing a decrease in Na^+ influx. Cu^{2+} and Ag^+ will also target CA by inhibiting the hydration of CO_2 to produce H^+ and HCO^-_3, which are needed for the apical H^+/Na^+-exchanger and Cl^-/HCO^-_3 exchanger, respectively. Inhibitation of CA would then result in ion disruption by reducing Na^+ and Cl^- influx. Cu^{2+} could also increase the diffusive loss of Na^+ by displacing Ca^{2+} in the tight junctions and disrupt the permeability of the branchial epithelium (Lauren and Macdonald, 1985). Depending on the exposure concentration, Ag^+ or Cu^{2+} may bind to NKA and CA more readily and influence/enhance the adverse effects of the other. Inhibition of these enzymes by Cu^{2+} and Ag^+ would cause a decrease in plasma Na^+ and Cl^-, which leads to a sequence of events that results in death due to circulatory collapses (Hogstrand and Wood, 1998). Processes involved with maintaining ion homeostasis during Cu^{2+} and Ag^+ toxicity may include an up-regulation of NKA and CA activity to increase ion uptake from water. However, this up-regulation for Na^+

uptake instead may facilitate further Cu^{2+} and Ag^{+} uptake into the gills and cause greater ion disruption.

Additional uptake routes that may lead to greater accumulation of metal may include a Na^{+}/H^{+}-exchanger and the high-affinity Cu-transporter (ctr1) found on the apical side of the gill cell. In mammalian cells, Ag^{+} has been shown to be a substrate for ctr1 uptake but this has not yet been described in fish (Bertinato et al., 2010). In the 10-day mixture test, Ag accumulation was less in mixture-exposed fish than in Ag^{+}-only exposed fish on day 1, and this may have been due a high Cu^{2+} concentration in the exposure where uptake of Cu^{2+} was favoured. By day 3, Ag accumulation in the gills of mixture-exposed fish were similar to those exposed to Ag^{+}-only, where Ag^{+} could have been up taken by multiple routes that include apical Na^{+}-channels and Na^{+}/H^{+}-exchangers (Figure 4.1). In the 14-day mixture test, gill accumulation of both Cu and Ag were enhanced in mixture-exposed fish but it is unclear how Cu^{2+} or Ag^{+} may have stimulated the uptake of the other (Figure 3.3). This enhanced accumulation may involve an unknown apical channel and/or exchanger on the gill cell that acts to facilitate either Cu^{2+} or Ag^{+} uptake. In the subcellular fractions of the gills, significant accumulation of Cu was observed in MRG, HSP (e.g. MT and MTLP), and HDP (e.g. cytosolic enzymes) of mixture-exposed fish on day 14. Since both Cu and Ag accumulation was enhanced in the gills, much of the Ag^{+} was perhaps directed at inhibiting NKA and CA while Cu^{2+} bound to a number of other cytosolic enzymes, hence the increased accumulation in HDP. The increase in MRG and HSP might be due to a process of detoxification where MT and MTLP sequesters Cu from the HDP and transports it sites of MRG formation (Wallace et al., 2003). With the observed physiological effects, it is speculated the majority of plasma ion loss is due to Ag^{+} inhibiting NKA and CA inside the gill cells. Cu^{2+} would contribute to additional ion loss by displacing Ca^{2+} in the tight junctions, increasing the diffusive Na^{+} loss (Figure 4.1). In a similar paracellular pathway, Cu^{2+} may also increase diffusive Cl^{-} loss

(Grosell, 2012). Combined, Ag^{+} and Cu^{2+} related effects would lead to a more than additive loss of plasma Na^{+} and Cl^{-}.

The toxicity of environmental contaminants has typically been defined one at a time. In reality, however, exposure to environmental contaminants occurs as mixtures. In these mixtures, interactions between contaminants can change the overall degree of toxicity. These interactions pose unique challenges to researchers since little is known on their health effects or environmental impact. Metal mixture studies like this one are integrative in nature and incorporate knowledge and concepts from other disciplines of Science into one overall study. For example, the chemical factors of a metal, such as speciation can affect the physical and biochemical aspects of the exposed organism from uptake, internal handling, metabolism, and toxicity. The collaboration of these scientific disciplines will improve our ability to better define the outcomes of metal mixture studies especially since the mechanisms of metal-metal interactions are not well understood. This and other studies have demonstrated the existences of metal-metal interactions (Xu et al., 2014; Naddy et al., 2015; Niyogi et al., 2015) where exposure to a mixture of metals can result in additive or more than or less than additive effects. The objective of this study was to form a linkage between tissue bioaccumulation to observed physiological effects.

There are several advantages in expressing physiological effects as a function of bioaccumulation, especially in the form of subcellular distribution. The subcellular distribution of metals in fish tissues can provide details concerning what portions of metals are detoxified (inactive metals) and which are freely available to cause toxicity, all of which better define the relationship between bioaccumulation and toxicity. Another critical aspect is that subcellular distribution of metals can demonstrate the existence of metal-metal interactions and reveal their mechanisms of accumulation dynamics and toxicity. The significance of these studies can be

used to improve metal mixture modeling in ecotoxicology and lead to better protective measures for aquatic environments.

4.2 Figure

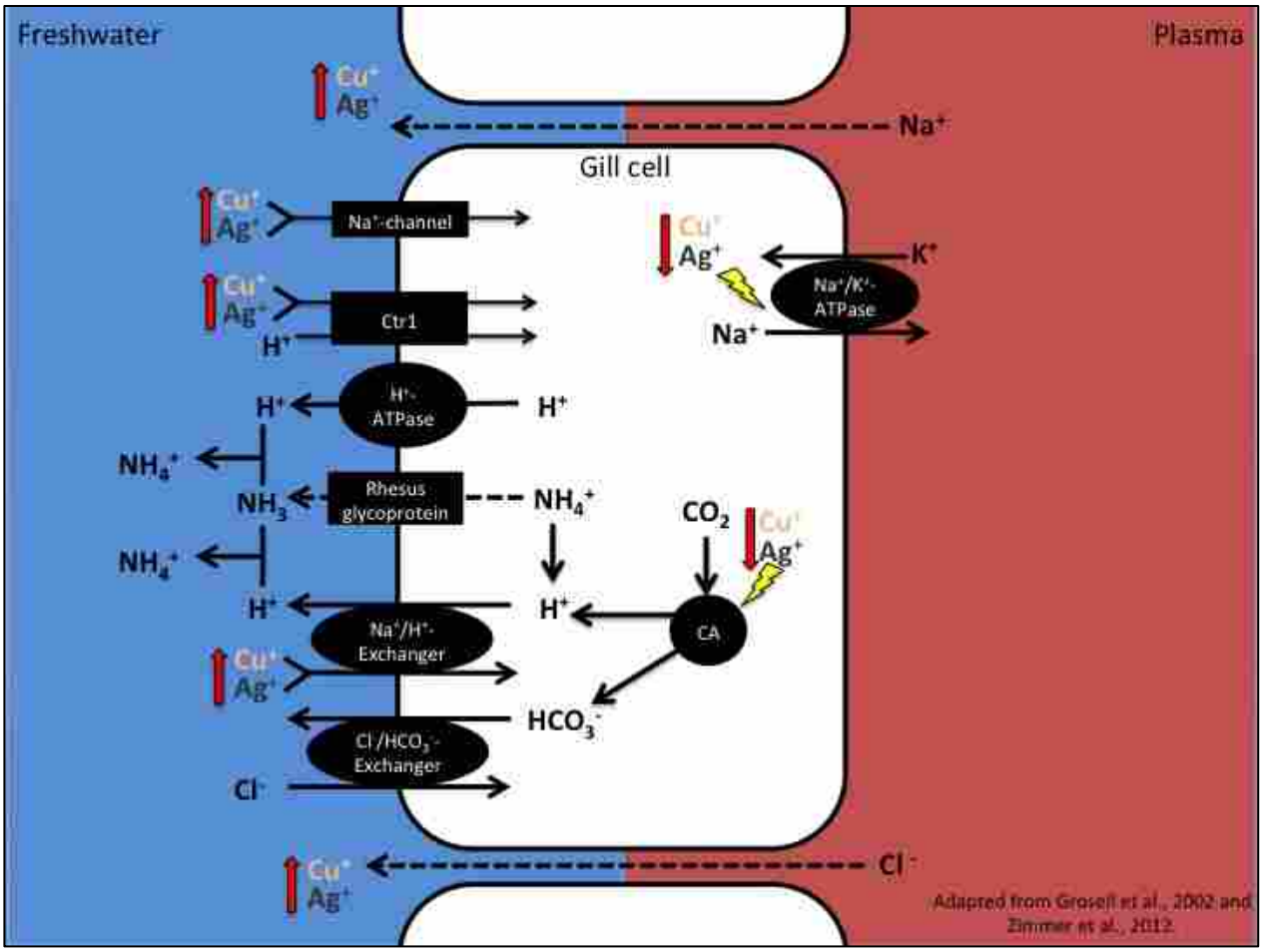
Freshwater
Plasma
Gill cell
Cu+
Ag+
Na+
Na+-channel
Ctr1
H+
H+-ATPase
K+
Na+/K+-ATPase
NH4+
NH3
Rhesus glycoprotein
CO2
CA
Na+/H+-Exchanger
HCO3-
Cl-/HCO3- Exchanger
Cl-
Adapted from Grosell et al., 2002 and Zimmer et al., 2012

Figure 4.1. Proposed model of the interactions between Cu and Ag in the gill cell of fishes. Solid lines donate active transport while dotted lines represent diffusive transport. Solid black shapes and include the sodium (Na^+)- channel, high-affinity Cu transport (ctr1), rhesus glycoprotein, hydrogen- adenosine triphosphatase (H^+-ATPase), Na^+/H^+- exchanger or chloride/bicarbonate (Cl^-/HCO_3^--exchanger). The red arrow pointing "up" or "down" denotes activation or inhibitation of the respective relevant processes by Ag^+ and/or Cu^{2+}. Symbols in yellow represent Cu^{2+} and Ag^+ targeting sodium/potassium-adenosine triphosphatase (Na^+/K^+-ATPase) and carbonic anhydrase (CA). Cu^{2+} and Ag^+ are proposed to compete with one another for uptake by can be taken up from the water and into the gill cell by Na^+ channel. The uptake of Cu and Ag could also occur through apical Na^+/H^+-exchanger and ctr1 pathways. Once in the gills Cu^{2+} and Ag^+ will cause toxicity by inhibiting Na^+/K^+-ATPase and CA activity, leading to reduced $Na^+ + Cl^-$ influx. Cu^{2+} could also increase diffusive loss of $Na^+ + Cl^-$ and combined with inhibition of Na^+/K^+-ATPase and CA, results in reduced plasma ion concentration. Another target site of Cu^{2+} and Ag^+ may include inhibiting rhesus glycoproteins, which results in increase plasma ammonia concentrations. Processes involved in maintain homeostasis may include an up-regulation of Na^+/K^+-ATPase and CA activity to increase ion uptake from the water. This instead may facilitate further Cu^{2+} and Ag^+ uptake into the cell by additional pathways including apical Na^+/H^+-exchanger.

Adapted from Grosell et al., 2002 and Zimmer et al., 2012.

4.3 References

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Appendix A: Raw data for (*Chapter 2*) 10-day mixture test

Table A.1 Means (± SEM (n)) for measured total and dissolved Cu^{2+} concentrations in water samples from control, Cu^{2+}-only and mixture exposures over 10 days. Values are represented in µmol/L (µM). Samples were taken directly from the head tank and exposure (in replicate) tanks.

	Water in Head Tank		**Water Exposure Tank**	
Exposure	**Total (µM)**	**Dissolved (µM)**	**Total (µM)**	**Dissolved (µM)**
Control (0 µM)	N/A	N/A	0.00 ± 0 (8)	0.00 ± 0 (8)
Cu^{2+}-only (1.0 µM)	0.88 ± 0.62 (4)	0.68 ± 0.34 (4)	0.77 ± 0.27 (8)	0.75 ± 0.25 (8)
Mixture (1.0 µM Cu^{2+} + 0.04 µM Ag^{+})	0.98 ± 0.69 (2)	0.53 ± 0.37 (2)	0.86 ± 0.49 (3)	0.52 ± 0.30 (3)

Table A2. Means (± SEM (n)) for measured total and dissolved Ag^{+} concentrations in water samples from control, Ag^{+}-only and mixture exposures over 10 days. Values are represented as nmol/L (nM). Samples were taken directly from the head tank and exposure (in replicate) tanks.

	Head Tank Water		**Exposure Tank Water**	
Exposure	**Total (nM)**	**Dissolved (nM)**	**Total (nM)**	**Dissolved (nM)**
Control (0 µM)	N/A	N/A	0.76 ± 0.27 (8)	0.64 ± 0.24 (8)
Ag^{+}-only (0.04 µM)	32.68 ± 16.34 (4)	23.75 ± 11.87 (4)	33.49 ± 12.66 (7)	21.20 ± 8.01 (7)
Mixture (1.0 µM Cu^{2+} + 0.04 µM Ag^{+})	35.95 ± 25.42 (2)	16.53 ± 11.69 (2)	28.53 ± 14.26 (3)	16.07 ± 8.04 (3)

Table A3. Percent (%) mortality of juvenile rainbow trout exposed to either 1.0 μM Cu^{2+}-only, 0.04 μM Ag^{+}-only or 1.0 μM Cu^{2+} + 0.04 μM Ag^{+} mixture for 10 days. Values are represented in percentage of mortality (number of mortalities) of an entire exposure group during the 10 days of the experiment.

Exposure	**% Mortality**
Control (0 μM)	4.17 % (1)
Cu^{2+}-only (1.0 μM)	33.33 % (8)
Ag^{+}-only (0.04 μM)	8.33 % (2)
Mixture (1.0 μM Cu^{2+} + 0.04 μM Ag^{+})	62.50 % (15)

Table A4. Bioaccumulation of Cu in the gill and liver of juvenile rainbow trout exposed to either 1.0 μM Cu^{2+}-only or 1.0 μM Cu^{2+} + 0.04 μM Ag^{+} mixture for 10 days. Values are represented as means (nM/g tissue wet weight) ± SEM (n). * represents a significant difference between exposed fish and controls on that sampling day while † represents a significant difference between mixture-exposed fish and Cu-only exposed fish (ANOVA $P < 0.05$).

		Exposure		
Tissue	**Day**	**Control**	**Cu^{2+}-only**	**Mixture**
Gill	0	21 ± 6 (6)	21 ± 6 (6)	21 ± 6 (6)
	1	37 ± 5 (6)	124 ± 29 (6)*	110 ± 5 (6)
	3	33 ± 4 (6)	217 ± 21 (4)*	182 ± 17 (3)*
	5	36 ± 6 (6)	532 ± 117 (4)*	N/A
	10	39 ± 11 (5)	320 ± 72 (2)*	N/A
Liver	0	3243 ± 266 (6)	3243 ± 266 (6)	3243 ± 266 (6)
	1	3960 ± 531 (6)	4947 ± 48 (6)	9552 ±1207 (6)
	3	5953 ± 979 (6)	8595 ± 288 (4)	3607 ± 1708 (3)†
	5	5429 ± 417 (6)	6481 ± 927 (4)	N/A
	10	4763 ± 907 (5)	7614 ± 279 (2)	N/A

Table A5. Bioaccumulation of Ag in the gill and liver of juvenile rainbow trout to either 0.04 μM Ag^{+}-only or 1.0 μM Cu^{2+} + 0.04 μM Ag^{+} mixture for 10 days. Values are represented as means (nM/g tissue wet weight) ± SEM (n). * represents a significant difference between exposed fish and controls on to that sampling day while † represents a significant difference between mixture-exposed fish and Ag-only exposed fish (ANOVA $P < 0.05$).

		Exposure		
Tissue	**Day**	**Control**	**Ag^{+}-only**	**Mixture**
Gill	0	0.15 ± 0.15 (6)	0.15 ± 0.15 (6)	0.15 ± 0.15 (6)
	1	0.03 ± 0.1 (6)	17.18 ± 6.04 (6)*	7.30 ± 2.87 (6)
	3	0.08 ± 0.03 (6)	10.27 ± 0.61 (6)*	12.79 ± 3.07 (3)*
	5	0.10 ± 0.02 (6)	13.39 ± 2.98 (6)*	N/A
	10	0.13 ±0.01 (5)	10.32 ± 6.89 (4)*	N/A
Liver	0	0.24 ± 0.03 (6)	0.24 ± 0.03 (6)*	0.24 ± 0.03 (6)
	1	0.21 ± 0.01 (6)	9.55 ± 2.49 (6)*	1.27 ± 0.43 (6)†
	3	0.10 ± 0.01 (6)	11.53 ± 2.13 (6)*	4.91 ± 0.38 (3)†
	5	0.08 ± 0.01 (6)	20.67 ± 9.05 (6)*	N/A
	10	0.24 ± 0.05 (5)	15.27 ± 4.35 (4)*	N/A

Table A6. Effect of either 1.0 μM Cu^{2+}-only, 0.04 μM Ag^{+}-only or 1.0 μM Cu^{2+} + 0.04 μM Ag^{+} mixture exposure on plasma Na^{+} and Cl^{-} regulation in juvenile rainbow trout over 10 days. Unexposed fish were grouped as "Control". Values are presented as means (± SEM (n)). * represents a significant difference between exposed fish and controls on that sampling day while † represents a significant difference between mixture-exposed fish and Cu-only exposed fish (ANOVA $P < 0.05$).

		Exposure			
Plasma Ion	**Day**	**Control**	**Cu^{2+}-only**	**Ag^{+}-only**	**Mixture**
Na^{+}	0	142 ± 11 (6)	142 ± 11 (6)	142 ± 11 (6)	142 ± 11 (6)
	1	187 ± 4 (6)	151 ± 7 (6)	150 ± 6 (6)	138 ± 2 (6)
	3	141 ± 2 (6)	141 ± 2 (4)	121 ± 8 (6)	52 ± 4 (3) *†
	5	109 ± 6 (6)	79 ± 8 (4)	97 ± 11(6)	N/A
	10	129 ± 2 (5)	121 ± 4 (2)	104 ± 5 (4)	N/A
Cl^{-}	0	113 ± 3 (6)	113 ± 3 (6)	113 ± 3 (6)	113 ± 3 (6)
	1	114 ± 4 (6)	90 ± 7 (6)*	104 ± 2 (6)	87 ± 4 (6) *†
	3	114 ± 5 (6)	83 ± 6 (4)*	97 ± 3 (6)	91 ± 9 (3)
	5	113 ± 3 (6)	70 ± 5 (4)*	85 ± 4 (6)*	N/A
	10	114 ± 5 (5)	88 ± 5 (2)*	71 ± 5 (4)*	N/A

Appendix B: Raw data for (*Chapter 3*) 14-day mixture test

Table B1. Means (± SEM (n)) for measured total and dissolved Cu^{2+} concentrations in water samples from control, Cu^{2+}-only and mixture exposures over 14 days. Values are represented in µmol/ L (µM). Samples were taken directly from the head tank and exposure (in replicate) tanks.

	Water in Head Tank		Water Exposure Tank	
Exposure	**Total (µM)**	**Dissolved (µM)**	**Total (µM)**	**Dissolved (µM)**
Control (0 µM)	0.11 ± 0.05 (4)	0.03 ± 0.01 (4)	0.06 ± 0.02 (8)	0.03 ± 0.01 (8)
Cu^{2+}-only (0.35 µM)	0.55 ± 0.28 (4)	0.45 ± 0.23 (4)	0.51 ± 0.18 (8)	0.40 ± 0.14 (8)
Mixture (0.35 µM Cu^{2+} + 0.04 µM Ag^{+})	0.64 ± 0.32 (4)	0.52 ± 0.26 (4)	0.56 ± 0.20 (8)	0.43 ± 0.15 (8)

Table B2. Means (± SEM (n)) for measured total and dissolved Ag^{+} concentrations in water samples from control, Ag^{+}-only and mixture exposures over 14 days. Values are represented in nmol/L (nM). Samples were taken directly from the head tank and exposure (in replicate) tanks.

	Water in Head Tank		Water Exposure Tank	
Exposure	**Total (nM)**	**Dissolved (nM)**	**Total (nM)**	**Dissolved (nM)**
Control (0 µM)	0.08 ± 0.04 (4)	0.00 ± 0.00 (4)	0.00 ± 0.00 (8)	0.00 ± 0.00 (8)
Ag^{+}-only (0.04 µM)	41.77 ± 20.89 (4)	40.15 ± 20.07 (4)	42.58 ± 15.06 (8)	37.53 ± 13.27 (8)
Mixture (0.35 µM Cu^{2+} + 0.04 µM Ag^{+})	56.03 ± 28.01 (4)	50.99 ± 05.50 (4)	49.58 ± 17.53 (8)	43.89 ± 15.52 (8)

Table B3. Percent (%) mortality of juvenile rainbow trout exposed to either 0.35 μM Cu^{2+}-only, 0.04 μM Ag^{+}-only or 0.35 μM Cu^{2+} + 0.04 μM Ag^{+} mixture for 14 days. Values are represented in percentage of mortality (number of mortalities) of an entire exposure group during the 14 days of the experiment.

Exposure	**% Mortality**
Control (0 μM)	0.00 % (0)
Cu^{2+}-only (0.35 μM)	0.00 % (0)
Ag^{+}-only (0.04 μM)	15.63 % (5)
Mixture (0.35 μM Cu^{2+} + 0.04 μM Ag^{+})	31.25 % (10)

Table B4. Bioaccumulation of Cu in the gill, liver and kidney of juvenile rainbow trout to either 0.35 μM Cu^{2+}-only or 0.35 μM Cu^{2+} + 0.04 μM Ag^{+} mixture for 14 days. Values are represented as means (nM/g tissue wet weight) ± SEM (n). * represents a significant difference between exposed fish and controls on that sampling day while † represents a significant difference between mixture-exposed fish and Cu-only exposed fish (ANOVA $P < 0.05$).

		Exposure		
Tissue	**Day**	**Control**	**Cu^{2+}-only**	**Mixture**
Gills	0	39 ± 3 (8)	39 ± 3 (8)	39 ± 3 (8)
	1	60 ± 7 (8)	79 ± 12 (8)*	93 ± 3 (8)*†
	3	44 ± 2 (8)	92 ± 20 (8)*	159 ± 17 (6)*†
	7	36 ± 1 (8)	63 ± 9 (8)*	123 ± 11 (5)*†
	14	44 ± 4 (8)	123 ± 35 (8)*	237 ± 32 (3)*†
Liver	0	3104 ± 618 (8)	3104 ± 618 (8)	3104 ± 618 (8)
	1	3002 ± 755 (8)	3355 ± 733 (8)	2817 ± 444 (8)
	3	4047 ± 407 (8)	4438 ± 548 (8)	2963 ± 640 (6)
	7	2398 ± 377 (8)	2356 ± 378 (8)	6102 ± 1006 (5)*†
	14	3293 ± 328 (8)	3154 ± 426 (8)	6122 ± 1369 (3)*†
Kidney	0	91 ± 4 (8)	91 ± 4 (8)	91 ± 4 (8)
	1	88 ± 5 (8)	86 ± 6 (8)	75 ± 3 (8)
	3	80 ± 6 (8)	73 ± 1 (8)	104 ± 14 (6)*†
	7	95 ± 6 (8)	101 ± 7 (8)*	164 ± 19 (5)*†
	14	109 ± 9 (8)	130 ± 13 (8)*	191 ± 13 (3)*†

Table B5. Bioaccumulation of Ag in the gill, liver and kidney of juvenile rainbow trout exposed to either 0.04 μM Ag^{+}-only or 0.35 μM Cu^{2+} + 0.04 μM Ag^{+} mixture for 14 days. Values are represented as means (nM/g tissue wet weight) ± SEM (n). * represents a significant difference between exposed fish and controls on that sampling day while † represents a significant difference between mixture-exposed fish and Ag-only exposed fish (ANOVA $P < 0.05$).

		Exposure		
Tissue	**Day**	**Control**	**Ag^{+}-only**	**Mixture**
Gills	0	0.11 ± 0.02 (8)	0.11 ± 0.02 (8)	0.11 ± 0.02 (8)
	1	0.24 ± 0.02 (8)	1.70 ± 0.52 (8)*	0.79 ± 0.07 (8)
	3	0.11 ± 0.01 (8)	3.29 ± 0.88 (8)*	12.79 ± 5.33 (6)*†
	7	0.12 ± 0.01 (8)	1.69 ± 0.33 (7)	12.64 ± 0.81 (5)*†
	14	0.09 ± 0.02 (8)	3.3 ± 0.38 (4)*	18.93 ± 0.65 (3)*†
Liver	0	0.12 ± 0.02 (8)	0.12 ± 0.02 (8)	0.12 ± 0.02 (8)
	1	0.20 ± 0.02 (8)	2.00 ± 0.91 (8)*	0.40 ± 0.08 (8)†
	3	0.14 ± 0.02 (8)	2.16 ± 0.59 (8)*	1.58 ± 0.24 (6)*†
	7	0.19 ± 0.01 (8)	2.15 ± 0.33 (7)*	3.65 ± 0.56 (5)*†
	14	0.15 ± 0.02 (8)	13.01 ± 2.01 (4)*	6.09 ± 2.32 (3)*†
Kidney	0	0.19 ± 0.04 (8)	0.19 ± 0.04 (8)	0.19 ± 0.04 (8)
	1	0.17 ± 0.02 (8)	1.50 ± 0.49 (8)*	1.41 ± 0.56 (8)
	3	0.15 ± 0.01 (8)	4.66 ± 1.45 (8)	2.77 ± 1.56 (8)
	7	0.15 ± 0.03 (8)	0.43 ± 0.02 (7)	5.54 ± 1.79 (5)*†
	14	0.12 ± 0.00 (8)	5.24 ± 2.82 (4)*	9.16 ± 0.31 (3)*†

Table B6. Citrate synthase activity in mitochondrial fraction and cytosol of rainbow trout gills and liver homogenate. Values are represented as mean (nM/ min/ mg protein) ± 1 SEM (6). * represents a significant difference between mitochondrial fraction and cytosol (ANOVA $P < 0.05$).

Fraction	Tissue	Citrate Synthase Activity
Mitochondria	Gills	2.22 ± 0.28 *
	Liver	1.48 ± 0.42 *
Cytosol	Gills	0.13 ± 0.02
	Liver	0.03 ± 0.00

Table B7. Bioaccumulation of Cu in gill-subcellular fractions of juvenile rainbow trout exposed to either 0.35 μM Cu^{2+}-only or 0.35 μM Cu^{2+} + 0.04 μM Ag^{+} mixture for 14 days. Values are represented as means (nM/g tissue wet weight) ± SEM (n). * represents a significant difference between exposed fish and controls on that sampling day while † represents a significant difference between mixture-exposed fish and Ag-only exposed fish (ANOVA $P < 0.05$).

		Exposure	
Fraction	**Day**	**Cu^{2+}-only**	**Mixture**
MRG	0	1.42 ± 0.58 (6)	1.42 ± 0.58 (6)
	3	5.02 ± 2.05 (6)	16.58 ± 7.41 (6)
	7	2.07 ± 0.84 (6)	36.08 ± 16.13 (5)
	14	13.79 ± 5.63 (6)	264.89 ± 152.94 (3)*†
Mitochondria	0	30.57 ± 12.48 (6)	30.57 ± 12.48 (6)
	3	42.17 ± 17.21 (6)	47.89 ± 19.55 (6)
	7	57.53 ± 23.49 (6)	73.80 ± 33.00 (5)
	14	68.62 ± 28.01 (6)	140.17 ± 80.93 (3)
Microsomes & Lysosomes	0	13.12 ± 5.36 (6)	13.12 ± 5.36 (6)
	3	46.16 ± 18.84 (6)	9.63 ± 3.93 (6)
	7	46.09 ± 18.82 (6)	15.85 ± 7.09 (5)
	14	27.60 ± 11.27 (6)	11.19 ± 6.46 (3)
HDP	0	4.94 ± 2.02 (6)	4.94 ± 2.02 (6)
	3	13.77 ± 5.62 (6)	3.79 ± 1.55 (6)
	7	8.01 ± 3.27 (6)	4.04 ± 1.81 (5)
	14	1.20 ± 0.49 (6)	57.34 ± 33.10 (3)*†
HSP	0	1.54 ± 0.63 (6)	1.54 ± 0.63 (6)
	3	9.50 ± 3.88 (6)	15.15 ± 6.19 (6)
	7	5.58 ± 2.28 (6)	11.65 ± 5.21 (5)
	14	6.69 ± 2.73 (6)	52.31 ± 30.20 (3)*†

Table B8. Bioaccumulation of Ag in gill-subcellular fractions of juvenile rainbow trout exposed to either 0.04 μM Ag^{+}-only or 0.35 μM Cu^{2+} + 0.04 μM Ag^{+} mixture for 14 days. Values are represented as means (nM/g tissue wet weight) ± SEM (n) (ANOVA $P < 0.05$).

		Exposure	
Fraction	**Day**	**Ag^{+}-only**	**Mixture**
MRG	0	9.45 ± 1.67 (6)	9.45 ± 1.67 (6)
	3	58.19 ± 16.91 (6)	125.45 ± 35.82 (6)
	7	109.52 ± 26.72 (6)	131.25 ± 40.57 (5)
	14	140.55 ± 128.39 (4)	195.46 ± 42.86 (3)
Mitochondria	0	0.76 ± 0.01 (6)	0.76 ± 0.01 (6)
	3	12.72 ± 5.19 (6)	7.06 ± 2.88 (6)
	7	2.17 ± 0.89 (6)	5.68 ± 2.54 (5)
	14	18.36 ± 9.18 (4)	15.51 ± 8.96 (3)
Microsomes &	0	1.24 ± 0.51 (6)	1.24 ± 0.51 (6)
Lysosomes	3	9.69 ± 3.96 (6)	10.39 ± 4.24 (6)
	7	7.23 ± 3.23 (6)	8.01 ± 3.58 (5)
	14	7.39 ± 3.70 (4)	8.20 ± 4.73 (3)
HDP	0	3.54 ± 1.45 (6)	3.54 ± 1.45 (6)
	3	5.99 ± 2.44 (6)	6.43 ± 2.63 (6)
	7	4.94 ± 2.02 (6)	9.84 ± 4.40 (5)
	14	2.84 ± 1.42 (4)	7.17 ± 4.14 (3)
HSP	0	0.26 ± 0.11 (6)	0.26 ± 0.11 (6)
	3	5.45 ± 2.23 (6)	5.24 ± 2.14 (6)
	7	2.19 ± 0.90 (6)	2.74 ± 1.23 (5)
	14	4.81 ± 2.41 (4)	3.60 ± 2.08 (3)

Table B9. Bioaccumulation of Cu in liver-subcellular fractions of juvenile rainbow trout exposed to either 0.35 μM Cu^{2+}-only or 0.35 μM Cu^{2+} + 0.04 μM Ag^{+} mixture for 14 days. Values are represented as means (nM/g tissue wet weight) ± SEM (n) (ANOVA $P < 0.05$).

		Exposure	
Fraction	**Day**	**Cu^{2+}-only**	**Mixture**
MRG	0	1624 ± 663 (6)	1624 ± 663 (6)
	3	552 ± 247 (6)	322 ± 144 (6)
	7	639 ± 452 (6)	337 ± 151 (5)
	14	745 ± 304 (6)	350 ± 202 (3)
Mitochondria	0	1111 ± 453 (6)	1111 ± 453 (6)
	3	1435 ± 586 (6)	567 ± 232 (6)
	7	1800 ± 735 (6)	1598 ± 715 (5)
	14	798 ± 326 (6)	1507 ± 870 (3)
Microsomes &	0	891 ± 364 (6)	891 ± 364 (6)
Lysosomes	3	395 ± 161 (6)	458 ± 187 (6)
	7	3928 ± 1604 (6)	662 ± 296 (5)
	14	501 ± 205 (6)	264 ± 153 (3)
HDP	0	33 ± 13 (6)	33 ± 13 (6)
	3	36 ± 15 (6)	137 ± 56 (6)
	7	171 ± 70 (6)	118 ± 53 (5)
	14	352 ± 157 (6)	51 ± 30 (3)
HSP	0	472 ± 193 (6)	472 ± 193 (6)
	3	368 ± 150 (6)	401 ± 164 (6)
	7	62 ± 25 (6)	186 ± 83 (5)
	14	205 ± 84 (6)	810 ± 468 (3)

Table B10. Bioaccumulation of Ag in liver-subcellular fractions of juvenile rainbow trout exposed to either 0.04 μM Ag^{+}-only or 0.35 μM Cu^{2} + 0.04 μM Ag^{+} mixture for 14 days. Values are represented as means (nM/g tissue wet weight) ± SEM (n). * represents a significant difference between exposed fish and controls on that sampling day (ANOVA $P < 0.05$).

		Exposure	
Fraction	**Day**	**Ag^{+}-only**	**Mixture**
MRG	0	4.28 ± 0.31 (6)	4.28 ± 0.31 (6)
	3	26.47 ± 3.55 (6)	33.47 ± 5.99 (6)
	7	71.34 ± 17.52 (6)*	108.38 ± 12.78 (5)*
	14	25.83 ± 1.29 (4)	55.64 ± 24.35 (3)
Mitochondria	0	0.55 ± 0.01 (6)	0.55 ± 0.01 (6)
	3	9.01 ± 3.68 (6)	6.87 ± 2.81 (6)
	7	1.70 ± 0.69 (6)	2.71 ± 1.21 (5)
	14	5.19 ± 2.59 (4)	5.23 ± 3.02 (3)
Microsomes &	0	0.60 ± 0.25 (6)	0.60 ± 0.25 (6)
Lysosomes	3	12.34 ± 5.04 (6)	6.09 ± 2.48 (6)
	7	11.01 ± 4.49 (6)	12.06 ± 5.40 (5)
	14	20.95 ± 12.10 (4)	16.20 ± 9.36 (3)
HDP	0	3.22 ± 1.31 (6)	3.22 ± 1.31 (6)
	3	7.75 ± 3.17 (6)	6.47 ± 2.64 (6)
	7	3.98 ± 1.63 (6)	6.52 ± 2.91 (5)
	14	5.67 ± 2.83 (4)	4.42 ± 2.55 (3)
HSP	0	0.27 ± 0.11 (6)	0.27 ± 0.11 (6)
	3	5.04 ± 2.06 (6)	0.82 ± 0.34 (6)
	7	2.50 ± 1.02 (6)	1.56 ± 0.70 (5)
	14	2.50 ± 1.44 (4)	3.06 ± 1.77 (3)

Table B11. Effects of either 0.35 μM Cu^{2+}-only, 0.04 μM Ag^{+}-only or 0.35 μM Cu^{2+} + 0.04 μM Ag^{+} mixture exposure on plasma Na^{+} and Cl^{-} regulation in juvenile rainbow trout over 14 days. Unexposed fish were grouped as "Control". Values are presented as means (± SEM). * represents a significant difference between exposed fish and controls on that sampling day while † represents a significant difference between mixture-exposed fish and Cu-only exposed fish. ‡ represents a significant difference between mixture-exposed fish and both single metal exposed fish (ANOVA $P < 0.05$).

		Exposure			
Plasma Ion	**Day**	**Control**	**Cu^{2+}-only**	**Ag^{+}-only**	**Mixture**
Na^{+}	0	146 ± 7 (8)	146 ± 7 (8)	146 ± 7 (8)	146 ± 7 (8)
	1	140 ± 2 (8)	142 ± 1 (8)	137 ± 3 (8)*	133 ± 3 (8)†
	3	151 ± 2 (8)	147 ± 1 (8)	136 ± 3 (8)*	125 ± 2 (6)*‡
	7	150 ± 1 (8)	152 ± 1 (8)	124 ± 3 (7)*	112 ± 8 (5)*†
	14	145 ± 4 (8)	144 ± 4 (8)	108 ± 0 (4)*	93 ± 6 (3)*
Cl^{-}	0	116 ± 2 (8)	116 ± 2 (8)	116 ± 2 (8)	116 ± 2 (8)
	1	119 ± 2 (8)	116 ± 1 (8)	112 ± 2 (8)*	103 ± 3 (8)*‡
	3	120 ± 1 (8)	117 ± 1 (8)	108 ± 1 (8)*	98 ± 2 (6)*‡
	7	119 ± 1 (8)	117 ± 2 (8)	97 ± 4 (7)*	85 ± 2 (5)*‡
	14	115 ± 2 (8)	108 ± 4 (8)	78 ± 6 (4)*	69 ± 7 (3)*†