The sublethal effects of Cu exposure on the osmoregulatory and swimming performance in juvenile rainbow trout (Oncorhynchus mykiss)

by

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B.Sc., Simon Fraser University, 2011

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Abstract

Cu is a widely occurring contaminant in aquatic systems and is acutely toxic to fish. The current paradigm of copper’s toxic mechanism of action in fish is believed to be through direct effects on several important functions of the teleost gill; therefore experiments were conducted to examine Cu effects on the osmoregulatory ability and swimming performance of juvenile rainbow trout (*Oncorhynchus mykiss*) in water of varying hardness. Fish were exposed to three Cu concentrations (0, 20 and 60 µg/L in hard water [100 mg/L CaCO₃], and 0, 6 and 16 µg/L in soft water [6 mg/L CaCO₃]), for 4, 8 and 16 d and then tested for their ability to osmoregulate and burst swim. Burst swimming speed (*Uₜ₉₉*) was not different between control and Cu-exposed fish in either soft or hard water. Osmoregulatory ability following Cu exposure was examined through several biochemical measurements related to osmoregulation and a seawater challenge. Cu exposure did not elicit a change in any osmoregulatory-related measurement or result in mortalities during the seawater challenge. These results indicate that the current paradigm of Cu toxicity may not reflect the mechanism of sublethal Cu toxicity or that compensatory mechanisms are offsetting major physiological disturbances caused by Cu, at concentrations that are near those which typically cause mortality in salmonids.

**Keywords:** Rainbow trout; copper; sublethal toxicity; swimming performance; *Uₜ₉₉*, osmoregulation
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# Table of Contents

Approval .................................................................................................................................................. ii
Ethics Statement ...................................................................................................................................... iii
Abstract ................................................................................................................................................... iv
Dedication ................................................................................................................................................ v
Acknowledgements ................................................................................................................................. vi
Table of Contents ................................................................................................................................... vii
List of Tables ............................................................................................................................................ vii
List of Figures .......................................................................................................................................... x
List of Acronyms and Abbreviations ....................................................................................................... xiii

## Chapter 1. General Introduction ........................................................................................................... 1
1.1. The chemistry of Cu ............................................................................................................................... 1
1.2. Sources ................................................................................................................................................ 2
1.3. Environmental concentrations ............................................................................................................. 2
1.4. Cu chemistry in water ............................................................................................................................ 3
1.5. Cu bioavailability and modifying factors ............................................................................................. 3
1.6. Toxicokinetics ..................................................................................................................................... 6
1.7. Mechanism of action ............................................................................................................................ 10
1.8. Acute toxicity ...................................................................................................................................... 11
1.9. Sublethal toxicity ............................................................................................................................... 14
1.10. Sublethal endpoints .......................................................................................................................... 17
1.11. Research objectives .......................................................................................................................... 19

## Chapter 2. Materials and Methods ....................................................................................................... 20
2.1. Chemicals .......................................................................................................................................... 20
2.2. Fish .................................................................................................................................................... 20
2.3. Acute toxicity tests ............................................................................................................................. 21
2.4. Sublethal Cu exposures ....................................................................................................................... 21
2.5. Cu exposures for swim experiments .................................................................................................. 22
2.6. Swim performance protocol ............................................................................................................. 23
2.7. Seawater challenge .............................................................................................................................. 24
2.8. Osmoregulatory indicators ................................................................................................................ 25
2.9. Calculations and statistics .................................................................................................................. 26

## Chapter 3. Results .................................................................................................................................. 28
3.1. General .............................................................................................................................................. 28
3.2. LC50 determinations .......................................................................................................................... 28
3.3. Swimming performance ...................................................................................................................... 30
3.4. Osmoregulation ................................................................................................................................. 31
  3.4.1. Gill Na"/K"-ATPase (NKA) activity ................................................................................................. 31
  3.4.2. Plasma ion concentrations and osmolality ................................................................................... 33
  3.4.3. Seawater challenge ...................................................................................................................... 37
3.5. Biochemical stress response .............................................................................................................. 37
Chapter 4. Discussion ................................................................. 41

Chapter 5. Conclusion and Future Directions.............................. 51

References ............................................................................. 53
List of Tables

Table 1.1. Summary of LC50 values across a range of species. N/A indicates information not available. Relatable indicates whether study is relevant to the present research (based on duration, life stage and other variables). (Modified from: Eisler, 2000) ........................................ 12

Table 2.1. Summary of off-site well water parameters (analyte concentration, water quality measurements). Values are mean ± SEM. ......................... 22
List of Figures

Figure 1.1. Schematic diagram of the biotic ligand model (From: Di Toro et al., 2001) ................................................................. 6

Figure 1.2. Schematic diagram of Cu uptake in a teleost gill cell. CR = copper reductase, ENaC = epithelial Na⁺ channel, NHE3 = Na⁺/H⁺ antiporter, Na-K pump = Na⁺/K⁺-ATPase, CTR1 = copper transporter 1, GN = golgi network, MC = metallochaperone, MBP = metal binding protein. (Image courtesy of Ryan Lebek, adapted from: Bury et al., 2003 & Parker and Boron, 2013). .................................................. 8

Figure 2.1 Schematic representation of the swim tunnel apparatus (From: Mackinnon and Farrell, 1992). .................................................. 24

Figure 3.1 Mortality (%) of fish exposed to different Cu concentrations after 96 h, in hard water (A) and soft water (B). Mean values reported. N=10 ........................................................................................................ 29

Figure 3.2 Burst swimming speed (BL/s) of fish exposed to Cu. 4, 8 and 16 refers to days of Cu exposure, H = hard water (100 mg/L CaCO₃), S = soft water (6 mg/L CaCO₃). Low Cu treatments are 6 and 20 µg/L Cu in soft and hard water, respectively. High Cu treatments are 16 and 60 µg/L Cu in soft and hard water, respectively. Box indicates 25th and 75th quartiles, whiskers indicate 10th and 90th percentiles. Medians are indicated by a black line and means are indicated by a +. N=1 for each treatment group. Treatments connected by the same letter are not significantly different (p < 0.05). Tukey’s multiple comparisons test was used to determine differences between treatment groups .................................................. 31

Figure 3.3 Gill Na⁺/K⁺-ATPase activity (µmole ADP/mg protein/h) of fish exposed to Cu. 4, 8 and 16 refers to days of Cu exposure, H = hard water (100 mg/L CaCO₃), S = soft water (6 mg/L CaCO₃). Low Cu treatments are 6 and 20 µg/L Cu in soft and hard water, respectively. High Cu treatments are 16 and 60 µg/L Cu in soft and hard water, respectively. Box indicates 25th and 75th quartiles, whiskers indicate 10th and 90th percentiles. Medians are indicated by a black line and means are indicated by a +. N=2 for each treatment group. Treatments connected by the same letter are not significantly different (p < 0.05). Tukey’s multiple comparisons test was used to determine differences between treatment groups ....... 33
Figure 3.4  Plasma Cl\(^-\) concentration (mmol/L) of fish exposed to Cu. 4, 8 and 16 refers to days of Cu exposure, H = hard water (100 mg/L CaCO\(_3\)), S = soft water (6 mg/L CaCO\(_3\)). Low Cu treatments are 6 and 20 µg/L Cu in soft and hard water, respectively. High Cu treatments are 16 and 60 µg/L Cu in soft and hard water, respectively. Box indicates 25\(^{th}\) and 75\(^{th}\) quartiles, whiskers indicate 10\(^{th}\) and 90\(^{th}\) percentiles. Medians are indicated by a black line and means are indicated by +. N=2 for each treatment group. Treatments connected by the same letter are not significantly different (p < 0.05). Student’s t-test was used to determine differences between treatment groups. ............................. 34

Figure 3.5  Plasma Na\(^+\) ion concentration (mmol/L) of fish exposed to Cu. 4, 8 and 16 refers to days of Cu exposure, H = hard water (100 mg/L CaCO\(_3\)), S = soft water (6 mg/L CaCO\(_3\)). Low Cu treatments are 6 and 20 µg/L Cu in soft and hard water, respectively. High Cu treatments are 16 and 60 µg/L Cu in soft and hard water, respectively. Box indicates 25\(^{th}\) and 75\(^{th}\) quartiles, whiskers indicate 10\(^{th}\) and 90\(^{th}\) percentiles. Medians are indicated by a black line and means are indicated by a +. N=2 for each treatment group. Treatments connected by the same letter are not significantly different (p < 0.05). Tukey’s multiple comparisons test was used to determine differences between treatment groups. ....... 36

Figure 3.6  Plasma osmolality (mmol/kg) of fish exposed to Cu. 4, 8 and 16 refers to days of Cu exposure, H = hard water (100 mg/L CaCO\(_3\)), S = soft water (6 mg/L CaCO\(_3\)). Low Cu treatments are 6 and 20 µg/L Cu in soft and hard water, respectively. High Cu treatments are 16 and 60 µg/L Cu in soft and hard water, respectively. Box indicates 25\(^{th}\) and 75\(^{th}\) quartiles, whiskers indicate 10\(^{th}\) and 90\(^{th}\) percentiles. Medians are indicated by a black line and means are indicated by a +. N=2 for each treatment group. Treatments connected by the same letter are not significantly different (p < 0.05). Student’s t-test was used to determine differences between treatment groups. ................................................................. 37

Figure 3.7  Hematocrit (%) in fish exposed to Cu. 4, 8 and 16 refers to days of Cu exposure, H = hard water (100 mg/L CaCO\(_3\)), S = soft water (6 mg/L CaCO\(_3\)). Low Cu treatments are 6 and 20 µg/L Cu in soft and hard water, respectively. High Cu treatments are 16 and 60 µg/L Cu in soft and hard water, respectively. Box indicates 25\(^{th}\) and 75\(^{th}\) quartiles, whiskers indicate 10\(^{th}\) and 90\(^{th}\) percentiles. Medians are indicated by a black line and means are indicated by a +. N=2 for each treatment group. Treatments connected by the same letter are not significantly different (p < 0.05). ......................................................... 38
Figure 3.8  Plasma cortisol concentration (ng/ml) of fish exposed to Cu. 4, 8 and 16 refers to days of Cu exposure, H = hard water (100 mg/L CaCO$_3$), S = soft water (6 mg/L CaCO$_3$). Low Cu treatments are 6 and 20 µg/L Cu in soft and hard water, respectively. High Cu treatments are 16 and 60 µg/L Cu in soft and hard water, respectively. Box indicates 25$^{th}$ and 75$^{th}$ quartiles, whiskers indicate 10$^{th}$ and 90$^{th}$ percentiles. Medians are indicated by a black line and means are indicated by a +. N=2 for each treatment group. Treatments connected by the same letter are not significantly different (p < 0.05). Tukey’s multiple comparisons test was used to determine differences between treatment groups. ....... 40
# List of Acronyms and Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>m</td>
<td>month</td>
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<tr>
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<td>liter</td>
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<tr>
<td>NKA</td>
<td>Na⁺/K⁺-ATPase</td>
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<td>SFU</td>
<td>Simon Fraser University</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<td>Cu</td>
<td>copper</td>
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Chapter 1.

General Introduction

1.1. The chemistry of Cu

Metals can be classified as being either essential or nonessential. Nonessential metals have no known biological role and include tin (Sn), aluminum (Al), cadmium (Cd), mercury (Hg) and lead (Pb). For these metals, an increase in concentration corresponds to an increase in toxicity. The essential metals have a known biological function and include copper (Cu), zinc (Zn) and iron (Fe) (Kennedy, 2011). Essential metals are incorporated into various proteins and participate in redox reactions (Vaishaly et al., 2015). They are required at low concentration, and deficiencies can result in adverse outcomes. At elevated concentrations these metals can be extremely toxic (Hassan, 2011). Metals can also be classified as either light or heavy (exceeding 50-Da molecular weight), and can be further categorized with respect to their toxicity and essentiality. Class A heavy metals are essential at high concentrations (e.g. Fe) and class B heavy metals are nonessential and are nontoxic at low concentrations (e.g. strontium [Sr]). Class C metals are essential though toxic at high concentrations (e.g. Cu, Zn), and Class D metals are nonessential and toxic at low concentrations (e.g. Hg and Pb) (Kennedy, 2011).

Copper is a heavy metal with a molecular mass of 63.54. It is a transition metal found in Group 1B on the periodic table, existing in four oxidation states (Cu$^0$, Cu$^{1+}$, Cu$^{2+}$, Cu$^{3+}$) with Cu$^{2+}$ being the most common (CCME, 1997). Elemental copper (Cu$^0$) is not oxidized in water, and cuprous copper (Cu$^{1+}$) only exists in water when involved in a complex (Schroeder et al., 1966, Aaseth and Nortseth, 1986). The cupric ion (Cu$^{2+}$) is the ion most commonly found in water, and the trivalent ion (Cu$^{3+}$) does not occur

1.2. Sources

Cu is naturally occurring and a widespread element, found in many minerals and as an uncombined metal (Eisler, 2000). The weathering, oxidation and bacterial breakdown of Cu minerals primarily release Cu$^{2+}$ into the environment (Flemming and Trevors, 1989, Georgopoulos and Roy, 2001). The input of Cu into aquatic systems has increased drastically due to anthropogenic use; freshwater systems can be heavily impacted by industrial discharges and the dissolution of Cu from bedrock (Nriagu, 1979, Buhl and Hamilton, 1990, Martin and Goldblatt, 2007). Sources of industrial discharge include mining, smelting, and the production of leather, textiles, electrical equipment and plumbing fixtures (Spry et al., 1981, Patterson et al., 1998, ATSDR, 1990, Morrissey and Edds, 1994). Residential sources release Cu into aquatic systems as well, primarily via storm water runoff (Davis et al., 2001, McIntyre et al., 2012). This release arises through Cu use in break pads, roofing material, and pesticide and fertilizer formulations used in agriculture (Alva et al., 1995, Eisler, 2000).

1.3. Environmental concentrations

Cu is naturally present in freshwater systems in concentrations ranging from 0.2 to 30 µg/L, though rarely exceeding 5 µg/L (Bowen, 1985). Due to anthropogenic use, Cu concentrations have been reported that greatly exceed background levels. Freshwater systems receiving anthropogenic input have reported concentrations ranging from background to 100 µg/L, although areas of urban and highway runoff can reach concentrations as high as 200 µg/L (Davis et al., 2001, WHO, 2004, Martin and Goldblatt, 2007). In areas of extreme contamination (e.g. mining areas), concentrations up to 200 mg/L have been reported (Rock et al., 1985, Davis and Ashenberg, 1989).
1.4. **Cu chemistry in water**

In freshwater systems, free Cu is primarily found in its most stable state, as the cupric ion (Cu$^{2+}$), which can form complexes with many other compounds. It may associate with water to form the aquo ion [Cu(H$_2$O)$_6$]$^{2+}$, with organic or inorganic ligands, or adsorb onto organic particulates (e.g. humic and fulvic acids), clays and sediments (Spear and Pierce, 1979, Eisler, 2000, Georgopoulos and Roy, 2001). The majority of Cu in waterbodies partitions to sediments, with Cu binding being dependent primarily on particle size, although the type of sediment and redox potential are also important (Singleton, 1987). Fine-grained sediments typically contain a higher Cu concentration than coarse-grained sediments, due to the larger surface area of small particulates (Stancil, 1980, Erickson et al., 1996).

In freshwater, Cu solubility is modified by pH, hardness and temperature (Nriagu, 1979). Under acidic conditions (pH < 5.0) the cupric ion (Cu$^{2+}$) is the major species present, and as water pH decreases, Cu carbamates (e.g. CuCO$_3$, Cu(CO$_3$)$_2^{2-}$) and copper hydroxides (eg. CuOH$^+$) predominate (Spear and Pierce, 1979). The chemical form of Cu in freshwater is crucial for determining the toxicity of Cu to organisms as the cupric ion and Cu hydroxyl species are highly toxic to aquatic life, while the carbonate species (e.g. CuHCO$_3^+$, CuCO$_3$, Cu(CO$_3$)$_2^{2-}$) are far less toxic (Meador, 1991).

1.5. **Cu bioavailability and modifying factors**

The toxicity of a metal is dependent on its potential to be taken up from the environment and the resulting internal dose. Metal uptake rates and accumulation is concentration-dependent and varies with metal species and their properties. Bioavailability is defined as the fraction of a chemical that is transferred from the environment into an organism, making it available for distribution to target tissues and organs (USEPA, 2003a; USEPA, 2007). The differing uptake rates of each Cu species will affect Cu bioavailability and resulting accumulations at target sites vary accordingly. As well, the environmental concentrations of other metals or compounds that share the same uptake mechanism or surface membrane target sites can result in competition and reduce uptake, and therefore modify toxicity.
The properties of water (including pH and temperature) can affect Cu solubility, uptake, and toxicity. Several other physical and chemical characteristics of water may also affect Cu bioavailability and toxicity, and include alkalinity and the concentrations of dissolved organic carbon compounds, suspended particulates, and inorganic cations and anions (including those that contribute to water hardness (Meador, 1991). Of these, pH is the most important for Cu speciation (Meador, 1991). The effect of pH on speciation is due to Cu’s interaction with hydrogen ions (Peterson et al., 1984). Concentrations of free Cu decrease roughly by one order of magnitude for every 0.5 increase in pH above pH 6 (Stumm and Morgan, 1981, Meador, 1991). Increasing dissolved organic carbon (DOC) decreases Cu availability due to complexation with the metal ions (Winner, 1985). Alkalinity has also been shown to decrease Cu bioavailability (Chakoumakos et al., 1979). Research shows that Cu bound to organic ligands is not bioavailable, therefore Cu toxicity is attributed to dissolved forms of Cu that are present in the aquatic environment (Sprague, 1968, Chakoumakos et al., 1979, Erickson et al., 1996, Eisler, 2000).

Water hardness is a measure of dissolved polyvalent metallic ions, primarily calcium and magnesium, though strontium, manganese, iron and barium can also contribute (USEPA, 1976). Hardness is usually expressed as the concentration of calcium carbonate (CaCO₃) in the solution. In harder waters, Ca²⁺ ions can be at concentrations that outcompete metals for binding sites on the gills (one of the primary uptake sites in fish), thus affecting their bioavailability and uptake of metals (Laurén and McDonald, 1986, Saglam et al., 2013).

The biotic ligand model (BLM) has been used to predict the bioavailability of metals and their accumulation at target sites, by taking into account the major factors that affect metal bioavailability including DOC concentrations and water hardness. As will be addressed in the section below on mechanism of action, the gills are the primary target site of Cu toxicity and the site of the BLM. The BLM is derived from two earlier models, the free-ion-activity model ([FIAM] Morel, 1983), and the gill surface interaction model ([GSIM] Pagenkopf, 1983) (USEPA, 2003b). The FIAM states that the chemical activity of the aquo ion of a given metal is a better predictor of its toxicity than the total dissolved concentration. This model is considered limited as it does not take
interactions between organic and inorganic complexes and the biotic ligand into account (Meyer et al., 2007). The GSIM estimates the concentration of a metal that accumulates on the gill surface based on the competitive binding of various cations (e.g. Ca^{2+}, Mg^{2+}, H^+) (Meyer et al., 2007). The BLM integrates components from these two earlier models and is generally based on the hypothesis that metal bioavailability is not only determined by the total metal concentration, but is related to the physical and chemical properties of the water that affect the metal bioavailability at the target site (Di Toro et al, 2001). Metal toxicity is a result of free metal ions interacting with binding sites in the target tissue (i.e. the gill surface). Organic (e.g. DOC) and inorganic (e.g. OH^-, HCO_3^- and Cl^-) complexes can form with metals and render them inert, and cations (e.g. Ca^{2+} and H^+) can compete with free metal ions for binding at the target sites (Figure 1.1).
1.6. Toxicokinetics

By acting as a cofactor for numerous proteins, Cu is an essential heavy metal and is required in low concentrations, within the range of 1 to 4 mg Cu/kg (dry mass) in teleost fish (Lanno et al., 1985b, Bury et al., 2003). At elevated concentrations, Cu is a potent toxicant, and so body Cu concentrations are subject to tight homeostatic control to prevent both deficiency and toxicity (Kamunde et al., 2002b).

Teleost fish, including rainbow trout, can obtain Cu either from their diet or from water. Metals are present in their diet since they can be incorporated in prey organisms (i.e. as metals bound in proteins or other organic molecules such as chitin) (Bryan, 1976; Clearwater, et al., 2002). The uptake of Cu by teleosts following dietary exposure occurs across the pyloric caeca and intestines (Handy et al., 1999). The molecular mechanism of uptake has not been elucidated, however evidence supports its similarity with that which occurs in mammalian systems (Handy, 1996; Handy et al., 2000).
Potential uptake pathways include entering enterocytes from the mucosal side by a non-specific, ATP driven metal-ion transporter, DCT1 (Gunshin et al., 1997), a Cu transporter, CTR1 (Dancis et al., 1994, Zhou and Gitschier, 1997) or by passive diffusion through Na$^+$ channels (Wapnir, 1991). On the basolateral membrane of enterocytes, a Cu-ATPase or Cu/anion symporter are thought to be involved in Cu uptake (Harrison and Dameron, 1999, Handy et al., 2000).

The observation that elevated aqueous Cu concentrations leads to increased Cu concentrations in the gills provided the first evidence that the gills served as a Cu uptake route (Buckley et al., 1982). Prior to transport across membranes, Cu$^{2+}$ is reduced to Cu$^+$, potentially via a copper reductase (Bury et al., 2003). Grosell and Wood (2002) identified two apical branchial uptake mechanisms in rainbow trout: a Na$^+$-sensitive and a Na$^+$-insensitive pathway. Data suggests that Cu shares a similar uptake pathway as Na$^+$ since elevated aqueous Cu concentrations also results in impaired branchial Na$^+$ uptake (via the Na$^+$-sensitive pathway (Laurén and McDonald, 1985). This occurs through an epithelial Na$^+$ channel (ENaC) or an H$^+$-ATPase-coupled Na$^+$ (NHE3) channel (Fenwick et al., 1999; Fig 1.2). This pathway is likely as Cu uptake is reduced following the administration of phenamil, a known ENaC inhibitor (Kleymann and Cragoe, 1988). Additionally, silver (Ag$^+$), a known Cu analogue is taken up into fish by the apical Na$^+$ pathway, further supporting the Na$^+$-sensitive pathway (Bury and Wood, 1999). Na$^+$-insensitive uptake is due to a high-affinity Cu importer, the Ctr family of proteins (Grosell and Wood, 2002). After entering epithelial cells, Cu can bind with metallochaperones, bringing it to the Golgi network. Cu is then incorporated into metal binding proteins and brought to the basolateral membrane for release into plasma via exocytosis (Bury et al., 2003). On the basolateral membrane, if Cu remains free (i.e. doesn't enter the aforementioned pathway in the Golgi), Cu enters the interstitial fluid via Na$^+$/K$^+$-ATPase (NKA) pumps (Wood, 2001).
Following uptake, Cu is transported in plasma bound to either ceruloplasmin, a plasma protein that binds tightly and with high affinity to Cu, or albumin and other Cu binding amino acids, which bind less tightly and are believed to transport Cu from uptake sites (Gubbler et al., 1953; Marceau and Aspin, 1973; Grosell et al., 1998). In fish the liver is the primary organ involved in Cu homeostasis (Grosell et al., 2000; Kamunde et al., 2002a). The liver accumulates Cu absorbed from either dietary or branchial
exposure, and is the site of ceruloplasmin synthesis (Bury et al., 2003). The accumulation of Cu in internal organs varies with the route of exposure and Cu concentration (Clearwater et al., 2002). Dietary uptake initially results in accumulation in the intestines and liver, and eventually (after 3 d to 1 w) is found throughout the body. The highest concentrations are reported in the intestines, liver and gall bladder (in bile) and in lower concentration in the gills, muscle tissue and kidney (Lanno et al., 1985a; Clearwater et al., 2000; Kamunde et al., 2001). Waterborne Cu entering through the gills achieve higher Cu concentrations in the gills and are lower throughout the gastrointestinal (GI) tract (Clearwater et al., 2002).

Detoxification mechanisms for Cu primarily involve inducing metallothioneins, allowing long-term retention after absorption without eliciting a toxic effect (Harrison, 1986; Carbonell and Tarazona, 1994). Metallothioneins (MT) are metal binding proteins that help to regulate free metal ion concentrations by binding with essential metals such as Cu and Zn. An increase in intracellular metal concentration induces MT production to allow for binding and sequestration of excess metals (above essential concentrations) (De Boeck et al., 2003). Differing capacities of MT induction is one potential explanation for the varying tolerance to metals in fish (Olsson and Kille, 1997). As the liver is the major detoxification organ in fish, it accumulates the highest levels of MTs in the body (Hauser-Davis et al., 2014).

Fecal and hepatobiliary excretion are the two primary routes for Cu elimination in fish, though branchial and renal excretion also occur (Clearwater et al., 2002). Within the intestines, free Cu increases mucus production. This was determined using everted gut sacs; when Cu was added to the incubation media, mucus production increased and sloughed off the tissue (Handy et al., 2000). Mucus can complex with Cu, essentially “trapping it” and allowing for fecal elimination (Clearwater et al., 2002). Handy et al. (2000) found approximately 76% of Cu formed complexes with mucus. Cu exposure increases the turnover rate and apoptosis of enterocytes, increasing the number of cells sloughing off in the GI tract, possibly to increase fecal elimination in fish (Kamunde et al., 2001). While renal excretion in fish does occur, the loss of Cu via urine is much lower than in bile (Grosell et al., 1988). Research by Grosell et al. (2001) showed that some Cu may also be lost via the gills, although the mechanism is still unknown.
1.7. Mechanism of action

As explained above, Cu is essential for cellular metabolism at low concentrations, but at higher concentrations is extremely toxic (Hassan, 2011). The specific toxic mechanism of action of Cu, however, is not fully elucidated. In general, metals affect the respiratory and osmoregulatory (i.e. water and ion balance) systems of teleost fish at both the physiological and biochemical levels, with the gills being the primary target organ (Beaumont et al, 1995; Mazon, 2002; Saglam et al, 2013). This occurs through physical changes to the gill epithelia and by affecting the activity of ion ATPases (Laurén and McDonald, 1985; Wilson and Taylor, 1993a; Beaumont et al, 1995).

Gills are particularly vulnerable to waterborne pollutants that can cause significant structural changes to the epithelial cells. Both epithelial cells and mucocytes (mucus producing cells) of trout can undergo hyperplasia (cell proliferation) following metal exposure (Beaumont et al., 2003). Mucus production is a general defence mechanism against heavy metal exposure, as the mucus binds to metals and decreases their rate of diffusion across the gills (Pärt and Lock, 1983; De Boeck et al., 1995). Additionally, epithelial cells may swell and lift off the basement membrane, in addition to the fusion or curling of gill lamellae upon exposure to metals such as Cu (Wilson and Taylor, 1993a; Karan et al., 1998; Beaumont et al., 2003). Epithelial lifting and hyperplasia are thought to be general defense mechanisms, increasing the diffusion distance between aqueous toxicants and the blood (Karan et al., 1998). Lamellar fusion and curling is likely due to heavy metal cations disrupting the charges on glycoproteins located in the plasma membrane causing attraction between cells of adjacent lamellae (Daoust et al., 1984). While hypothesized to be a general defense mechanism this is likely a toxic effect from metal exposure. These effects all serve to disrupt the gill’s effectiveness as a gas and ion exchange organ, through increasing the gas diffusion distances and decreasing the rate of oxygen uptake (De Boeck et al., 2006). This can be exacerbated during periods of exercise; Cu exposure (50-250 mg/L CaCO\textsubscript{3}) results in reduced oxygen consumption (from 30-60% reduction) during swimming performance trials in trout (Beaumont et al., 2003, McKenzie et al., 2003, De Boeck et al., 2006).
As previously mentioned, Cu shares an uptake pathway with Na⁺; it acts as a Na⁺ analogue and can out-compete Na⁺ for Na⁺/K⁺-ATPase transports that are found in the both the gills and GI tract of fish including rainbow trout (Clearwater et al., 2002, De Boeck et al, 2007). Disruption of Na⁺/K⁺-ATPase transports following Cu exposure reduces ion uptake in freshwater fish. The opposite occurs in saltwater fish where Cu exposure causes an increase in ion uptake resulting in ionoregulatory (i.e. ion balance) failure (Wood, 2001, Grosell et al., 2002, Grosell et al., 2004). Cu exposure primarily affects Na⁺ homeostasis via the disruption ion transporters; Na⁺ and Cl⁻ influx is inhibited in fresh water fish, including rainbow trout (Grosell et al., 2002, Taylor et al, 2003). Chronically impaired ion transporters from Cu exposure are unable to maintain Na⁺ homeostasis, which can result in ionoregulatory failure. This involves reduced ion concentrations decreasing plasma volume (and therefore it’s viscosity), which decreases oxygen transport and heart rates, which can eventually lead to death (species include rainbow trout and fathead minnows [Pimephales promelas]) (Reid and McDonald, 1991, Wilson and Taylor, 1993a, Kolok et al., 2002).

1.8. Acute toxicity

Cu is acutely toxic to fish, with LC50 values typically ranging from 10-1000 µg/L (Eisler, 2000). This broad range of values is due to biotic factors (e.g. age, species and size) and abiotic factors related to water quality (e.g. hardness, pH, DOC, temperature) (Spear and Pierce, 1979, Pilgaard et al., 1994, Gensemer et al., 2002). In soft freshwater, Cu is most acutely toxic with LC50 values between 10 and 20 µg/L (NAS 1977). Generally as alkalinity and hardness increase there is a decrease in Cu’s acute toxicity, as complexes with carbonates are formed or competition with Ca²⁺ cations for uptake at the gill occurs (Meyer et al., 2007). As a result, metal exposures in soft water are more toxic than exposures in hard water. In all the taxonomic groups of fish studied to date, there is an increase in LC50 values as water hardness increases (Spear and Pierce, 1979, Laurén and McDonald, 1985). In soft water (30 mg/L CaCO₃), the 96-h LC50 for rainbow trout is between 20 and 30 µg/L (Howarth and Sprague, 1978). The 96-h LC50 rainbow trout exposed to Cu increases as hardness increases; for example the 96-h LC50 is 90 µg/L in 120 mg/L CaCO₃ (Taylor et al., 2000).
Cu binds to DOC present in solution, rendering the free metal inert; Cu toxicity therefore decreases as DOC increases (Brown et al., 1974). Waterborne metals including Na\(^+\) and cobalt can also protect against acute lethal Cu toxicity (Spear and Pierce, 1979, Marr et al., 1998). Temperature has a variable effect on Cu toxicity. Cairns et al. (1978) reported an increase in toxicity with a temperature increase from 5 to 30 °C whereas Hansen et al. (2002) reported a decrease in toxicity as temperature increased (from 8 to 16 °C). Contrary to this, Carvalho and Fernandes (2006) reported no effect of changing temperature on the acute toxicity of Cu.

Fish species vary in their sensitivity to Cu in acute tests (as measured by LC50 values). Salmonids (e.g. salmon and trout) appear to be the most sensitive, followed by cyprinids (e.g. carps and minnows); anguillids (e.g. eels), centrarchids (e.g. bass) and percids (e.g. perch) are the most resistant families of fish (Spear and Pierce, 1979). A summary table of LC50 values for fish are found below in Table 1.1.

Table 1.1. Summary of LC50 values across a range of species. N/A indicates information not available. Relatable indicates whether study is relevant to the present research (based on duration, life stage and other variables). (Modified from: Eisler, 2000)

<table>
<thead>
<tr>
<th>Taxonomic group, organism species</th>
<th>Cu concentration of LC50</th>
<th>Duration</th>
<th>Life stage/age</th>
<th>Other variables</th>
<th>Relatable</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Topsmelt, Atherinops affinis</td>
<td>238 µg/L</td>
<td>96 h</td>
<td>Larvae</td>
<td>n/a</td>
<td>No</td>
<td>Anderson et al., 1991</td>
</tr>
<tr>
<td>Topsmelt, Atherinops affinis</td>
<td>365 µg/L</td>
<td>7 d</td>
<td>Larvae</td>
<td>n/a</td>
<td>No</td>
<td>McNulty et al., 1994</td>
</tr>
<tr>
<td>Goldfish, Carassius auratus</td>
<td>36 µg/L</td>
<td>96 h</td>
<td>n/a</td>
<td>20 mg/L CaCO(_3)</td>
<td>No</td>
<td>USEPA, 1980</td>
</tr>
<tr>
<td>Goldfish, Carassius auratus</td>
<td>300 µg/L</td>
<td>96 h</td>
<td>n/a</td>
<td>52 mg/L CaCO(_3)</td>
<td>No</td>
<td>USEPA, 1980</td>
</tr>
<tr>
<td>Catfish, Clarias sp.</td>
<td>425 µg/L</td>
<td>96 h</td>
<td>n/a</td>
<td>Nonresistant strain of fish</td>
<td>No</td>
<td>Daramola and Oladimeji, 1980</td>
</tr>
<tr>
<td>Species</td>
<td>LC50 (µg/L)</td>
<td>Exposure Time</td>
<td>Life Stage</td>
<td>Temperature</td>
<td>CaCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Results</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>-------------</td>
<td>---------------</td>
<td>------------</td>
<td>-------------</td>
<td>-----------------</td>
<td>---------</td>
</tr>
<tr>
<td>African sharp-tooth catfish, <em>Clarias gariepinus</em></td>
<td>1240</td>
<td>96 h</td>
<td>Juvenile</td>
<td>Between 21-28 °C</td>
<td>No</td>
<td>Van der Merwe et al., 1993</td>
</tr>
<tr>
<td>Pacific herring, <em>Clupea harengus pallasi</em></td>
<td>33</td>
<td>6 d</td>
<td>Embryo</td>
<td>n/a</td>
<td>No</td>
<td>USEPA, 1980</td>
</tr>
<tr>
<td>Mummichog, <em>Fundulus heteroclitus</em></td>
<td>8000</td>
<td>96 h</td>
<td>n/a</td>
<td>n/a</td>
<td>No</td>
<td>Lin and Dunson, 1993</td>
</tr>
<tr>
<td>Brown bullhead, <em>Ictalurus nebulosus</em></td>
<td>170</td>
<td>96 h</td>
<td>Juvenile</td>
<td>n/a</td>
<td>No</td>
<td>Brungs et al., 1973</td>
</tr>
<tr>
<td>Bluegill, <em>Lepomis macrochirus</em></td>
<td>1100</td>
<td>96 h</td>
<td>Larvae</td>
<td>n/a</td>
<td>No</td>
<td>Benoit, 1975</td>
</tr>
<tr>
<td>Tidewater silverside, <em>Menidia peninsulae</em></td>
<td>140</td>
<td>96 h</td>
<td>Larvae</td>
<td>n/a</td>
<td>No</td>
<td>Mayer, 1987</td>
</tr>
<tr>
<td>Striped bass, <em>Morone saxatilis</em></td>
<td>150</td>
<td>96 h</td>
<td>Fingerling</td>
<td>68 mg/L CaCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>No</td>
<td>USEPA, 1980</td>
</tr>
<tr>
<td>Cutthroat trout, <em>Oncorhynchus clarki</em></td>
<td>37</td>
<td>96 h</td>
<td>n/a</td>
<td>18 mg/L CaCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Yes</td>
<td>USEPA, 1980</td>
</tr>
<tr>
<td>Cutthroat trout, <em>Oncorhynchus clarki</em></td>
<td>232</td>
<td>96 h</td>
<td>n/a</td>
<td>204 mg/L CaCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>No</td>
<td>USEPA, 1980</td>
</tr>
<tr>
<td>Coho salmon, <em>Oncorhynchus kisutch</em></td>
<td>31.9</td>
<td>96 h</td>
<td>Juvenile</td>
<td>n/a</td>
<td>Yes</td>
<td>Buhi and Hamilton, 1990</td>
</tr>
<tr>
<td>Rainbow trout, <em>Oncorhynchus mykiss</em></td>
<td>13.8</td>
<td>96 h</td>
<td>Juvenile</td>
<td>n/a</td>
<td>Yes</td>
<td>Buhi and Hamilton, 1990</td>
</tr>
<tr>
<td>Rainbow trout, <em>Oncorhynchus mykiss</em></td>
<td>20</td>
<td>96 h</td>
<td>n/a</td>
<td>32 mg/L CaCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Yes</td>
<td>USEPA, 1980</td>
</tr>
<tr>
<td>Rainbow trout, <em>Oncorhynchus mykiss</em></td>
<td>514</td>
<td>96 h</td>
<td>n/a</td>
<td>370 mg/L CaCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>No</td>
<td>USEPA, 1980</td>
</tr>
<tr>
<td>Fathead minnow, <em>Pimephales promelas</em></td>
<td>23</td>
<td>96 h</td>
<td>n/a</td>
<td>20 mg/L CaCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>No</td>
<td>USEPA, 1980</td>
</tr>
</tbody>
</table>
1.9. Sublethal toxicity

In addition to being acutely toxic to fish, a multitude of effects occur in fish exposed to sublethal concentrations of Cu. At concentrations as low as 4 µg/L, effects on growth and metabolism can occur, and at around 10 µg/L reproduction and survival can be affected in sensitive species (Hodson et al., 1979). Copper has been shown to reduce disease resistance (Anderson, 1990, Dethloff and Bailey, 1998), alter migration (Lorz and McPherson, 1977), impair respiration and osmoregulation (Clearwater et al., 2002, Grosell et al., 2002, Taylor et al, 2003, De Boeck et al, 2007), cause tissue structure changes to the gills (Wilson and Taylor, 1993a, Karan et al., 1998, Beaumont et al., 2003), disrupt lateral line mechanoreception (Linbo et al., 2006, Webb et al., 2014), alter swimming performance (Beaumont et al., 1995, Kolok et al., 2002, Waser et al., 2009), impair olfaction (Palm and Powell, 2010, McIntyre et al., 2012) and alter blood chemistry (Christensen et al., 1972, Heath, 1995).

The primary target site of sublethal Cu toxicity fish is the gills, more specifically the ion transport ATPases (Hansen et al., 1993). As discussed in the section on mechanisms of action, Cu exposure alters ion homeostasis, primarily via disruption of the Na⁺/K⁺-ATPases. This occurs either by Cu acting as a Na⁺ analogue and out-competing Na⁺ for ATPases, or by Cu binding and inhibiting the ATPase pump (Laurén and McDonald, 1986, De Boeck et al, 2007). The overall result is an impaired ion exchange system with altered ion concentrations in fish. As a result of the altered ion transport activity, Cu exposure in freshwater generally results in decreased plasma osmolality, decreased plasma ion concentrations (e.g. Na⁺ and Cl⁻) and increased hematocrit. (Lewis and Lewis, 1971, Christensen et al., 1972, Courtois, 1976). Hematocrit is inversely related to plasma volume; a change to plasma ion concentrations can change plasma volume and therefore hematocrit (Brauner et al., 1994).
Fish growth can be used as a sensitive and reliable endpoint in sublethal toxicity testing, due to decreases in food intake or increases in metabolic demands due to detoxification processes (De Boeck et al., 1997). Cu exposure has been shown to decrease appetite and alter energy stores (fat and glycogen), leading to a reduction in growth in various fish species (Cu exposure concentrations vary based on species) (Benoit, 1975, Buckley et al., 1982, De Boeck et al., 1997). Additionally, hematological evaluation of fish blood can provide information about the physiological responses to changes in the environment. As mentioned, altered plasma ion concentrations are reported due to Cu’s ability to alter the activity of ion transport pumps. Cu exposure decreases red blood cell numbers: hemoglobin, mean corpuscular volume (MCV; average volume of red cells) and can increase white blood cell numbers (McLeay, 1973, Larsson et al., 1985, Van Vuren et al., 1994). Blood glucose, lactate, bilirubin, urea, cholesterol and acetylcholine increase in response to Cu exposures (Christensen et al., 1972, Schrek and Lorz, 1978, Oikari and Nakari, 1982, Nemsock and Hughes, 1988, Singh and Reddy, 1990, Heath, 1995). The mechanism underlying the alteration of energy stores and haematological parameters is not fully elucidated to date. It is likely due to the release of stress hormones including cortisol, inducing the secondary stress response to provide physiological compensation during periods of stress, including Cu exposure (Mazeaud et al., 1977, MacFarlane and Benville, 1986, De Boeck et al., 1997).

Activities reliant on swimming performance include migration, prey capture, spawning, predator avoidance and maintenance of position in a current (Nikl & Farrel, 1993, Reidy et al., 1995). The effects of Cu on critical swimming speed is well documented in many fish species including rainbow trout, brown trout and fathead minnows (Beaumont et al., 1995, Kolok et al., 2002, Waser et al., 2009). Exposure to Cu results in a significant reduction in maximum and sustained swimming speeds, which is hypothesized to be caused by alterations to the gill structure or an increase in ammonium production (Beaumont et al., 1995, Beaumont et al., 2003, van Heerden et al., 2004, De Boeck et al., 2006).

Cu can also affect the peripheral olfactory system; olfactory neurons are continuously exposed to water and are therefore highly vulnerable to contaminants (McIntyre et al., 2012). Scent detection in fish is important as chemical cues released
either by conspecifics or other organisms can be crucial to survival, including the
detection of food, conspecific alarm scents or predator presence (Beyers and Farmer,
2001, Baldwin et al., 2003, Palm and Powell, 2010). In addition, Cu can interfere with
fish migration by either disrupting their ability to detect their natal stream or altering the
odour of the stream (Hasler and Scholtz, 1983).

Cu can affect the immune system of fish. Cu has been show to increase
infection and death rates by suppressing resistance to both viral and bacterial pathogens
(Hetrick et al., 1979, Rougier et al., 1992, Rougier et al., 1994). Fish exposed to 40 µg/L
Cu required fewer pathogens to induce a fatal infection (Baker et al., 1983). Macrohage
function including phagocytosis is affected, impairing the first line of
defense against foreign matter in the body (Secombes and Fletcher, 1992). The effects
on blastogenic (i.e. increase in number) and antibody-production response of
lymphocytes (i.e. white blood cells) is mixed (Cu range of 5-30 µg/L), with both
increases and decreases reported, affecting the magnitude of humoral and cell-mediated
immunity (Khangarot and Tripathi, 1991, Roche and Boge, 1993, Dethloff and Bailey,
1998). The mechanism has not been elucidated to date.

The lateral line is a sensory organ that receives and integrates environmental
stimuli (e.g. water flow and pressure changes) (Webb, 2014). It is comprised of
neuromasts that have the mechanosensory hair cells, containing cilia to detect water
movement (Webb, 2014). Exposure to low Cu concentrations (10-635 µg/L) for short
durations (3 h) is reported to be toxic to hair cells (Hernandez et al., 2006, Linbo et al.,
2006). Initially the number of hair cells diminishes, followed by complete elimination of
all hair cells; at higher concentrations the accessory cells and regeneration ability of
neuromasts are also compromised (Hernández et al., 2006, Linbo et al., 2006, Linbo et
al., 2009). Other metals do not elicit similar effects to the lateral line, so it appears to be
particularly susceptible to Cu exposure (Hernandez et al., 2006). The information
provided by the lateral line is used for prey acquisition, predator avoidance and
schooling behaviours including navigation and communication: destruction of the hair
cells can have profound effects (Webb, 2014).
There is limited data, and with mixed results, relating Cu exposure to reproductive endpoints, though generally reproductive success decreases. In one study, during a 22 min exposure, concentrations of 32.5 µg/L affected the number of viable eggs produced and hatchability in the brook trout (*Salvelinus fontinalis*). Cu concentrations of 17 µg/L affected the survival and growth of alevins and juvenile trout, and adult survival and growth was diminished at 32.5 µg/L (McKim and Benoit, 1971). This is supported by research by Hodson et al. (1979) who reported effects on survival and reproduction at 20 µg/L. Another study conducted over two generations in brook trout reported no effects on reproductive endpoints at Cu concentrations up to 9.4 µg/L (McKim and Benoit, 1974). During a chronic exposure (22 months) spawning success measured as number of eggs of adult bluegill (*Lepomis macrochirus*) was reduced to 0 (no eggs survived) at 162 µg/L Cu (Benoit, 1975).

1.10. Sublethal endpoints

Sublethal effects of pollutants can be detected using various endpoints including those at higher levels of biological organization such as swim performance and behaviour (Beaumont et al., 1995). While not detected in standard acute toxicity tests (e.g. LC50), sublethal toxicity can have severe effects on a fish’s ability to perform essential life functions (e.g. swim, procure food, avoid predators and reproduce), which can then affect survival at the population level (Smith and Weis, 1997). Whole animal assays are useful for detecting sublethal effects of stressors as they may integrate multiple physiological systems; effects on any subcomponent may result in changes in whole animal performance.

Swimming performance has been widely used to detect the sublethal effects of environmental contaminants (Beaumont et al., 1995, Jain et al., 1998, Kolok et al., 2002, Tierney et al., 2007, Waser et al., 2009, Goulding et al., 2013). It can be studied relatively easily in lab using a swim tunnel to measure swimming speed. Swimming performance is organized into three categories: sustained, prolonged or burst, and various exercise protocols have been established to evaluate each type of swimming. These typically measure the swimming speed achieved within a set interval of time (min or h) after experiencing water velocity changes (Hammer, 1995, Plaut, 2001). Sustained
swimming is the velocity that a fish can maintain for a long period of time (>200 min) and is typically used for foraging and migration behaviour, as these only require low velocities. Sustained swimming is measured as the maximum speed sustained or $U_{\text{max}}$ (Sepulveda and Dickson, 2000). Prolonged swimming is shorter in duration than sustained swimming and is the most commonly studied (Brett, 1964). A common measure of prolonged swimming is critical swimming speed ($U_{\text{crit}}$) (Brauner et al., 1994, Reidy et al., 1995, MacNutt et al., 2004, Kennedy and Farrell, 2006, Farrell, 2008). Sustained and critical swimming speed have been extensively used, particularly with salmonids, to assess the impact of environmental factors including ammonia, crude oil, hypoxia, pesticides, metals, pulp and paper effluents, and temperature (Brett and Glass, 1973, Beamish, 1978, Waiwood and Beamish, 1978, Kumaraguru and Beamish, 1981, Thomas and Rice, 1987, Nikl et al., 1993, Beaumont et al., 1995, Kennedy et al., 1995, Shingles et al., 2001, Kolok et al., 2002, Wicks et al., 2002, Kennedy and Farrell, 2006, Landman et al., 2006, Waser et al., 2009, Goulding et al., 2013, Yu et al., 2015).

Burst swimming is the maximum velocity a fish can maintain for a short period of time, involving rapid sprint swimming (Nadeau et al., 2009). It is typically used for predator avoidance and catching prey and typically lasts for up to 20 s. Burst swimming uses anaerobic metabolism and results in fatigue for the fish. These swimming assays involve a constant acceleration in the test chamber and measure the burst swimming speed ($U_{\text{burst}}$) (Reidy et al., 1995, Pedersen & Malte, 2004, Farrell, 2008, Pedersen et al., 2008, Nadeau et al., 2009). Far less research has been conducted assessing the impact of environmental factors using burst swimming as the endpoint. Contaminants studied to date include ammonia, pesticides, temperature and xenoestrogens (Tudorache et al., 2010, Goulding et al., 2013, Osachoff et al., 2014).

As described in detail earlier (Mechanism of Action), the gill epithelium is the primary osmoregulatory organ in fish, with the gastrointestinal tract and kidneys also contributing (Wendelaar Bonga and Lock, 2008). Freshwater fish absorb ions from water at the gills, reduce ion loss by decreasing paracellular ion movement at the gills and absorb ions at the kidneys to reduce ion loss via urine (Evans et al., 2005). Gills are composed of a variety of cell types, though the mitochondrion-rich (MR) cells (e.g. chloride cells) are directly involved in osmoregulation. These cells are associated with
ion uptake in fish as they are the location of ion transport proteins on both the apical and basolateral membranes (Chang et al., 2001, Hwang and Lee, 2007). Exposure to toxicants can damage MR cell integrity (structural changes, necrosis) or alter ion transport protein function, which can be detected using protein activity assays (Wendelaar Bonga and Lock, 2008). Disrupted ion transporters can disturb ion permeability, which will eventually lead to alterations in plasma ion levels and osmolality (Wendelaar Bonga and Lock, 2008). Changes to ion ATPases (e.g. Na⁺/K⁺-ATPase and Ca²⁺-ATPase) and plasma ion concentrations have been demonstrated in many fish species following exposure to metals and other contaminants including: ammonia, pesticides, polycyclic aromatic hydrocarbons, pulp and paper mill effluents and xenoestrogens (Davis and Wedemeyer, 1971, Dangé, 1986, Laurén and McDonald, 1987, Schoenmakers et al., 1992, Pratap and Wendelaar Bonga, 1993, Twitchen and Eddy, 1994, Tuvikene, 1995, Li et al., 1998, Berntsson et al., 1999, Pane et al., 2003, Rogers et al., 2003, Matsuo et al., 2004, Parvez et al., 2006, Lerner et al., 2007, Firat et al., 2011).

1.1.1. Research objectives

The overall aim of this study was to further examine the effects of Cu on osmoregulation and swimming performance in rainbow trout. While the effects of Cu on critical swimming speed in several species has been examined, its effects on burst swimming have not been investigated. The importance of water quality to Cu toxicity suggests that a link between alterations in swimming performance and osmoregulation will exist. Endpoints at both the biochemical and whole animal levels of organization were selected to assess these relationships. Juvenile rainbow trout were selected due to increased species and life stage sensitivity to Cu exposure. As well, experiments were performed to examine the effects of exposure duration on the magnitude of Cu toxicity.
Chapter 2.

Materials and Methods

2.1. Chemicals

Copper chloride (CuCl₂·2H₂O) was purchased from Thermo Fisher Scientific (Mississauga, ON) and the various salts used to synthetically harden water (CaSO₄·½H₂O; CaCl₂; MgCl₂·6H₂O; KCl, NaCl, NaHCO₃) were purchased from Sigma-Aldrich; (Oakville, ON). Ferric nitrate (Fe(NO₃)₃) and mercuric thiocyanate (Hg(SCN)₂) for plasma chloride assays were purchased from Sigma-Aldrich (Oakville, ON). Sodium deoxycholate, lactate dehydrogenase, pyruvate kinase, phosphoenolpyruvate, NADH, ATP, ouabain and ADP were purchased from Sigma-Aldrich (Oakville, ON). Tricaine methanesulfonate (MS222) was purchased from Syndel International Inc. (Vancouver, BC).

2.2. Fish

Juvenile rainbow trout (Onchorhynchus. mykiss) with a fork length of 11.34 ± 0.07 cm (mean ± SEM) and a mass of 16.35 ± 0.35 g of mixed sex in an undetermined ratio were obtained from Miracle Springs Hatchery (Mission, BC). Fish were maintained in 500 L tanks, on a 12 h light:12 h dark photoperiod, supplied with dechlorinated municipal water (oxygen saturation > 90%, water hardness 6.3 mg/L CaCO₃ and pH ~7.0 ± 0.2) at 8 ± 1 °C. Fish were acclimated for a minimum of 2 weeks prior to starting any experiment. Fish were fed ad libitum daily but were fasted for approximately 24 h prior to use. Fish use and experimental protocols were approved by the Simon Fraser University Animal Care Committee in accordance with Canadian Council on Animal Care (CCAC) guidelines.
2.3. Acute toxicity tests

An initial 96-h LC50 test was conducted following the Environment Canada protocol (EPS 1/RM/9, 1990) to determine the sublethal Cu concentrations for use in subsequent experiments. Exposures were conducted in 170 L aerated fiberglass tanks on a 12 h light:12 h dark photoperiod, and fish acclimated for approximately 24 h in the exposure tanks in either hard or soft water prior to the addition of CuCl_2. Fish (n=10) in each treatment were exposed in duplicate tanks (n=5 per tank) to a range of Cu concentrations in both hard (100 mg/L CaCO_3, 0, 12.5, 25, 50, 100, 200, 250, 300 µg/L Cu) and soft water (6 mg/L CaCO_3, 0, 6.25, 12.5, 25, 50, 100 µg/L Cu). Fish mortality was recorded at 5, 10, 20, 40, 80, 160 m, and 24, 48, 72 and 96 h. Water quality was measured at the beginning and end of exposure (pH, D.O., temperature and conductivity). LC50 values were calculated using CETIS (Tidpool Scientific Software) and mortality vs. concentration curves were generated using GraphPad Prism version 5.0.

2.4. Sublethal Cu exposures

All sublethal Cu treatments were conducted in duplicate (including controls) as static renewal exposures in 170 L aerated fiberglass tanks. Exposures were conducted in either soft or artificially hardened water. Municipal dechlorinated water with a baseline hardness of 6 mg/L CaCO_3 was used as the soft water. Salts were added to dechlorinated municipal water to attain a value of 100 mg/L CaCO_3 for the artificially hardened water. Salts (CaSO_4 ·½H_2O; CaCl_2; MgCl_2 ·6H_2O; KCl, NaCl, NaHCO_3) were dissolved in deionized water, before being added to municipal dechlorinated water to achieve the appropriate hardness values. The water was hardened using this methodology to match off-site well water used for studies done in collaboration with Environment Canada (Pacific Environmental Science Centre [PESC], North Vancouver, BC). A summary table of the well water parameters from PESC is found in Table 2.1. Fish were acclimated for 24 h in exposure tanks in either hard or soft water prior to the addition of CuCl_2. Water samples were taken to measure water hardness and Cu concentrations in the artificially hardened water and water analyses were conducted at PESC. Water quality measurements (temperature, pH, dissolved oxygen and
conductivity) were measured at the start and end of an exposure in each tank. Dissolved oxygen (D.O.) and temperature were measured using an oxygen meter (YSI 58 Dissolved Oxygen Meter, Yellow Springs, OH), pH with a pH pen (S96275B, Fisher Science Education, Mississauga, ON) and conductivity with a pocket conductivity meter (Oakton ECTestr 11 dual range, Vernon Hills, IL).

Table 2.1. Summary of off-site well water parameters (analyte concentration, water quality measurements). Values are mean ± SEM.

<table>
<thead>
<tr>
<th>Well Water Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloride (Cl⁻)</td>
<td>94 ± 5 mg/L</td>
</tr>
<tr>
<td>Sulphate (SO₄²⁻)</td>
<td>7.9 ± 0.5 mg/L</td>
</tr>
<tr>
<td>Calcium (Ca²⁺)</td>
<td>36.7 ± 0.1 mg/L</td>
</tr>
<tr>
<td>Magnesium (Mg²⁺)</td>
<td>3.1 ± 0.1 mg/L</td>
</tr>
<tr>
<td>Potassium (K⁺)</td>
<td>3.7 ± 0.1 mg/L</td>
</tr>
<tr>
<td>Sodium (Na⁺)</td>
<td>44 ± 0.1 mg/L</td>
</tr>
<tr>
<td>Overall Hardness</td>
<td>100 ± 0.5 mg/L CaCO₃</td>
</tr>
<tr>
<td>Conductivity</td>
<td>468.33 ± 1.83 µS</td>
</tr>
<tr>
<td>pH</td>
<td>7.73 ± 0.04</td>
</tr>
<tr>
<td>Temperature</td>
<td>14.65 ± 0.07 °C</td>
</tr>
</tbody>
</table>

2.5. Cu exposures for swim experiments

Fish (n=16 per treatment group) were exposed to one of three CuCl₂ concentrations: control, low (15% of the 96-h LC50 concentration) or high (40% of the 96-h LC50 concentration). Nominal low and high concentrations in hard water were 20 and 60 µg/L, and soft water concentrations were 6 and 16 µg/L, respectively. Exposures were 4, 8 and 16 d in duration. For the 8 and 16 d exposures, 50% of water was renewed every 4 d (Report EPS 1/RM/44, 2004) without disturbing the fish. Water was replaced with water of the appropriate hardness and CuCl₂ concentrations for each treatment.
2.6. Swim performance protocol

The swim tunnel apparatus used to measure swim performance (burst swimming speed, or $U_{burst}$) was similar to that described in Mackinnon and Farrell (1992). It consisted of a 2400 L fiberglass oval raceway containing an enclosed test chamber along the long axis (Figure 2.1). A condensing cone directed water into the test chamber which contained fish, and flow vanes at either curved end of the raceway maintained the appropriate water velocities and laminar flow within the test chamber. Two electric motors (Minn-Kota Endura C2-40, Poco Marine Ltd., Port Coquitlam, BC) were used to generate the water current. These were connected to a voltage regulator to control the velocity of the water in the raceway. The water level of the swim tunnel was maintained at a constant volume and temperature ($11.9 \pm 0.1^\circ C$). Calibration curves for voltage vs. water velocity were generated before each test using a current meter (Forestry Suppliers Inc., Jackson, MI).

Following Cu exposure, fish were transferred to the test chamber in the swim tunnel and acclimated for 20 min at a water speed of 10 cm/s (approximately 1 body length per second (BL/s)) (Farrell, 2008). The burst-swimming test ($U_{burst}$) consisted of 1 m stepwise water velocity increments of 5 cm/s (~0.5 BL/s) until fish were fatigued (Farrell, 2008, Nendick et al., 2009). Fish were considered fatigued when they rested on the rear net of the test chamber and did not respond to mechanical stimulation. Once fatigued, fish were removed and euthanized in 0.5 g/L buffered MS222. Fork length ($L_F$) and mass ($M$) of each fatigued fish were then measured.
2.7. Seawater challenge

Fish were exposed to Cu in either soft or hard water for 4 or 16 d at 3 Cu concentrations (control, low and high) as described above. At the end of the exposure period, n = 14 fish per treatment were subjected to a seawater challenge measuring mortality, and n = 10 fish were used for biochemical measures of osmoregulation and stress.

For the seawater challenge, fish (n=14 per exposure treatment) were placed in duplicate tanks containing 170 L seawater at 28 ppt (Bills and Kenworthy, 1986). The seawater challenge occurred over 24 h; at the end of this period, fish mortality was recorded. The remaining fish were euthanized in 0.5 g/L buffered MS222. Fork length ($L_F$) and mass ($M$) were measured for all fish.
2.8. Osmoregulatory indicators

At the end of the Cu exposure period, n = 10 fish per exposure treatment were euthanized in buffered MS222 and blood and gill tissue were sampled. To obtain plasma, the caudal peduncle was severed (Congleton and LaVoie, 2001) and blood was collected in heparinized capillary tubes (Chase Scientific Glass, Inc., Rockwood, TN). Blood was centrifuged for 3 min in a microcapillary centrifuge (International Equipment Company, Chattanooga, TN) at 13000 x g. Hematocrit was measured in duplicate and plasma was then separated and placed in 1.5 mL centrifuge tubes and stored on dry ice until being transferred to -80 °C for future analysis.

Gill filaments (10-15 filaments per sample) were collected in duplicate from the left side and placed in 1.5 mL capillary tubes containing 125 µL SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3; McCormick, 1993). Two stainless steel 2 mm beads (Retsch, Newtown, PA) were added to the tube and stored on dry ice until they were transferred to -80 °C. These samples were later analyzed for gill Na⁺/K⁺-ATPase (NKA) activity.

Plasma samples were measured in duplicate for osmolality, sodium ion (Na⁺), chloride ion (Cl⁻) and cortisol concentrations. Osmolality was measured using a vapor pressure osmometer, which measures the concentration of particles that reduce the vapour pressure of a solution (Wescor Vapro 5520, EliTech Group, Logan, UT). Plasma [Na⁺] was determined in duplicate using flame photometry on a Varian AA240FS Atomic Absorption Spectrometer (Palo Alto, CA). Flame photometry is an atomic emission method to determine the concentration of metal salts. Solutions are aspirated into a flame, which causes the metals to atomize, and electrons to excite to a higher and unstable energy state. The subsequent return to a stable energy state emits light at a specific wavelength for each element, with light intensity being proportional to concentration of the element in solution (Overman and Davis, 1947). Plasma [Cl⁻] was measured in triplicate following the protocol in Osachoff et al. (2014). Chloride concentration is determined via competition between Hg²⁺ and Fe²⁺ for 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ). In the presence of Cl⁻, Hg²⁺ complexes to form HgCl₂, allowing Fe²⁺ to complex with TPTZ. While the Hg-TPTZ complex is colourless, Fe-
TPTZ results in a colorimetric product that is proportional to the concentration of Cl\(^-\) present and can be measured on a spectrophotometric plate reader. Cortisol concentration was measured in duplicate using an ELISA (enzyme-linked immunosorbent assay) kit (Neogen Corp., Lansing, MI). Cortisol competes with an enzyme conjugate for binding sites on the plate, and the plate is subsequently washed to remove any unbound materials. A substrate is added which binds to the enzyme conjugate to generate a colour (inversely proportional to cortisol concentration), which can be read on a spectrophotometric plate reader.

Gill NKA activity was determined following the protocol of McCormick (1993), with the exception that gill homogenates were prepared using a bead mill homogenizer (Mixer Mill 300; Qiagen, Mississauga, ON) set to a program of 20 Hz for 30 seconds. Each sample was homogenized twice. Briefly, each gill samples (containing 10-15 filaments) was homogenized in SEID buffer (100 mL of SEI buffer with 0.5 g of deoxycholic acid) and centrifuged (5000 x g) for 30 s. Each sample was analyzed in microplates, with two sets of duplicates; the first set contained the assay mixture (4 U/mL lactate dehydrogenase, 5 U/mL pyruvate kinase, 2.8 mM phosphoenolpyruvate, 0.7 mM ATP, 0.22 mM NADH, 50 mM imidazole), and the second contained the assay mixture with ouabain (0.5 mM). Microplates were read as a kinetic assay, every 30 s over a 10 min period. For each sample, the difference in slopes between the two sets, and standardized for protein content using a BCA protein assay kit (Thermo Fisher Scientific, Mississauga, ON) resulted in the final ATPase activity, expressed as µmoles ADP/mg protein/h. Plates for assays were read using a Bio-tek PowerWave Reader (Bio-Tek, Winooski, VT) at the wavelength specified in the protocols. Standard curves were run concurrently on each plate (R\(^2\) > 0.97).

2.9. Calculations and statistics

LC50 values were calculated via probit conversion followed by linear regression in CETIS (Tidepool Scientific Software, v1.8.4). The percent mortality is converted to probits (probability units) and the concentrations are log transformed. Plotting probits vs. log concentrations transforms the sigmoid mortality curve to a linear relationship, to which a regression line can be fit for calculation of the LC50 value. The \(U_{burst}\) water
velocity (the velocity fish fatigued at) for each fish was standardized by dividing by the fish fork length, resulting in a final fatigue velocity in body lengths per second (BL/s) (Goulding et al., 2013). To ensure overall fish health, condition factor (K) was calculated using: \(100 \times (M + L_F^3)\), with \(M = \) mass (g) and \(L_F = \) fork length (cm). Graphs were constructed using GraphPad Prism version 5.0 and statistical analyses were performed using JMP version 10. For each endpoint, the statistical analyses were conducted on the mean values from each exposure tank. Analysis for effects of Cu, duration of exposure and water hardness (and their interactions) was performed using a three-factor ANOVA. This was followed by Tukey’s multiple comparisons test (≥2 comparisons) or Student’s t-test (1 comparison) to compare between treatments. Statistical significance was set at \(p < 0.05\). Grubbs’ test was used to detect and remove outliers (GraphPad Software Inc., La Jolla, CA, USA). Coefficient of variation (CV) between duplicates <20% were deemed acceptable (Reed et al., 2002). All data are presented as mean ± standard error of the mean (SEM), with the number of fish (n) noted.
Chapter 3.

Results

3.1. General

The water temperature in the exposure tanks was 11.8 ± 0.09 °C, dissolved oxygen was 10.4 ± 0.04 mg/L and pH was 7.5 ± 0.03. Tanks with soft water had a conductivity of 9.5 ± 0.76 µS, and 436.8 ± 3.63 µS in hard water. The water hardness and concentration Cu in hard water exposures were measured. Artificially hardened water had a nominal concentration of 100 mg/L CaCO₃ and measured average concentration of 108.40 ± 0.76 mg/L CaCO₃. Control exposure water had an average Cu concentration of 1.02 ± 0.18 µg/L, due to background Cu in municipal water. Low Cu treatment water had a nominal target concentration of 20 µg/L Cu in hard water, and an average measured concentration of 20.00 ± 1.70 µg/L Cu. High Cu treatment water had a nominal target concentration of 60 µg/L Cu in hard water, and an average measured concentration of 58.93 ± 3.36 µg/L Cu.

Condition factor (K), a general measure of fish health, was not significantly different between exposed and control fish at any time point (p = 0.2012). In most cases sample size was equal across treatments, however experimental conditions affected sample size between groups. In swim trials, fish (≤2 fish per trial) escaped from the swim chamber, could not be retrieved and were therefore not included in data analysis.

3.2. LC50 determinations

The 96-h LC50 values for the exposure conditions in this study, calculated by probit analysis, were 40 and 150 µg/L Cu, for soft (6 mg/L CaCO₃) and hard (100 mg/L CaCO₃).
CaCO₃) respectively. The mortality curves showing the average percent mortality at each Cu concentration for hard and soft water are found in Fig 3.1

Figure 3.1  Mortality (%) of fish exposed to different Cu concentrations after 96 h, in hard water (A) and soft water (B). Mean values reported. N=10
3.3. **Swimming performance**

Burst swimming performance ($U_{burst}$) is the maximum velocity a fish can maintain over a short period of time, and was assessed following Cu exposure using a swim tunnel according to the methods of Osachoff et al. (2014). Juvenile fish (n=16 per treatment; due to tank effects: n=1 for analyses) were exposed to either control (municipal dechlorinated water), low (6 and 20 µg/L in soft [6 mg/L CaCO$_3$] and hard water [100 mg/L CaCO$_3$], respectively) or high (16 and 60 µg/L in soft and hard water, respectively) Cu concentrations for 4, 8 or 16 d.

Across all treatment groups, $U_{burst}$ values ranged from 6.13 ± 0.11 to 7.53 ± 0.14 BL/s. Exposure to Cu did not alter burst swimming speed in any treatment group ($p = 0.1120$), therefore Cu treatment groups were pooled together for all further comparisons of the effects of water hardness and exposure duration on $U_{burst}$ values (Fig 3.2). The duration of exposure, regardless of water hardness had no significant effect on $U_{burst}$ ($p = 0.2960$). $U_{burst}$ values at 4, 8 and 16 d of exposures were 6.88 ± 0.05, 6.75 ± 0.05 and 6.86 ± 0.05 BL/s, respectively. There was an interaction between the duration of exposure and water hardness ($p = 0.0011$). Burst swimming speed for 4 d exposures were significantly higher in soft water (7.43 ± 0.07 BL/s) compared to hard water (6.33 ± 0.07 BL/s; $p = 0.0028$). There was no difference in burst swimming speed in 8 d exposures conducted in either soft or hard water (6.67 ± 0.07 and 6.84 ± 0.07 BL/s respectively; $p = 0.6047$) or 16 d exposures conducted in either soft or hard water (6.67 ± 0.07 and 7.04 ± 0.07 BL/s, respectively; $p = 0.1305$). For exposures conducted in hard water, $U_{burst}$ values were significantly lower for 4 d exposures (6.33 ± 0.07 BL/s) compared to 8 (6.84 ± 0.07 BL/s; $p = 0.0461$) and 16 d (7.04 ± 0.07 BL/s; $p = 0.0147$). For exposures conducted in soft water, $U_{burst}$ velocities were significantly slower for 4 d exposures (7.43 ± 0.07 BL/s) compared to 8 (6.67 ± 0.07 BL/s; $p = 0.0109$) and 16 d (6.67 ± 0.07 BL/s; $p = 0.0112$).
Figure 3.2  Burst swimming speed (BL/s) of fish exposed to Cu. 4, 8 and 16 refers to days of Cu exposure, H = hard water (100 mg/L CaCO₃), S = soft water (6 mg/L CaCO₃). Low Cu treatments are 6 and 20 µg/L Cu in soft and hard water, respectively. High Cu treatments are 16 and 60 µg/L Cu in soft and hard water, respectively. Box indicates 25th and 75th quartiles, whiskers indicate 10th and 90th percentiles. Medians are indicated by a black line and means are indicated by a +. N=1 for each treatment group. Treatments connected by the same letter are not significantly different (p < 0.05). Tukey’s multiple comparisons test was used to determine differences between treatment groups.

3.4. Osmoregulation

3.4.1. Gill Na⁺/K⁺-ATPase (NKA) activity

Gill NKA activity was assessed in juvenile fish (n=10 per treatment; due to tank effects, n=2 for analyses) following Cu exposure to either control (municipal dechlorinated water), low Cu concentrations (6 and 20 µg/L in soft or hard water, respectively) or high Cu concentrations (16 and 60 µg/L in soft or hard water,
respectively) for 4 and 16 d. NKA activity was assessed following the protocol of McCormick (1993).

Gill NKA activity ranged from $0.27 \pm 0.11$ to $1.79 \pm 0.09$ µmole ADP/mg protein/h across all treatment groups. Exposure to Cu did not alter gill NKA activity in any treatment group ($p = 0.2717$), therefore Cu treatment groups were pooled together for all further comparisons of the effects of water hardness and exposure duration (Fig 3.3). There was a significant interaction effect between the duration of exposure and water hardness ($p < 0.0001$). Gill NKA activity for 4 d exposures were significantly higher in soft water ($1.70 \pm 0.05$ µmole ADP/mg protein/h) compared to that in hard water ($0.67 \pm 0.05$ µmole ADP/mg protein/h; $p < 0.0001$). For 16 d exposures, gill NKA activity was significantly higher in hard water ($1.23 \pm 0.05$ µmole ADP/mg protein/h) compared to soft water ($0.46 \pm 0.05$ µmole ADP/mg protein/h; $p < 0.0001$). In hard water (Fig 3.3A), gill NKA activity was significantly higher for 16 d ($1.23 \pm 0.05$ µmole ADP/mg protein/h) compared to 4 d exposures ($0.67 \pm 0.05$ µmole ADP/mg protein/h; $p < 0.0001$). For exposures conducted in soft water (Fig 3.3B), gill NKA activity was significantly elevated at 4 d ($1.70 \pm 0.05$ µmole ADP/mg protein/h) compared to 16 d ($0.46 \pm 0.05$ µmole ADP/mg protein/h; $p < 0.0001$).
Figure 3.3  Gill Na¹/K¹-ATPase activity (µmole ADP/mg protein/h) of fish exposed to Cu. 4, 8 and 16 refers to days of Cu exposure, H = hard water (100 mg/L CaCO₃), S = soft water (6 mg/L CaCO₃). Low Cu treatments are 6 and 20 µg/L Cu in soft and hard water, respectively. High Cu treatments are 16 and 60 µg/L Cu in soft and hard water, respectively. Box indicates 25th and 75th quartiles, whiskers indicate 10th and 90th percentiles. Medians are indicated by a black line and means are indicated by a +. N=2 for each treatment group. Treatments connected by the same letter are not significantly different (p < 0.05). Tukey’s multiple comparisons test was used to determine differences between treatment groups.

3.4.2. Plasma ion concentrations and osmolality

Exposures were conducted as described above. Briefly, fish (n=10 per treatment; due to tank effects, n=2 for analyses) were exposed to either control (municipal dechlorinated water), low Cu concentrations (6 and 20 µg/L in soft or hard water, respectively) or high Cu concentrations (16 and 60 µg/L in soft or hard water, respectively) for 4 and 16 d.

Across all treatment groups, plasma Cl⁻ concentrations ranged from 98.20 ± 2.85 to 155.86 ± 2.89 mmol/L. Cu exposure did not alter Cl⁻ concentration in any treatment group (p = 0.6566), therefore Cu treatment groups were pooled together for all further
comparisons of the effects of exposure duration and water hardness (Fig 3.4). Water hardness did not affect Cl\(^-\) concentration in any treatment group \((p = 0.0969)\), with plasma concentrations of 116.82 ± 3.10 and 124.72 ± 3.10 mmol/L in hard and soft water, respectively. The duration of exposure had a significant effect on Cl\(^-\) concentration. Fish exposed for 16 d (126.61 ± 3.10 mmol/L) had a significantly higher concentration of Cl\(^-\) in plasma than fish exposed for 4 d (114.93 ± 3.10 mmol/L; \(p = 0.0208\)).

**Figure 3.4** Plasma Cl\(^-\) concentration (mmol/L) of fish exposed to Cu. 4, 8 and 16 refers to days of Cu exposure, H = hard water (100 mg/L CaCO\(_3\)), S = soft water (6 mg/L CaCO\(_3\)). Low Cu treatments are 6 and 20 µg/L Cu in soft and hard water, respectively. High Cu treatments are 16 and 60 µg/L Cu in soft and hard water, respectively. Box indicates 25\(^{th}\) and 75\(^{th}\) quartiles, whiskers indicate 10\(^{th}\) and 90\(^{th}\) percentiles. Medians are indicated by a black line and means are indicated by +. N=2 for each treatment group. Treatments connected by the same letter are not significantly different \((p < 0.05)\). Student’s t-test was used to determine differences between treatment groups.

Plasma Na\(^+\) concentrations ranged from 91.56 ± 10.78 mmol/L to 123.24 ± 2.60 mmol/L across all treatment groups. Exposure to copper had no significant effect on plasma Na\(^+\) concentrations \((p = 0.1335)\), therefore Cu treatment groups were pooled together for all further comparisons of the effects of water hardness and exposure.
duration (Fig 3.5). Water hardness did not affect Na\(^+\) concentration in any treatment group \((p = 0.3023)\), with plasma concentrations of 117.77 ± 1.39 and 115.65 ± 1.39 mmol/L in hard and soft water, respectively. There was a significant interaction between duration of exposure and water hardness \((p = 0.0195)\). Plasma Na\(^+\) concentration after 4 d of exposure was not significantly different between hard water exposures \((118.19 ± 1.97\) mmol/L) and soft water exposures \((110.76 ± 1.97\) mmol/L; \(p = 0.0835)\). After 16 d exposures, there was no significant difference in plasma Na\(^+\) between hard \((117.35 ± 1.97\) mmol/L) and soft water \((120.54 ± 1.97\) mmol/L; \(p = 0.6711)\). There was no significant difference for exposures conducted in hard water between 4 d \((118.19 ± 1.97\) mmol/L) and 16 d \((117.35 ± 1.97\) mmol/L; \(p = 0.9900\); Fig 3.5A). For exposures conducted in soft water, 16 d exposures had significantly elevated plasma Na\(^+\) concentration \((120.54 ± 1.97\) mmol/L) than 4 d exposures \((110.76 ± 1.97\) mmol/L; \(p = 0.0194\); Fig 3.5B).
Figure 3.5  Plasma Na\(^+\) ion concentration (mmol/L) of fish exposed to Cu. 4, 8 and 16 refers to days of Cu exposure, H = hard water (100 mg/L CaCO\(_3\)), S = soft water (6 mg/L CaCO\(_3\)). Low Cu treatments are 6 and 20 µg/L Cu in soft and hard water, respectively. High Cu treatments are 16 and 60 µg/L Cu in soft and hard water, respectively. Box indicates 25\(^{th}\) and 75\(^{th}\) quartiles, whiskers indicate 10\(^{th}\) and 90\(^{th}\) percentiles. Medians are indicated by a black line and means are indicated by a +. N=2 for each treatment group. Treatments connected by the same letter are not significantly different (p < 0.05). Tukey’s multiple comparisons test was used to determine differences between treatment groups.

Across all treatment groups, plasma osmolality ranged from 263.20 ± 11.49 mmol/kg to 312.63 ± 3.94 mmol/kg (Fig 3.6). Exposure to copper did not alter osmolality in any treatment group (p = 0.2148), therefore Cu treatment groups were pooled together for all further comparisons of the effects of exposure duration and water hardness. The duration of exposure had a significant effect on plasma osmolality (p = 0.0452). Osmolality after 4 d exposures (297.00 ± 2.12 mmol/kg) were significantly higher than 16 d (290.30 ± 2.12 mmol/kg). Water hardness had a significant effect on plasma osmolality (p < 0.0001); exposures in hard water (302.48 ± 2.12 mmol/kg; Fig 3.6A) were significantly higher than soft water exposures (284.83 ± 2.12 mmol/kg; Fig 3.6B).
3.4.3. Seawater challenge

Fish were subjected to a seawater challenge following the exposure protocol described above. Following 24 h in seawater, no mortalities occurred in any treatment group.

3.5. Biochemical stress response

The stress response was assessed in fish (n=10 per treatment; due to tank effects, n=2 for analyses) that were exposed to either control (municipal dechlorinated...
water), low (6 and 20 µg/L in soft and hard water, respectively) or high (16 and 60 µg/L in soft and hard water, respectively) Cu concentrations for 4 or 16 d.

Hematocrit values ranged from 35.06 ± 1.44 to 43.31 ± 1.56. Exposure to copper resulted in no significant change to hematocrit (\( p = 0.4266 \)), therefore Cu treatment groups were pooled together for all further comparisons of the effects of exposure duration and water hardness (Fig 3.7). The duration of exposure had no effect on hematocrit (\( p = 0.0772 \)), with 4 and 16 d exposures having hematocrit values of 38.46 ± 0.58 and 40.04 ± 0.58, respectively. Water hardness had no effect on hematocrit (\( p = 0.0545 \)), with hard (Fig 3.7A) and soft (Fig 3.7B) having values of 38.35 ± 0.58 and 40.15 ± 0.58, respectively.

**Figure 3.7** Hematocrit (%) in fish exposed to Cu. 4, 8 and 16 refers to days of Cu exposure, H = hard water (100 mg/L CaCO₃), S = soft water (6 mg/L CaCO₃). Low Cu treatments are 6 and 20 µg/L Cu in soft and hard water, respectively. High Cu treatments are 16 and 60 µg/L Cu in soft and hard water, respectively. Box indicates 25th and 75th quartiles, whiskers indicate 10th and 90th percentiles. Medians are indicated by a black line and means are indicated by a +. N=2 for each treatment group. Treatments connected by the same letter are not significantly different (\( p < 0.05 \)).
Plasma cortisol values ranged from $1.54 \pm 0.37$ to $131.01 \pm 28.34$ ng/ml. Copper exposure did not alter plasma cortisol concentrations in any treatment groups ($p = 0.1441$), therefore Cu treatment groups were pooled together for all further comparisons of the effects of water hardness and exposure duration (Fig 3.8). There was a significant interaction between the duration of copper exposure and water hardness ($p = 0.0021$). Fish exposed for 4 d had significantly elevated cortisol concentrations in soft water ($68.67 \pm 8.31$ ng/ml) compared to hard water ($8.07 \pm 8.31$ ng/ml; $p = 0.0012$). For exposures conducted over 16 d, there was no significant difference between hard ($7.45 \pm 8.31$ ng/ml) and soft water ($3.16 \pm 8.31$ ng/ml; $p = 0.9826$). Hard water exposures were not significantly different comparing 4 d ($8.07 \pm 8.31$ ng/ml) and 16 d exposures ($7.45 \pm 8.31$ ng/ml; $p = 0.9999$; Fig 3.8A). Soft water exposures resulted in significantly elevated plasma cortisol concentrations after 4 d of exposure ($68.67 \pm 8.31$ ng/ml) compared to 16 d ($3.16 \pm 8.31$ ng/ml; $p = 0.0006$; Fig 3.8B).
Figure 3.8 Plasma cortisol concentration (ng/ml) of fish exposed to Cu. 4, 8 and 16 refers to days of Cu exposure, H = hard water (100 mg/L CaCO₃), S = soft water (6 mg/L CaCO₃). Low Cu treatments are 6 and 20 µg/L Cu in soft and hard water, respectively. High Cu treatments are 16 and 60 µg/L Cu in soft and hard water, respectively. Box indicates 25th and 75th quartiles, whiskers indicate 10th and 90th percentiles. Medians are indicated by a black line and means are indicated by a +. N=2 for each treatment group. Treatments connected by the same letter are not significantly different (p < 0.05). Tukey’s multiple comparisons test was used to determine differences between treatment groups.
Chapter 4.

Discussion

The gills, specifically the ion transport ATPases, are believed to be the primary site of Cu toxicity in fish (Hansen et al., 1993). Exposure to Cu affects osmoregulation by impairing ion exchange: Cu outcompetes Na\(^+\) for ATPase transporters (Laurén and McDonald, 1986, De Boeck et al, 2007). Water hardness can affect metals toxicity, including that of Cu. In hard water, calcium ions are at a high enough concentration that they outcompete metals for binding sites on the gills and therefore decrease metal bioavailability (Laurén and McDonald, 1986, Saglam et al., 2013). The link between water hardness and Cu effects on osmoregulation is obvious. Proper gill function is crucial for cardiovascular and respiratory function, and therefore a potential link between swimming performance, water quality and Cu toxicity is also likely to exist. The experiments in this study were conducted to determine the effects of Cu exposure on several endpoints related to swimming performance and osmoregulation in a representative salmonid species, the rainbow trout. Juveniles were selected due to their sensitivity to Cu. The effects water hardness and the duration of exposure on the magnitude of effects of sublethal Cu exposure were also examined. Durations were selected that had previously shown effects to Cu in literature.

While Cu is an essential metal at low concentrations, it is extremely toxic at elevated concentrations (Hassan, 2011). Cu is acutely toxic to fish in the range of 10-1000 \(\mu\)g/L Cu, with a broad range due to biotic (e.g. fish species and age) and abiotic (e.g. water hardness, temperature, pH, DOC) factors (Spear and Pierce, 1979, Pilgaard et al., 1994, Eisler, 2000). LC50 values are significantly lower in soft water than hard water. In hard water, with Ca\(^{2+}\) ions at a high enough concentration to outcompete Cu for uptake at the gills, there is a reduction in Cu bioavailability resulting in reduced lethality in harder water (Laurén and McDonald, 1986, Saglam et al., 2013). In the present study, the LC50 values for Cu were 40 and 150 \(\mu\)g/L in soft and hard water,
respectively. These values fall within the range of published LC50 values for salmonids. In soft freshwater (~30 mg/L CaCO$_3$), Cu is most acutely toxic with 96 h LC50 values between 10 and 30 µg/L (NAS 1977, Howarth and Sprague, 1978). In hard freshwater (95-200 mg/L CaCO$_3$), LC50 values range from 75 to 230 µg/L Cu, depending on species (Lorz and McPherson, 1977, US EPA, 1980, Eyckmans et al., 2010).

In this study the exposure to sublethal concentrations of Cu had no significant effect on any of the osmoregulatory endpoints selected, including gill Na$^+$/K$^+$-ATPase activity, plasma [Na$^+$] and [Cl$^-$], plasma osmolality, or mortality during a seawater challenge. Control values in this study were 1.04 ± 0.04 µmol/mg protein/h (gill Na$^+$/K$^+$ ATPase activity), 117.00 ± 171 mmol/L (plasma Na$^+$), 119.50 ± 3.80 mmol/L (plasma Cl$^-$) and 291.93 ± 2.60 mmol/kg (osmolality), respectively. The control values for these endpoints are within published ranges for acute sublethal exposure in freshwater fish. Gill NKA activity in freshwater fish range from 0.5-4 µmol/mg protein/h (Monteiro et al., 2005, Eyckmans et al., 2010, Kulac et al., 2013). Plasma Na$^+$ ion values range from 120-197 mmol/L, plasma chloride ion values range from 120-150 mmol/L and osmolality ranges from 280-300 mmol/kg. (McKim et al., 1970, Wilson and Taylor, 1993a, Pelgrom et al., 1995, Monteiro et al., 2005, Eyckmans et al., 2010).

Copper is well known to disrupt osmoregulation and ionoregulation in fish. Acute exposure in the range of 15 to 2000 µg/L Cu (large range due to species differences) (31.3 µmol/L) has been shown to inhibit Na$^+$/K$^+$-ATPase activity (between 33 and 65% reduction) and increase gill permeability to Na$^+$ (between 20 and 60% ion loss) for exposures conducted from 1 h to 14 d, in a range of water hardness values (1 – 250 mg/L CaCO$_3$) (Laurén and McDonald, 1985, 1987a, 1987b, Wilson and Taylor, 1993a, Monteiro et al., 2005, Hashemi et al., 2008, Eyckmans et al., 2010). Stimulation of ion (Na$^+$ and Cl$^-$) efflux (between 18 and 30% ion loss) in freshwater fish can also occur, likely occurring through the tight junctions in the branchial epithelium (Cu concentration of ~5 µg/L, within 24 h of exposure) (Nieboer and Richardson, 1980, Wilson and Taylor, 1993b). Tight junction permeability is at least partially controlled by bound Ca$^{2+}$ and Cu is known to displace Ca$^{2+}$ from negatively charged groups on the plasma membrane (Nieboer and Richardson, 1980, Laurén and McDonald, 1985). As dissolved Cu concentrations increase, Cu may displace Ca$^{2+}$ and increase ion permeability through
the junctions (Laurén and McDonald, 1985). Together these disruptions (inhibited Na⁺/K⁺-ATPase activity and stimulated ion efflux) lead to a loss of Na⁺ and Cl⁻, decreasing plasma concentrations for both the ions (Laurén and McDonald, 1985, Pelgrom et al., 1995, Eyckmans et al., 2010). The increased permeability of the gills after Cu exposure leading to ion efflux may also be due to a disruption of the membrane integrity in gill cells as concluded by Wilson and Taylor (1993b) (6 µg/L Cu for 24 h in rainbow trout, unknown hardness) and Taylor et al. (2000) (2 & 60 µg/L, 30 d exposure in rainbow trout, hardness 20 and 120 mg/L). Laurén and McDonald (1985) found that water hardness had no effect on the net loss of Na⁺ and Cl⁻ caused by exposure to 200 µg/L Cu (i.e. no significant difference in ion loss between hard [83 mg/L CaCO₃] and soft water [2 mg/L CaCO₃]). Similar results were found by Taylor et al. (2000), with no significant difference in ion loss after Cu exposure in hard and soft water (hard water = 120 mg/L CaCO₃, 60 µg/L Cu, soft water = 20 mg/L CaCO₃, 2 µg/L Cu).

In addition to the reduction in plasma [Na⁺] and [Cl⁻], it is well documented that there is a decrease in plasma osmolality, due to reduced ion concentrations. Six and 21 d exposures to concentrations of 39 and 68 µg/L Cu (unknown hardness) significantly reduced osmolality in yearling brook trout (between 5 and 13% reduction) (McKim et al., 1970). The same effect is noted in other species; a 96 h exposure at 121 µg/L Cu (unknown hardness) in the common carp (C. carpio) reduced osmolality significantly by 7% (De Boeck et al., 2001) and over the course of 21 d at 400 µg/L (75 mg/L CaCO₃), osmolality was significantly reduced by 40% in Oreochromis niloticus (Monteiro et al., 2005).

While the previously cited literature indicates a reduction in Na⁺/K⁺-ATPase activity, plasma [Na⁺] and [Cl⁻] following exposure to Cu in freshwater, a study conducted by Eyckmans et al., (2010) support the findings of the present research. They noted a reduction from control of branchial NKA activity only at 12 h of exposure to 20 µg/L Cu (250 mg/L CaCO₃) (10% the 96 h LC50) in rainbow trout; at all other time points (1 h, 24 h, 3 d, 1 w, 1 m) a response was not detected. This suggests the potential for a transient reduction of NKA activity with a return to baseline values under some circumstances. Based on these findings, the sampling times used in the present study would not detect alterations in gill NKA activity if it occurred at an earlier point to
sampling. Compensatory mechanisms that returned values to baseline levels by the first sampling period may be occurring. Eyckmans et al., (2010) also detected a reduction in plasma [Na\(^+\)] and [Cl\(^-\)] within the first 24 h of exposure (including 1, 12 and 24 h), however exposures of a longer duration (3 d, 1 w and 1 m) showed no difference from baseline values.

A seawater challenge is a whole-animal performance assay that is used to determine if freshwater fish can osmo- and ionoregulate in seawater (Bills and Kenworth, 1986, Marshall and Grosell, 2005). As anadromous or catadromous fish transition from freshwater to seawater (e.g. during migration) the change in salinity can subject some fish to severe osmotic stress (Eddy, 1981). Following exposure to a stressor (e.g. Cu), fish are challenged by being transferred from freshwater directly into saltwater. It was hypothesized that fish exposed to Cu would be unable to maintain normal ionoregulation and would be unable to successfully survive the transition from freshwater to seawater. No mortalities were recorded during the seawater challenge, supporting the findings from the biochemical endpoints (NKA activity, plasma Na\(^+\) and Cl\(^-\) concentrations and osmolality) that Cu under these exposure conditions was not an ionoregulatory stressor. Salmonids have been widely used to study osmoregulatory disturbance during transfer to seawater; to our knowledge, the present study is the first time a seawater challenge has been used following Cu exposure (Handeland et al., 1998, Shrimpton et al., 1994, Türker et al., 2004, Shrimpton et al., 2005)

In the present study, control hematocrit values were 40.0 ± 0.7% across all exposure conditions, and was unchanged after 4 and 16 d exposure to Cu. Hematocrit has been shown in several studies to increase with Cu exposure. Studies have shown an increase in hematocrit between 20 and 25% after Cu exposure (ranging from 30-70 µg/L Cu over 4-6 d) (McKim et al., 1970, Mazon et al., 2002, Bagdonas and Vosyliene, 2006, Carvalho and Fernandes, 2006). Hematocrit and plasma volume are inversely related, and plasma ion concentrations can alter plasma volume (Brauner et al., 1994). As there was no change to plasma ion concentrations in the present study, no change in hematocrit would be expected.

Metal exposure, including Cu are known stressors in fish and can trigger the
release of stress hormones, most notably cortisol, as well as both adrenaline and noradrenaline (Wendelaar Bonga, 1993). Cortisol aids in water and ion homeostasis and can trigger the biosynthesis of metallothioneins in fish for metal detoxification (Wendelaar Bonga, 1993). In fish, plasma cortisol concentrations <30 ng/ml are considered to indicate an unstressed condition; concentrations in the range of 30 to 300 ng/ml are typically found in fish exposed to acute stressors (Barton, 2002). Baseline cortisol concentration in the present study across all exposure conditions was 9.45 ± 7.20 ng/ml, which is within the published range for unstressed fish (De Boeck et al., 2001, Gagnon et al., 2006, Osachoff et al., 2014).

In this study, Cu exposure did not result in an elevation of plasma cortisol concentrations in any treatment group, indicating that Cu was not acting as an acute stressor under the experimental conditions. These results differ from much of the published literature, as Cu has been well documented to increase plasma cortisol following exposure. For example, cortisol was significantly increased from 10 to 115 ng/ml following a 96 h exposure of common carp to 120 µg/L Cu (De Boeck et al., 2001). In Oreochromis niloticus, a 3 d exposure to 40 and 400 µg/L (75 mg/L CaCO₃) Cu increased plasma cortisol concentrations from a control value of 28 ng/ml to 199 and 258 ng/ml, respectively (Monteiro et al., 2005). Increases in plasma cortisol concentrations are usually transient. For example, Dethloff et al. (1999) noted a significant increase in cortisol concentrations following 3 d of Cu exposure (to 29 µg/L Cu; 45 mg/L CaCO₃), which returned to control values by 21 d. The initial spike in cortisol may have missed in our study as the first time point (4 d) could be too late for detection. This theory is supported by the results of Pickering and Pottinger (1989) and Kennedy and Farrell (2008), with an acute stressor resulting in an initial (between 1 to 24 h) cortisol spike (plasma cortisol concentrations between 40 to 100 ng/ml, depending on stressor) in rainbow trout, with a return to baseline after 24 h.

The current paradigm of metal toxicity, including Cu, is supported by a broad literature base and hundreds of studies. The apparent lack of effect on common endpoints involved in the Cu toxicity mechanism (particularly at such high Cu concentrations near the LC50 value) is unclear but several possible explanations exist. Typically, when fish are exposed to a toxicant such as sublethal Cu, they enter into a
cycle of damage and repair in order to develop a tolerance to metals, that is comprised of three phases (McDonald and Wood, 1993, McGeer et al., 2000, Tate-Boldt and Kolok, 2008). The initial ‘shock phase’ results in physical damage to the gill and disruptions to the osmoregulatory and respiratory systems. These include reductions in Na⁺/K⁺-ATPase activity, reduced Na⁺ influx and stimulated Na⁺ efflux in freshwater species (Laurén and McDonald, 1985, 1987a, Pelgrom et al. 1995, Kolok et al., 2002). This is followed by the ‘damage phase’ during which the effects from the ‘shock phase’ become apparent, resulting in a net decrease in whole-animal Na⁺ concentrations (Kolok et al., 2002). Following this second phase, fish can enter a ‘repair phase’ where Na⁺ concentrations return to pre-exposure (baseline) concentrations (Laurén and McDonald, 1987a, McGeer et al., 2000) which is achieved by increasing the biosynthetic pathways responsible for repairing damage and correcting the physiological effects from exposure, including an increase in metallothioneins for metal binding and up-regulation of pathways that restore ion homeostasis (Laurén and McDonald, 1987a, Hogstrand and Wood, 1996, McGeer et al., 2000). The time frame for each phase is not fully elucidated and a general consensus has not been reached on the timing of each phase. It is likely dependent on both exposure duration and concentration.

The paradigm of damage and repair is supported by several studies (Dethloff et al., 1999, Eyckmans et al., 2010). They found that alterations to NKA activity, ion concentrations, and plasma cortisol concentrations in rainbow trout were most evident from 24 h (NKA activity and plasma ion concentrations) to 3 d (plasma cortisol) of Cu exposure (Cu exposures between 20 and 50 µg/L for exposures up to 21 d), after which all parameters returned to baseline values (after 3 d). This indicates that in the present study, the initial shock and damage phases may have occurred by the first sampling period of 4 d and that the sampling times of 4 and 16 d were well into the repair phase, when altered endpoints have returned or are returning to baseline values. Species differences may also dictate the length of each phase (along with exposure concentration and duration). For example, common carp (Cyprinus carpio) and gibel carp (Carassius auratus gibelio) exhibited altered gill NKA and ion concentrations following Cu exposure, values which never returned to baseline levels (Eyckmans et al., 2010). The present study indicates that following the initial shock phase, rainbow trout may be better able to restore homeostatic values than some other freshwater species.
One of the postulated mechanisms involved in restoring osmoregulatory regulation following metal exposure resides in the chloride cells, where the majority of the NKA transport proteins reside within the gills. These are mitochondria-rich cells that also contain branchial Na⁺/Ca²⁺ exchangers (Flik et al., 1995, Perry, 1997). One process involved in the restoration of ion homeostasis following Cu exposure involves the turnover and proliferation of chloride cells, increasing their density in the gills (McDonald and Wood, 1993, Pelgrom et al., 1995, Li et al., 1998, Tate-Boldt and Kolok, 2008, Eyckmans et al., 2010). While some chloride cells degenerate due to necrosis following Cu exposure, their increase in numbers after acute (< 6 d) Cu exposure may help compensate for reductions in NKA activity and aid in maintaining ion balance (Pelgrom et al., 1995).

In addition to gills, kidneys and intestines are also involved in osmoregulation. Both organs contain Na⁺/K⁺-ATPases, Ca²⁺-ATPases and Na⁺/Ca²⁺ exchangers for ion transport and ion homeostasis (Schoenmakers et al., 1993). Cu affects these ion transporters in a similar manner as in the gills, although an increase in renal Na⁺ uptake has been reported following sublethal Cu exposure (Laurén and McDonald, 1985). This is believed to be a physiological response that compensates for reduced branchial NKA activity, suggesting that renal NKA pumps may provide better protection against disturbance than branchial NKA pumps to the effects of Cu. Renal Na⁺ reabsorption aids to counterbalance the reduced Na⁺ absorption at the gills following sublethal Cu exposure (Laurén and McDonald, 1987a, 1987b, Grosell et al., 1998). In the present study, plasma Na⁺ and Cl⁻ ion concentrations and osmolality were unaffected by Cu exposure after 4 and 16 d exposure, potentially due, in part, to the upregulation of renal NKA activity. As NKA activity was not measured in any tissue other than the gills, the contribution of renal ion transport to ion homeostasis in the face of metal challenge remains unknown.

Swimming performance has been utilized as an endpoint to determine the effects of various stressors on fish, as it requires the integration of multiple organ systems to function optimally (Osachoff et al., 2014). These include the respiratory and cardiovascular systems as well as the musculature and skeletal systems, among others (Bone, 1978, Jones and Randall, 1978). Adjustments to both the respiratory and cardiac
systems of fish occur during intense exercise in order to meet increased energetic demand brought on by increased oxygen and energy demands, and it is during such periods of maximum performance that all subsystems need to be working optimally (Jones and Randall, 1978).

The respiratory system is responsible for gas exchange and is limited by factors including the surface area over which gas exchange occurs (i.e. the gills), the surface’s permeability to gases, and the distance across the exchange surface (Jones and Randall, 1978). During exercise the amount of water flowing over the gills increases; there is an increase in the contractions and expansions of both the buccal and opercular pumps to achieve this, aided by the forward swimming motion of the fish (Kiceniuk and Jones, 1977). This increased ventilation of the gills allows for increased oxygen uptake necessary for swimming (Brett, 1964). Exercise in fish results in an increase in peripheral blood flow in order to oxygenate muscles involved in locomotion (Jones and Randall, 1978). This can occur by increasing the heart rate and stroke volume (i.e. the volume of blood pumped out of the ventricle per beat; Jones and Randall, 1978). Typically the increased cardiac output required for more rapid oxygen transport is associated with changes to the stroke volume rather than heart rate (Randall, 1970). Changes to heart rate vary greatly by species; at the onset of exercise both bradycardia and tachycardia have been reported (Priede, 1974). Additionally, levels of hematocrit, hemoglobin and plasma proteins increase during exercise to increase oxygen transport (Cameron and Davis, 1970).

As the gills are in direct contact with water, they are vulnerable to many waterborne toxicants including Cu (Beaumont et al., 2003). Cu exposure at concentrations ranging from 5 to 100 µg/L and exposure from 6 to 96 h can interfere with respiration by causing physical damage to gills, including swelling and lifting of epithelial cells, fusion and collapse of gill lamellae, and an increase in mucus production (De Boeck et al., 2001, Beaumont et al., 2003). The net effect of this physical damage is a disruption of gas exchange through an increase in diffusion distance that decreases the rate of oxygen uptake (De Boeck et al., 2006). The accumulation of carbon dioxide results in an acidosis and an increase in blood viscosity. The combination of reduced oxygen uptake and increased blood viscosity decreases O₂ delivery during Cu exposure.
(300 µg/L Cu for 24 h; Wilson and Taylor, 1993a). Oxygen consumption has been shown to significantly decrease immediately (1 h) following exposure to Cu at concentrations of 20 and 50 µg/L. After 7 d of continuous exposure to these concentrations, oxygen consumption returned to control values for the 20 µg/L exposure group, however the 50 µg/L group did not recover in this respect, suggesting the possibility of a compensatory mechanism, especially in low Cu concentration range (De Boeck et al., 1995).

Typically, a large proportion of a fish’s mass is muscle, and in salmonids approximately 60% of their total body mass consists of locomotory muscles (Bainbridge, 1962). The most common swimming performance measure is critical swimming speed ($U_{crit}$) which is a long duration assay and is regarded as a measure of aerobic swimming, utilizing primarily red muscle, which are slow twitch fibres that contain numerous mitochondria (Bone, 1966, Farrell, 2008). As described earlier, an increase in blood viscosity could decrease oxygen delivery to the locomotory muscles, affecting aerobic cellular metabolism (Beaumont et al., 2000). Blood viscosity affects oxygen transport by reducing heart stroke volume or reducing blood velocity through the capillaries (Randall and Brauner, 1991, Wells and Weber, 1991). Burst swimming ($U_{burst}$) is a test of shorter duration that measures the maximum speed a fish can sustain for a short period of time. It is regarded as primarily anaerobic swimming, utilizing mostly white muscle or fast twitch fibres, which contain fewer mitochondria (Bone, 1966, Farrell, 2008). This type of exercise (exhaustive) is fuelled by the breakdown of glycogen to lactate; the depletion of white muscle glycogen stores can account for reductions in $U_{burst}$ (Beaumont et al., 2000).

Few studies have examined the effects of Cu on swimming performance in fish, and the research has typically focused on critical swimming speed, or the determination of $U_{crit}$, a long duration swimming test. For example, 2 h exposure to Cu at 105 µg/L caused a significant reduction in critical swimming speed in trout (Brett, 1964, Waiwood and Beamish, 1978, Beaumont et al., 1995, Kolok et al., 2002, De Boeck et al., 2006, Waser et al., 2009). In addition to Cu, these studies altered several abiotic factors during exposure, including pH and temperature. Copper concentrations in these studies ranged between 5 and 30 µg/L (Brett, 1964, Beaumont et al., 1995) and between 10 and...
200 µg/L (Waiwood and Beamish, 1978). Copper significantly reduces swimming speed at low pH (6) vs. high pH (8) by 15% (Waiwood and Beamish, 1978) and by 40% in low temperature (5 °C) vs. high (15 °C) (Beaumont et al., 1995). Taylor et al. (2000) reported no change to critical swimming speed after a chronic (30 d) Cu exposure in hard (120 mg/L CaCO₃, 60 µg/L Cu) and soft water (30 mg/L CaCO₃, 2 µg/L Cu).

In the present study Cu exposure did not elicit a change in burst swimming speed. Across treatments, \( U_{\text{burst}} \) values (6.13 to 7.53 BL/s) were all within published burst swimming speed values (Pedersen and Malte, 2004, Pedersen and Malte, 2008, Goulding et al., 2013, Osachoff et al., 2014). The lack of Cu effect is likely due to \( U_{\text{burst}} \) being primarily fuelled by anaerobic metabolism. There is evidence of Cu exposure decreasing muscle glycogen stores (Cu concentrations between 80 and 1400µg/L, depending on species selected: carp and danionins), which could potentially result in a reduced burst swimming speed (Radhakrishnaiah et al., 1992, Vutukuru et al., 2005, Reddy et al., 2008). Reductions in oxygen uptake and consumption are more likely to affect the longer duration swimming tests (such as \( U_{\text{crit}} \)) as they are predominantly fuelled by aerobic metabolism. As \( U_{\text{burst}} \) is predominately fuelled by anaerobic metabolism, changes to the gills and decreased oxygen consumption would not have as much of an effect on burst swimming as it would on critical swimming. To our knowledge, this is the first study measuring \( U_{\text{burst}} \) following acute sublethal exposure to copper in juvenile rainbow trout. In a study by Taylor et al. (2000) did not find an effect on burst swimming after a chronic exposure (30 d) in hard (120 mg/L CaCO₃; 60 µg/L Cu) or soft water (20 mg/L CaCO₃; 2 µg/L). While the Taylor et al. (2000) study is a chronic Cu exposure, it does support the findings from the present study, with no effect on \( U_{\text{burst}} \) following Cu exposure, and that water hardness did not affect swimming speed. This could indicate \( U_{\text{burst}} \) may not be a suitable endpoint for Cu or heavy metal toxicity.
Chapter 5.

Conclusion and Future Directions

It has been well documented since the 1970’s that Cu causes ionoregulatory disturbances in fish through several mechanisms, most of which occur at the levels of the gills. There is a small but growing body of evidence that suggests that some fish species can acclimatize to, or compensate for the effects of Cu and under some circumstances, the concentrations that incur toxicity may not be as straightforward as previously thought. The present study aimed to examine the effects of Cu exposure on swimming performance and osmoregulation in juvenile rainbow trout. The concentrations selected were sublethal and environmentally relevant and the experiments were conducted in both soft and artificially hardened water (4 and 16 d) in order to determine how these additional factors might modulate Cu toxicity. It is possible that the sampling time points selected were after damage had occurred and would therefore not have been detected. It is also possible that under these conditions, Cu was not acting as a stressor as there was no alteration to any swimming or osmoregulatory endpoints.

There are multiple studies that could be conducted to follow up on these findings. Histological examinations of the gill structure following Cu exposure would be a first step in determining if physical damage is occurring. It is believed that the first 24 h of exposure is when gill damage occurs, leading to ionoregulatory disruption. In order to validate the proposed damage-repair model for compensation, selecting shorter exposure durations and measuring endpoints would be desirable, as this would allow for the examination of alterations that would occur in the ‘damage phase’ of Cu exposure. While branchial Na⁺/K⁺-ATPase activity is the ionoregulatory endpoint typically studied, measuring the activity of the branchial H⁺-ATPase-coupled Na⁺ channel would be useful to determine Na⁺ flux into the gills of these fish. As we noted no change in branchial
NKA activity on the basolateral membrane, it would be interesting to determine the activity of a sodium pump on the apical membrane. Additionally, as the kidney NKA pump is thought to be involved with maintaining ion balance, determining the activity of that pump after Cu exposure would provide further evidence for the kidney’s involvement in Cu acclimatization.

In BC, the current standards for Cu exposure to aquatic life under the Contaminated Sites Regulation (CSR) are 30 and 50 µg/L in soft water (<50 mg/L CaCO$_3$) and hard water (100-125 mg/L CaCO$_3$), respectively (BC MoE, 2014). Based on the data in the present study, these standards are protective to fish, as our LC50 values of 40 and 150 µg/L Cu (soft and hard water, respectively) are both higher than those in the CSR. Aquatic life water quality guidelines in BC are determined using a number of endpoints (lethal concentrations, effective concentrations and inhibitory concentrations) across many species of aquatic life in an attempt to be protective for all aquatic life (Meays and Nordin, 2013). As the sublethal endpoints in our study may not be useful for setting regulatory guidelines, as no sublethal effects were found at concentrations very close to lethal, our data provides further support to the quality of the standards in protecting fish, based on acute toxicity. This is of importance as Cu remains a contaminant of concern in BC, in part, due to extensive historical and current mining sites (e.g. Britannia, Mount Polley, Highland Valley), and regulators must ensure the standards set out in Regulations and Acts are protective of the fauna found in our province.
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