# PHYSIOLOGICAL INDICATORS OF WATERBORNE COPPER TOXICITY IN FRESHWATER FISH

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

Doctor of Philosophy (2002) McMaster University

(Biology) Hamilton, Ontario

TITLE: Physiological Indicators of Waterborne Copper Toxicity in Freshwater Fish

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NUMBER OF PAGES: XXI, 224

# ABSTRACT

Application of safety factors to water quality guidelines are often employed in the environmental management of waterborne metals. These factors extrapolate the available science into areas not reliably defined, such as: 1) acute-to-chronic toxicity, 2) laboratory-to-field exposures, and 3) model-to-resident species. Scientific advancements require modifications to the derivation process of water quality guidelines to minimize the arbitrary nature of safety factors. One advancement is the Biotic Ligand Model (BLM), which uniquely incorporates toxicology and physiology. This model, along with the mechanism of acute copper toxicity, forms the foundation of the thesis, with the main objective to reduce the uncertainty associated with the three areas in which safety factors are applied. The research employed laboratory experiments to understand the responses of freshwater fish to waterborne copper. This work was extended to a field situation with wild perch. In conclusion, the usual effect indicators of acute toxicity may be of limited value, in their current form, in evaluating chronic toxicity. The complexity of chronic toxicity reaches beyond the simple connections between acute effects and mortality. It now requires the translation of subtle physiological effects into an impact on growth, reproduction or fecundity, thereby affecting fish populations or aquatic food chains. The influence of water chemistry on copper bioavailability is one area most advanced in the last ten years, at least in part due to the development of the BLM. However, the influence of water constituents are likely to cause different effects on acute toxicity, rapid

gill surface binding, metal uptake, and metal accumulation. Lastly, the laboratory and field studies using yellow perch, a species endemic to metal contaminated lakes, show that although the mechanism of toxicity may be identical to that of a model species (i.e., rainbow trout), the mechanism of tolerance can greatly alter the physiological indicators used to detect copper toxicity.

# ACKNOWLEDGEMNENTS

At this time (November, 2002), I would like to acknowledge the people who helped me along the way. Number one is my family. Their continual emotional support was never outweighed by their financial contributions. I thank my mother for her famous words of encouragement, like "if Ph.D.s were easy, everyone would have one" and my father for his diplomatic, rational and calming advice which always managed to pull me back from the edge. My sister always came through for me, especially letting me live with her to save money and for the usual sarcastic-sisterly words of wisdom, such as "you can't always be a free-loader". I also thank my brother, who was lucky to be out West for most of the process, but paid for many trips to the movies upon his return to Ontario.

Secondly, I recognize my friends. I am extremely fortunate to have supportive and patient friends. I thank Lesley Novak, who encouraged me to go back to school and who always believed in me. My friend Suzanne Lang was unfailing in her support and is still my role model for an assertive, confident and sensitive woman (and now mother). My fish buddies, Wendy (Splendid or Bendy) MacFarlane, Sara (Oh dear) Croke and Jeffrey (Bogo) Richards, always made me laugh till it hurt and continually put thesis-woes in perspective. I also thank Danielle (not Marcy) McDonald for our long beer-drinking, nacho-eating sessions at the Phoenix.

Lastly, I thank Gord McDonald for the opportunity and to my supervisory committee (Chris Wood, Rick Playle and Pat Chow-Fraser) who came through for me in the end. I thank-you all.

## THESIS ORGANIZATION AND FORMAT

This thesis is presented in an 'open-faced' format approved by McMaster University and my supervisory committee. This thesis consists of seven chapters; the first provides the background, major findings and broad implications of the research. Chapters two, four and five have either been published or submitted for publication in peer-reviewed scientific journals.

**Chapter 1: Background and implications of the research.**

**Chapter 2: Physiological effects of chronic copper exposure to rainbow trout (*Oncorhynchus mykiss*) in hard and soft water: evaluation of chronic indicators.**

**Authors:** L.N. Taylor, J.C. McGeer, C.M. Wood and D.G. McDonald.

**Date Accepted:** February 8, 2000

**Journal:** *Environmental Toxicology and Chemistry*. 19: 2298-2308. (2000).

**Comments:** This study was conducted and written by L.N.T. under the supervision of D.G.M. J.C.M. and C.M.W. contributed to the study design.

**Chapter 3: Can two independent indicators of susceptibility be used to predict the sublethal and lethal effects of waterborne copper in trout (*Oncorhynchus mykiss*)?**

**Authors:** L.N. Taylor, C.M. Wood and D.G. McDonald.

**Comments:** This study was conducted and written by L.N.T. under the supervision of D.G.M and C.M.W.

**Chapter 4: An in vitro approach for modelling branchial copper binding in rainbow trout.**

**Authors:** L.N. Taylor, D.W. Baker, C.M. Wood and D.G. McDonald.

**Date Accepted:** July, 2002.

**Journal:** *Comparative Biochemistry and Physiology (C).* In press. (2002). Copyright Elsevier Science Inc. Ireland.

**Comments:** This study was conducted by L.N.T. and D.W.B., and written by L.N.T. under the supervision of D.G.M. and C.M.W.

**Chapter 5: An evaluation of sodium loss and gill metal binding properties to explain species differences in copper tolerance.**

**Authors:** L.N. Taylor, C.M. Wood and D.G. McDonald.

**Date Accepted:** September 2002.

**Journal:** *Environmental Toxicology and Chemistry.*

**Comments:** This study was conducted and written by L.N.T. under the supervision of D.G.M. and C.M.W.

**Chapter 6: The use of swim performance and gill binding characteristics in measuring the impact of chronic Cu exposure in wild yellow perch (*Perca flavescens*).**

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**Comments:** This study was performed jointly by L.N.T., W.J.M. and G.P.P. with P.C. at Laurentian University, Sudbury, ON. Each contributed equally in the design and completion of the study. The manuscript was written by L.N.T. under the supervision of D.G.M.

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# CHAPTER 1

# BACKGROUND, SCOPE, AND IMPLICATIONS OF THE STUDY

## BACKGROUND

There are three fundamental guiding principles in the development and application of the Canadian environmental quality guidelines (Gaudet *et al.*, 1995). The first has as its goal an environmental quality in which there are no observable adverse effects on aquatic and terrestrial ecosystems over the long-term. This task requires conservative assumptions, such as the protection of the most sensitive species and life stage. Second, these guidelines are developed for major uses of land and water (termed 'resource based use'), examples being recreation and agriculture. The resource-based use system provides a comprehensive framework for environmental management, encompassing both 'social' and economic considerations. The third principle is to provide generic guidance based on the available scientific information so as to set national guidelines and also establish site-specific objectives, which are agreed upon by the affected parties and reflect site-specific scientific information.

In Canada, the national water quality guidelines for copper have, for the most part, remained unchanged since 1987 [Canadian Council of Resource and Environment Ministries (CCREM), 1987, (CCME), 1999]. The concentration of copper that can be discharged into the environment is based on total recoverable copper and only takes into consideration the hardness of the receiving water. The guideline for total copper in fresh

water ranges from 2 to 4 $\mu$g/L, depending on water hardness (i.e., 2 $\mu$g Cu/L at 0-120 mg/L $CaCO_3$, 3 $\mu$g Cu/L at 120-180 mg/L $CaCO_3$ and 4 $\mu$g Cu/L at >180mg/L $CaCO_3$; CCME, 1999).

In the United States, the Clean Water Act establishes the regulatory framework that controls the discharge of metals into the aquatic environment. The United States Environmental Protection Agency (U.S. EPA) is required to develop Ambient Water Quality Criteria (AWQC) for the protection of aquatic organisms, based on the best available scientific information. The U.S. criteria are designed to protect most of the nation's aquatic species most of the time, whereas the Canadian guidelines aim to protect the most sensitive species all the time. In 1985, the U.S. EPA established 1-hour and 4-day limits which may be exceeded only once every 3 years. These limits are expressed as formulae requiring an applicable hardness value. At hardnesses of 50, 100 and 200 mg/L as $CaCO_3$, the 4-day average total copper concentrations were 6.5, 12 and 21 $\mu$g/L, respectively (i.e., the "chronic" AWQC). At the same 3 hardness levels, the 1-hour average copper concentrations were 9.2, 18 and 34 $\mu$g/L, respectively (i.e., the "acute" AWQC). Individual states, depending on their resources, can develop their own more protective Water Quality Standard (WQS) to reflect their aquatic species and receiving water characteristics.

In 1993, the US EPA recommended the use of dissolved metal concentrations to set and measure compliance with WQS. Each state can then convey the standards into National Pollutant Discharge Elimination System (NPDES) permits to provide effluent limits for point source discharges to surface waters. These permit limits are generally

established using worst case scenarios (e.g., low effluent dilution). These scenarios, in general, require the application of safety factors (also termed 'uncertainty' and 'application' factors), to extrapolate the available science into areas not yet reliably defined, such as, acute to chronic toxicity (via acute-to-chronic ratios), laboratory to field exposures, and model to resident species. These safety factors are examples of the Precautionary Principle applied to risk assessments and environmental management. At the Wingspread Conference in Racine, Wis. (1998), the Precautionary Principle was defined as: "when an activity raises threats of harm to human health or the environment, precautionary measures should be taken even if some cause and effect relationships are not fully established scientifically", in other words: caution first, science second (Appell, 2001).

The risk assessment process must somehow strike a balance between being over-protective and imposing unfair responsibility on industrial dischargers, or being under-protective and possibly threatening the aquatic environment. According to proponents of the Precautionary Principle, it is better to lean on the overprotective side. On the other hand, advancements in technology and scientific judgments require constant modifications to the derivation procedures from time to time. One such modification is the Water Effect Ratio (WER; US EPA, 1984) approach applied to site-specific WQC, whereby the characteristics of the site water, effluent and metal can combine to influence toxicity in a way not predicted by laboratory experiments in laboratory water. The WER is the ratio of toxicity from concurrent laboratory tests run in laboratory water, generally uncontaminated and of defined composition, *versus* the site water, determined for both

total and dissolved metal concentrations. Such an approach reduces the uncertainty for regulators and risk assessors by incorporating the influence of receiving water chemistry parameters [such as, pH, dissolved organic matter (DOM), hardness and alkalinity].

The Water Effect Ratio can provide one scientifically defensible safety factor to site-specific water quality criteria; however, the unempirical factors applied for acute to chronic and model-to-resident aquatic species assessments would still apply. Development of an approach which incorporates these two uncertainty factors would have to be based on both physiology and toxicology. Recently, such an approach for predicting metal toxicity, was put forward into the regulatory arena for validation. The method, termed the Biotic Ligand Model (BLM; DiToro *et al.*, 2001, Santore *et al.*, 2001), has been built with foundations in physiology, geochemical modelling and toxicology. At present, it is a predictive model for acute copper toxicity but has the capability of being adapted into a tool for also assessing chronic effects of metal exposure. In brief, the model incorporates the influence of both the abiotic (i.e., water chemistry constituents) and biotic (i.e., the organism's toxic receptor) ligands in an aquatic system. For the context of my thesis, the biotic ligand was the negatively charged surface on the fish gill and has been characterized mainly by Reid & McDonald (1991), Playle *et al.* (1993a,b), Playle, (1998), and MacRae *et al.* (1999). This copper model is the first to consider the mechanism of metal toxicity, which may be qualitatively and quantitatively different for individual metals and organisms.

The acute toxicity of copper has been well characterized in freshwater fish (for reviews, see Alabaster & Lloyd, 1982; Spear & Pierce, 1979; and Sorenson, 1991).

Nonetheless, the published acute toxicity values (i.e., LC50s or the concentrations which kill 50% of the test population in a specified time period) can range from 10 to 1000 $\mu$g total copper/L. This large range is mainly attributable to different fish species, size and water chemistry differences. The mechanism of acute copper toxicity in rainbow trout (*Oncorhynchus mykiss*) was well defined more than 15 years ago by Laurén and McDonald (1985, 1986, 1987) at McMaster University. Essentially, rainbow trout die as a result of an ionoregulatory disturbance, the stimulation of sodium loss and inhibition of sodium uptake, which is beyond homeostatic control. This mechanism of toxicity, along with the characteristics of the gill (mentioned above), formed the foundation on which my doctoral research was based.

While the BLM approach may not be the ultimate solution for deriving metals water quality criteria, it certainly stimulates research and advances scientific judgement, thereby reducing both the unfairness of the Precautionary Principle and the potential risk to the environment. The work presented in this thesis serves as an example of the critical scientific progression, with the main objective being to improve the available science upon which water quality regulations are derived. In each chapter, the research was placed in context with the large existing literature base of relevant copper knowledge, therefore, the following section serves to highlight key findings of my doctoral work.

## SCOPE AND IMPLICATIONS

My work for this thesis consisted mainly of laboratory-based experiments designed to understand the toxicological and physiological responses of freshwater fish to

waterborne copper. This work was then extended to the application of laboratory effects to a field situation. All five research chapters focus on three topics of copper toxicity, corresponding to the three areas in which the most uncertainty remains with respect to water quality criteria and the regulation of metals entering the aquatic environment. First, the chronic effects of copper in fish were studied, including the sensitivity of physiological toxicity indicators. Second, the influence of water chemistry was characterized to apply laboratory effects to field situations (i.e., site-specific conditions). Third, yellow perch (*Perca flavescens*), a species endemic to metal contaminated lakes, were compared to a reference species, rainbow trout (*Oncorhynchus mykiss*), for the effects of copper toxicity and mechanisms of metal tolerance.

*Chronic Copper Toxicity*

The main objective of Chapter 2 (Taylor *et al.*, 2000) was to document the effects of chronic copper exposure on a large suite of indicators and to propose a new rank order of sensitive effects. An earlier ranking, proposed almost 25 years ago (Spear & Pierce, 1979) was revised to include new indicators (i.e., target tissue metal concentrations, acclimation or increased lethal tolerance, and biotic ligand characteristics) and an improved standardization of metal exposure (e.g., acclimation to test water and diet/nutritional considerations). The indicators used were: acute toxicity (via LC50s), acclimation, growth, swimming performance, whole-body electrolytes, tissue concentrations and gill-copper binding characteristics. Exposure concentrations were chosen as a fraction of the LC50 and were based on the hypothesis that some mortality or

physical damage is necessary to elicit compensatory mechanisms (McDonald & Wood, 1993). To modify Cu bioavailability, the exposures were conducted at two water Cu concentrations in two distinct water types (i.e., hard and soft water).

This chapter led to a number of conclusions directly relevant to the task of assessing the risk of chronic copper exposure to wild fish populations. The sensitivity of each indicator was ranked by considering the number of statistical differences between Cu-exposed and control fish in the four exposure regimes (two Cu concentrations and two water hardness levels). By this criterion, the least sensitive indicators were growth, swim performance and initial electrolyte loss. In order of increasing sensitivity, the remaining indicators were: acclimation, gill and liver Cu concentration, and changes in gill-copper binding characteristics. Confirmation that gill-Cu burdens and binding characteristics were reliable indicators lends support to the BLM, and offers the most promise for assessing the effect of chronic copper exposure. The presence of increased resistance to Cu in wild populations of fish may also be a reliable indicator of chronic copper exposure in hard water; however it may not be relevant to fish inhabiting soft water environments. In soft water, the combined ionoregulatory stress of increased Cu toxicity and ion poor environments may favor lethality over acclimation. The physiological indicators which were deemed insensitive may be less reliable because of the important effect of ration quantity (and possibly quality) on their expression.

The main objective of Chapter 3 was to use two measures of susceptibility to predict the sublethal and lethal effects of copper in rainbow trout. The hypothesis tested was that healthier fish are less susceptible to the toxic effects of copper. The indicators

used to assess fish health were growth and sodium permeability measured before Cu exposure. Coincidentally, these two health measures are also the most prominent effect indicators of chronic Cu exposure documented in fish (e.g., DeBoeck *et al.*, 1997; Waiwood & Beamish, 1978; Marr *et al.*, 1996; Buckley *et al.*, 1982; Laurén & McDonald, 1985, 1987). All fish were implanted with passive integrative transponder (PIT) tags so that each fish was individually identifiable. A 'healthy' freshwater fish was operationally defined as one that exhibits a high growth rate and low sodium permeability. The sublethal effects of copper were evaluated using activity levels in the gills of an ion transport enzyme ($Na^+/K^+$-ATPase), tissue copper concentrations (in the gill, liver, gut and whole body), whole body sodium levels, and the specific growth rate during copper exposure. Acute toxicity (as measured by time to death) was assessed by the copper content in the gill, carcass and whole body, whole body sodium concentrations and the final weight of the fish. The measures of health were then related by correlation analysis to each indicator of copper toxicity.

Chapter 3 was novel in that it used biomarkers, not in the traditional sense to indicate effect or exposure, but to measure fish health and thereby predict the susceptibility of fish to copper toxicity. Under chronic exposure (i.e., one week at a sublethal Cu concentration), the growth rate of fish pre-exposure did influence their susceptibility to copper as shown by the traditional biomarker growth during the exposure. Also, a fish's permeability to sodium was predictive of gill Cu concentrations and the growth rate during the exposure to Cu. The measures of whole body sodium, carcass Cu, liver Cu and $Na^+/K^+$-ATPase activity were not sensitive indicators of effect

and would not improve the risk assessment procedure for Cu toxicity. In contrast, during an acute Cu exposure (i.e., lethal within 25 hours), a fish exhibiting a high growth rate was not less susceptible than a poorly growing fish; however, fish weight, whole body sodium and gill copper accumulation were more relevant to the lethal resistance time. In brief, the assessment of fish health was an effective measure of susceptibility for sublethal toxic effect, but not for lethal toxic effect (i.e., its predictive power was largely dependent on the severity of challenge).

*The Influence of Water Chemistry*

Fish gills are the initial target of waterborne copper toxicity (Lauren & McDonald, 1985, McDonald & Wood, 1993, Wood, 2001). The gill surface interaction model (GSIM) originally proposed by Pagenkopf (1983), aimed to explain the variability associated with trace metal toxicity by incorporating the differences in metal speciation, alkalinity, hardness and pH with gill-metal interactions. The 1983 study provided the necessary framework upon which the BLM was conceived. In brief, the BLM represents metal interactions with competing cations and complexing ligands found in natural waters and predicts metal accumulation at the gill. The level of accumulation is then used to predict acute toxicity and perhaps with future development of the BLM will eventually be used to predict chronic toxicity. Researchers have been well aware for many years of the influence of water chemistry parameters such as hardness, pH, alkalinity and DOM on copper toxicity (e.g., Zitko *et al.*, 1976, Howarth & Sprague, 1978, Chakoumakos, 1979, Miller & Mackay, 1979, Laurén & McDonald, 1986, and

Erickson *et al.*, 1996). In general, increases in water hardness, pH, alkalinity and DOM reduce the toxic effects of copper , presumably by reducing copper accumulation at the gill by either competition or complexation. Competition is when cations such as $Ca^{2+}$, $Na^{+}$ and $H^{+}$ compete with $Cu^{2+}$ for binding sites on the biotic ligand. In contrast, complexation is when ligands, such as $CO_3^{-2}$, $OH^{-}$ and DOM bind $Cu^{2+}$, thereby making copper less available for binding to the biotic ligand.

The main objective of Chapter 4 (Taylor *et al.*, 2002) was to characterize the individual effects of $Ca^{2+}$, $Na^{+}$, pH, alkalinity, and DOM on the initial (rapid) binding of copper to the gill surface. To focus primarily on the surface reactions of metal to tissue, a rapid 5-minute *in vitro* gill binding assay was employed. The use of isolated gill arches minimized any changes in water chemistry caused by the organism itself (i.e., through excretion, mucus sloughing, general body surface binding). The method, modified from that of Reid & McDonald (1991), involved using radiolabelled copper ($^{64}Cu$) at environmentally realistic and toxicologically relevant concentrations, in order to determine the 'newly accumulated' gill copper (against a background level). The method itself was validated before evaluating the effects of water chemistry, and this validation examined the changes in gill-Cu binding with time, the relationship between gill-Cu binding and water Cu concentrations, the displacement of copper from the gill surface, the resemblance to *in vivo* gill binding, and lastly, the influence of fish size on gill-Cu binding. The time-course study revealed a less variable and saturable response at exposure times of five minutes, beyond which the amount of binding increased as well as the variability. These saturable sites were identified as high affinity and low capacity,

thus confirming previous findings in Chapter 2 (Taylor *et al.*, 2000), and reflected at least 50% surface binding (the other 50% is presumed to have entered the gill tissue). Fish size had a large effect on gill-Cu binding, however the effect was largely evident in fish less than 25 grams. Coincidentally, the traditional *in vivo* gill binding techniques by Playle *et al.* (1993a, b) and MacRae *et al*, (1999), three studies used in the development of the Cu BLM, used fish ranging below this size.

Interestingly, water chemistry parameters influenced rapid gill-metal binding in a manner different from their influence on acute toxicity. This was evident by the absence of a protective effect from the competing ions ($Ca^{2+}$, $Na^{+}$ and $H^{+}$), no effect of alkalinity or high pH, and the fact that a commercial DOM did not completely prevent copper binding to the gills. Currently, the BLM uses the rapid increase of gill copper over 3-24 hours to model gill binding characteristics, since it is believed to reflect binding to physiologically active and toxicologically relevant receptor sites (DiToro *et al.*, 2000). The distinction between rapid surface binding and metal uptake plays an important role in determining the toxic effects of copper, which is especially relevant for setting site-specific water quality criteria based on the modifying effects of water chemistry.

*Species Differences*

It has long been recognized that different fish species can vary in tolerance to waterborne copper. Two families that are different in their sensitivity are Salmonidae and Percidae. According to Spear & Pierce (1979), salmonids can be up to one hundred times more sensitive than Perciformes. In rainbow trout (a salmonid species), the

mechanism of acute copper toxicity is clearly understood as an ionoregulatory disturbance. The yellow perch (*Perca flavescens*), a member of the Percidae family (but one not included in the 1979 review), is endemic to metal contaminated areas such as the Sudbury region in northern Ontario, a historical metal mining and smelting location. In yellow perch, the mechanism of Cu toxicity may be distinct from that in rainbow trout and in turn, may explain the documented difference in Cu tolerance between the two families.

The main objective of Chapter 5 (Taylor *et al.*, submitted) was to use a species comparison approach to understand differences in sensitivity to copper. First, the acute toxicity of copper (via 96 hour LC50s) was determined for each fish species in two different water types (hard and soft water), to confirm differences in tolerance. Next, the pattern of ion loss was examined in both species to understand the mechanism of toxicity and the mechanism of tolerance. Finally, gill-Cu binding characteristics and the accumulation of copper at the gill at the LC50 (termed the lethal accumulation at the concentration which caused 50% mortality or LA50) were compared between yellow perch and rainbow trout. Work in this chapter explicitly addressed three key questions: first, is the mechanism of copper toxicity different between rainbow trout and yellow perch? Second, what is the basis for the difference in tolerance? And third, does gill-Cu binding predict the difference in tolerance? The hypothesis was that species differences in toxicity would be reflected in the binding of copper to high affinity sites on the gill, the modeled target of the BLM.

In comparing the acute toxicity values for both fish species, copper was approximately four times more toxic to rainbow trout than to yellow perch in hard and soft water. However, the mechanism of copper toxicity is apparently not different between the two species. Specifically, the threshold for toxicity was reached when both species lost ~30-40% of their whole body sodium and at 60% sodium loss complete mortality ensued. As for the mechanism of tolerance, I demonstrated that yellow perch can resist $Na^+$ loss, however, this resistance was not a result of reduced copper binding at the gills. Rather, the concentration of 'newly accumulated' copper at the gills did not translate into toxicity, but more likely the surplus Cu was dealt with effectively by detoxification, elimination and storage mechanisms. The influence of water chemistry on acclimation (particularly soft water) and the binding properties of the gill demonstrates the dynamic nature of the gill in maintaining ionoregulatory homeostasis. This gill modification will become a key issue in the future development of the chronic BLM, especially since the process of soft water acclimation may only be relevant to laboratory fish used to advance the model, rather than to fish in their natural environment.

In Chapter 6, I applied lessons learned in the laboratory to a field population of copper contaminated yellow perch. The objective of this work was to evaluate the effects of chronic metal exposure on swim performance and gill-Cu binding characteristics in fish collected from lakes in the Sudbury region of northern Ontario. Different types of swim performance were evaluated using two different tests, namely the critical swimming speed or $U_{crit}$ test and the fixed velocity sprint test. Gill-copper binding was characterized using the short-term binding technique of Playle *et al.* (1993a,b), to

compare the results directly to the studies used in the development of the BLM. Yellow perch collected from metal contaminated lakes were tested in relation to perch populations from lakes containing low levels of metals (i.e., reference site lakes). The comparison of yellow perch populations from a contaminated and reference lake maximized the probability that any observed differences were caused by the environmental gradient. The hypothesis tested was that the acclimation processes which permit yellow perch to inhabit metal contaminated lakes would lead to improved swim performance and altered gill-Cu binding characteristics, compared to yellow perch living in reference lakes.

In total ten measures were used to describe the physiological state of the groups of yellow perch before any testing. These measures were of fish weight, length, condition factor, liver Cu, gill Cu, and carcass Cu concentrations, carcass sodium, plasma sodium and chloride, and muscle glycogen. Significant differences were detected among background tissue copper concentrations, with the perch from contaminated lakes having higher levels than the reference site fish. The concentration of copper in the liver roughly corresponded to the difference in water copper concentrations between lakes; however, the gill did not consistently show the same relationship. Condition factor, carcass sodium concentration and plasma ions were either not sensitive enough or were not consistent in their response among contaminated and reference site yellow perch. These three indicators, considered inadequate for identifying metal effects can vary with diet, which can be highly variable between lakes especially where metals have direct or indirect effects on invertebrate or other prey items. Muscle glycogen levels were also useful to

differentiate between the groups of wild yellow perch, but their significance may be limited to a relatively small effect on swim performance.

The swimming performance study revealed only a subtle effect of metal contamination on endurance because the effects of size could not be isolated from the effects of chronic metal exposure. Plasma ion regulation was not impaired after any exercise challenge. Yellow perch from the contaminated lake showed acclimation, as represented by a significantly longer survival time during an acute challenge to waterborne copper. Their resistance was in part due to a larger body size. The fact that these yellow perch were able to survive longer was supported further by physiological evidence, such as the absence of an increased whole body copper concentration and decreases in whole body sodium concentration from background levels. Surprisingly, these resistant perch had approximately three times higher gill copper concentrations at death than reference site fish. Apparently, wild yellow perch can tolerate copper on the gill without the usual toxic consequences, confirming previous findings in laboratory yellow perch (Chapter 5).

Perch from the contaminated lake also had lower rates of sodium loss during the exposure to a range of copper concentrations. The fact that these larger yellow perch were able to resist sodium loss was likely a contributing factor to their survival at acutely lethal concentrations. This resistance to copper-induced sodium loss was the same mechanism observed in laboratory yellow perch (Chapter 5; Taylor *et al.*, submitted). There was no effect of chronic metal exposure on the binding properties of the gill over the sublethal range of 10 to 350 $\mu$g/L (i.e., sublethal during the 3-hour gill binding assay),

but more importantly the accumulation of copper on the gill at death was affected. This distinction between the effect of chronic metal exposure on the accumulation of Cu at death and using binding properties to predict gill-Cu concentrations has strong implications for both the validation of the acute Cu BLM and the development of a chronic BLM.

In summary, my research reduced some of the uncertainty associated with the three main areas in which safety factors and the Precautionary Principle are applied to waterborne Cu. My thesis contributes to the available scientific information on chronic toxicity, the influence of site-specific water chemistry, and the extrapolation of laboratory model organisms to resident species. The usual effect indicators of acute toxicity may be of limited value, in their current form, in evaluating chronic toxicity. The complexity of chronic toxicity reaches far beyond the simple connections between acute effects and mortality. It now requires the translation of subtle physiological effects of Cu into an impact on fish growth, reproduction or fecundity, thereby affecting fish populations or aquatic food chains, and involves the assessment of waterborne and dietary routes of Cu exposure. The influence of water chemistry parameters on copper bioavailability is the one area most advanced in the last ten years, at least in part due to the development of the Biotic Ligand Model (Playle *et al.*, 1998, DiToro *et al.*, 2001, Santore *et al.*, 2001). The impact of water constituents, however, are likely different on acute toxicity, rapid gill surface binding, metal uptake, and metal accumulation. In my opinion, the two final research chapters, which contain the laboratory and field studies using yellow perch, provide the greatest contribution to the 'best available' science because my experiments

show that although the mechanism of toxicity may be identical to that of a model species (i.e., the rainbow trout), the mechanism of tolerance can greatly alter the physiological indicators used to detect copper toxicity.

## CHAPTER 2

# THE PHYSIOLOGICAL EFFECTS OF CHRONIC COPPER EXPOSURE TO RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) IN HARD AND SOFT WATER: AN EVALUATION OF CHRONIC INDICATORS


## ABSTRACT

Effects of chronic copper exposure on a suite of indicators were examined: acute toxicity, acclimation, growth, sprint performance, whole body electrolytes, tissue residues, and gill copper binding characteristics. Juvenile rainbow trout were exposed for 30 days to waterborne copper in hard water (hardness=120 mg/L as $CaCO_3$, pH = 8.0, Cu = 20 and 60 $\mu$g/L) and soft water (hardness=20 mg/L as $CaCO_3$, pH=7.2, Cu=1 and 2 $\mu$g/L). Significant acclimation to the metal occurred only in fish exposed to 60 $\mu$g/L, as seen by a ~ two fold increase in 96h LC50 (153 vs. 91 $\mu$g Cu/L). Chronic copper exposure had little or no effect on survival, growth or swimming performance in either water hardness, nor was there any initial whole body electrolyte loss ($Na^+$ and $Cl^-$). The present data suggest that the availability of food (3% wet body weight/day, distributed as three 1% meals) prevented growth inhibition and initial ion losses that usually result from Cu exposure. Elevated metal burdens in the gills and livers of exposed fish were measures of chronic copper exposure but not of effect. Initial gill binding experiments revealed the necessity of using radiolabelled Cu ($^{64}Cu$) to detect newly accumulated Cu against gill background levels. Using this method, we verified the presence of saturable

Cu binding sites in the gills of juvenile rainbow trout and were able to make estimates of copper binding affinity (log $K_{gill-Cu}$) and capacity ($B_{max}$). Furthermore, we showed that both chronic exposure to Cu and to low water calcium had important effects on the Cu binding characteristics of the gills.

**Key Words:** Copper, Rainbow trout, Toxicity

## INTRODUCTION

The acute toxicity of copper to freshwater fish has been well characterized (Alabaster and Lloyd, 1982; Spear and Pierce, 1979; Sorenson, 1991). Published values for 96-h LC50s range from as little as 10 $\mu$g/L to 10 000 $\mu$g/L total copper. Once species sensitivity differences are accounted for, most of the variation can be attributed to differences in water chemistry (particularly hardness), and secondarily to body size (Spear and Pierce, 1979). For example, Howarth and Sprague (1978) reported that the concentrations of total dissolved copper which produced 50% mortality in 96 hours (96 h LC50) for rainbow trout ranged from 20 $\mu$g/L in soft acidic water to 520 $\mu$g/L in hard alkaline water. In the same study, 10 g trout were 2.5 times more tolerant to Cu than were 0.7 g trout (Howarth and Sprague, 1978).

The effects of chronic copper exposure in fish have also been well documented in the literature (e.g. Dixon and Sprague, 1981; Farag *et al.*,1995; Marr *et al.*, 1996; Lett *et al.*, 1976; Buckley *et al.*, 1982) and include a whole variety of biochemical and physiological indicators, of which growth and ionoregulation are the most prominent. However, there is as yet no consensus as to the ranking of these effects in order of sensitivity. An early ranking by Spear and Pierce (1979) is now of limited value because it was drawn from several studies that each measured only a few indicators and where there was no standardization of Cu exposure (e.g. age of the organism, water quality, diet/nutritional status, temperature changes). Furthermore, there are at least three newer indicators of copper exposure that are worthy of inclusion in any rank-order. These indicators are tissue Cu residues, the presence/absence of acclimation, and gill Cu

binding characteristics. Tissue residues have proven to be a useful indicator of copper exposure in field collected fish (Farag *et al.*,1995; Miller *et al.*, 1992). Gill metal binding characteristics have been used to relate the fractional saturation of high affinity metal binding sites on gills directly to acute toxicity (the so called 'Biotic Ligand Modelling (BLM)' approach, Pagenkopf, 1983; Playle *et al.*, 1993a,b; Bergman *et al.*, 1997; MacRae, *et al.*, 1999) and may, in turn, be a sensitive indicator of chronic metal exposure. Acclimation can be used as a chronic indicator since it probably reflects copper exposure at a concentration which at least initially was sufficient to induce damage - the "damage-repair" hypothesis (McDonald and Wood, 1993).

Thus the objective of the present study was to document the effects of chronic copper exposure on a larger suite of indicators than has previously been attempted and to propose a new rank order of the effects of copper (c.f. Spear and Pierce, 1979). The indicators used were: acute toxicity, acclimation, growth, sprint performance, whole body electrolytes, tissue residues, and gill copper binding characteristics. Exposure concentrations were chosen in relation to initial 48- or 96 h-LC50s and were based on the hypothesis that some mortality or physical damage is necessary to elicit compensatory mechanisms (McDonald and Wood, 1993). To modify Cu bioavailability the exposures were conducted in hard and soft water. The hard water exposure provided an abundance of the necessary cations ($Ca^{2+}$ and $Mg^{2+}$) to compete with Cu for binding sites on the gill, and anions ($HCO_3^-$) to compete with gill sites for complexation of copper (Pagenkopf, 1983). The exposure in soft water provided an environment low in such ions and

therefore increased Cu toxicity. Exposure to soft water alone also provided a physiological challenge to fish acclimated to hard water (McDonald and Rogano, 1986).

## MATERIALS AND METHODS

### *Experimental Animals*

Juvenile rainbow trout (*Oncorhynchus mykiss,* 1-2 g) were obtained from Rainbow Springs Hatchery (Thamesford, ON, Canada), and were maintained in either dechlorinated Hamilton tap water of moderate hardness (14° C, pH 8, $Na^+$ 13.8, $Cl^-$ 24.8, $Ca^{2+}$ 40.0, DOC 3, hardness 120, alkalinity 95, all in mg/L) or soft water (17° C, pH 7.2, $Na^+$ 3.0, $Cl^-$ 3.5, $Ca^{2+}$ 5.2, DOC 0.4, hardness 20, alkalinity 15, all in mg/L) for a minimum of two weeks before experimentation. The 3°C temperature difference between the two experiments reflect ambient Lake Ontario water temperatures in winter and summer. Soft water was synthesized by mixing one part hard water to six parts ion-reduced water, the latter produced by reverse osmosis (Anderson Water Systems, Dundas, ON, Canada). Photoperiod was set to a light/dark cycle similar to the natural photoperiod for Western Lake Ontario.

### *Experimental Protocol*

i) Copper Exposure in Hard Water

Fish were exposed to two Cu concentrations (20 and 60 $\mu$g/L), with a control (background Cu ~3 $\mu$g/L) in a flow-through system for a minimum of 30 days. These concentrations were chosen from initial rangefinder 48h LC50 tests (Fig. 1) using naïve

fish, and were chosen to produce some mortality in the high Cu concentration and no mortality in the lower exposure. The high and low exposure concentrations correspond to approximately half and one-sixth of the initial 48h LC50 for Cu.

Fish were fed a dry ration of commercial trout pellets, at a rate of 3% wet body weight/day, distributed as three meals of 1% /day for the entire period of exposure. The feed contained ~3 $\mu$g/g Cu dry weight (Martins Feed Mill, Elmira, ON, Canada). Growth was monitored weekly through bulk weighing of fish in individual tanks, and the meal size was adjusted as the fish grew to maintain the 3% ration. Copper stock ($CuSO_4 \cdot 5H_2O$, Fisher Scientific, Toronto, ON, Canada) was metered into diluent water in two mixing head tanks. These tanks, in turn, fed two separate 200 L tanks at a rate of 1500 ml/min to each. Each exposure tank was aerated and initially contained 280 fish. Feces and organic matter were siphoned from the tanks daily. Copper concentrations in the fish tanks were measured twelve times throughout the exposure (Table 1). At Day 2, 10, 20, and 30, a subsample (N = 10) of fish from each tank (5 fish per tank, two tanks per concentration) were sacrificed. Gills were excised and rinsed for 10 s in clean control water to displace any surface bound Cu and were immediately frozen. Livers were also removed and together with the remaining carcass were frozen for subsequent metal-burden and electrolyte analysis.

ii) Copper Exposure in Soft Water

This exposure used the same flow-through system, feeding regime, and growth monitoring as outlined above. The two exposure concentrations of Cu were 1 and 2 $\mu$g/L

based on an initial 96h LC50 rangefinder using naïve fish acclimated to soft water for two weeks (Fig. 1). The trout had been acclimated to soft water for a total of seven weeks at the start of the exposure. The Cu exposures correspond to approximately one-half and one-quarter of the lethal concentration, respectively. A control (background Cu ~0.7 $\mu$g/L), was also run simultaneously with the copper exposed fish. Each tank held 225 fish and fresh soft water was supplied to the exposure tanks at a rate of 500 ml/min and a temperature of 17 °C. Water and tissue sampling was the same as in the hard water exposure except that three instead of five fish were sampled from each tank per interval (N = 6 for each concentration). Also, three intact whole bodies from each tank were sampled and frozen for subsequent metal-burden and electrolyte analysis.

*Analytical Techniques*

i) Sample analyses

Tissue samples were weighed and digested in five volumes of 1N $HNO_3$ (Fisher Scientific, Toronto, ON, Canada, trace metal analysis grade) for 3 h at 80°C. Copper was measured in whole bodies, liver and gills, after appropriate dilution of the supernatant with reagent grade de-ionized water, using flame and graphite furnace atomic absorption spectrophotometry (AAS, Varian AA-1275) against Fisher Scientific certified copper standards (Fisher Scientific, Toronto, ON, Canada). Whole body Cu burdens in hard water were calculated by adding the removed tissues and the carcass metal content. Sodium was analyzed from whole body digests following the same process as Cu. Chloride was determined from whole body digests without further dilution by titration

with a Radiometer-Copenhagen CMT-10 chloridometer (Copenhagen, Denmark). Water samples (15 ml) were acidified (50 $\mu$l concentrated $HNO_3$) and analyzed for Cu by either flame or graphite AAS, as appropriate.

ii) Sprint Performance

Fixed velocity sprint tests were conducted according to procedures described by McDonald *et al.* (1998) using a 100 L swim flume constructed of non-toxic polyethylene vinyl-chloride. Fish were not fed on the day of sprint trials and were transferred in batches of ten to the flume containing either hard or soft control water. Water temperature in the swim flume was identical to the exposure system from which fish were removed. After an initial orientation period of 5 min at 1 body length (BL)/s, water velocity was increased over 2 min to 6 or 8 BL/s in soft and hard water, respectively. Fish were removed when they became exhausted, and time to fatigue, fork length, and fish weight were recorded. Fish were considered exhausted when they became impinged on the back screen and would not swim after being manually reoriented towards the current.

iii) Gill Cu Binding Characteristics

Three different approaches were used to measure short term Cu uptake by the gills *in vivo:* i) 3 h exposure to hardwater Cu solutions ranging in concentration from 30 to 1050 $\mu$g/L Cu (N = 6 per exposure), ii) 0.5 to 2 h exposures to hardwater Cu solutions ranging from 120 to 430 $\mu$g/L Cu (N=4 per exposure and time interval) with radio-labelled $^{64}Cu$ (specific activity 3.9 to 16.1 KBq/$\mu$g), and iii) 3h exposures to $^{64}Cu$ labelled solutions ranging from 2 to 30 $\mu$g/L Cu (specific activity of 79 to 407 KBq/$\mu$g)

in hard water (N=4 per exposure) and 1 to 17 $\mu$g/L (specific activity of 73 to 578 KBq/$\mu$g) in soft water (N=10 per exposure). $^{64}Cu$ was supplied by the McMaster Nuclear Reactor, Hamilton, Ontario.

The first approach was derived from the method of Playle *et al.* (1992) for measuring gill surface metal binding *in vivo* in soft water. An exposure time of 3h was used because Playle *et al.* (1992) found that gill Cu levels *in vivo* had reached equilibrium at this time. Relatively high Cu levels were used to allow detection of gill Cu above background levels and also to ensure that sufficient free $Cu^{2+}$ ion was available in the water for binding to the gills in hard water. Background gill Cu (i.e. gill Cu before exposure) was subtracted from the amount found on the gill after the 3 h exposure to determine the 'newly' accumulated copper. The second approach was employed to increase the resolution of gill Cu measurement in hard water. The third approach was used because the first two approaches did not reveal saturable gill binding sites on the gills. Radioactivity in gill tissues, whole bodies, and acidified water samples was measured in a well-type gamma counter with a 3" NaI crystal (Packard Minaxi Auto-Gamma 5000 Series).

*Calculations*

i) Growth

Growth was calculated from periodic measurements of bulk weights. The data were best represented by the exponential curve:

$$wt(g) = ae^{bt}$$

where t = time (days), a = length (cm), b = growth coefficient, expressed as %/day. The 95% confidence limits for b were calculated using the statistical package SPSS for Windows, Release 8.0.0.

ii) Swimming Performance

The following steps were used to calculate fatigue times (FT) in the sprint tests according to procedures outlined in McDonald *et al.* ( 1998). Sprint times were first sorted in ascending order against rank. Time was converted to log time, and rank was converted to % fatigue and then to probit fatigue. A linear regression of probit fatigue (X) *vs.* log time (Y) yielded slope ± 95% CL and intercept ± 95% CL for the line. These data were used to calculate the time to 50% fatigue (FT50) with 95% confidence limits.

iii) Gill Cu Binding

Newly accumulated copper concentrations in the gill were calculated based on the accretion of radioactivity in the gill:

$$Cu_{gill} = \frac{^{64}Cu_{gill}}{(W \times SA)}$$

where $^{64}Cu_{gill}$ = radioactivity of the gill in KBq per minute, W = gill mass ($\mu$g), and SA = specific activity of the water in KBq/$\mu$g.

*Statistics*

All data presented are means $\pm$ 1 SEM (N) except for LC50, growth, and swim performance where means and 95% confidence limits are given. For the exceptions, a

Student's t-test (two tailed, unpaired) was used to test for significant differences, employing a Bonferroni adjustment for multiple comparisons to the t value. LC50 values were determined by linear functions relating the log concentration of copper to probit transformation of percent mortality (U.S. Environmental Protection Agency probit analysis program used for calculating lethal concentration/effect concentration values, Version 1.5). All other data were analyzed for statistical significance by ANOVA followed by a Student-Newman Keuls ranking test for means of equal and unequal sample size. Significance was set at $P < 0.05$.

## RESULTS

*Acute toxicity*

Based on published LC50 data for salmonids (Fig. 1), our rainbow trout population was particularly sensitive to Cu, especially in soft water. Both our hard water and soft water LC50s for control fish were well below the incipient lethal concentrations for other rainbow trout (Line 1, Fig. 1), and in soft water were even lower than the most sensitive salmonid species (Line 2, Fig. 1), as reported by Spear and Pierce (1979).

Furthermore, while we confirm that Cu was more toxic in soft water than in hard water, the effect of hardness was much greater in our study than in previous studies. Copper was approximately twenty times more toxic in soft water than in hard water for 1 to 2 g trout, whereas the literature predicts only a five fold difference (Fig. 1, Line 1 or 2). However, after ten weeks of further acclimation in soft water, trout were only six times more sensitive to Cu in soft water than in hard water.

*Chronic Toxicity*

i) Mortality

In hard water with 60 μg/L Cu, 3% of the fish died during the exposure period, whereas the controls and the fish exposed to 20 μg/L Cu had exhibited less than 0.5% mortality. Most of the Cu induced mortalities occurred within 5 days of exposure to copper in hard water. In contrast, in soft water, mortality was 10% after 15 days of exposure in all three treatments (i.e. independent of Cu exposure) and continued at a low rate until day 30.

ii) Acclimation

In hard water, only the 60 μg/L Cu group showed significant acclimation to Cu after 30 days exposure. These fish were approximately 1.7 times more resistant to Cu than were control fish (96h LC50 of 153.0 μg/L (95% confidence limits, 147.8 - 165.0) vs. 96h LC50 of 91.0 μg/L (65.9 - 107.6)). In contrast, neither exposure to Cu in soft water resulted in a significant increase in copper tolerance: the LC50s for trout in soft water averaged 16 μg/L after 30 days exposure.

*Performance Measures*

i) Growth

Even though our fish were particularly sensitive to copper in terms of acute toxicity, overall growth of the survivors was not affected throughout the metal exposure in either hard or soft water (Table 2.). There was also no evidence of an initial growth reduction during the first ten days of Cu exposure. Growth rates in hard water ranged

from 2.9 to 3.0 %/day from a starting weight of 1 to 2 g and from 3.1 to 3.4 %/day in soft water from a larger starting weight of 5 to 6 g. The slightly higher growth rate in soft water was probably due to the 3°C higher temperature.

ii) Sprint Performance

Sprint performance was not impaired by Cu exposure in either hard or soft water (Table 2). In fact, fish exposed to Cu sprinted for longer times than unexposed fish.

*Condition Measures*

i) Whole Body Electrolytes

Immediately before Cu exposure, whole body $Na^+$ and $Cl^-$ concentrations were identical between hard and soft water fish ($Na^+$ and $Cl^-$ ~50 and 40 $\mu$M/g, respectively). Upon Cu exposure, there was no initial or subsequent decline in whole body electrolytes in either hard or soft water.

ii) Tissue Residues

Before Cu exposure, the body Cu content and its distribution were similar in both hard- and softwater fish. The liver had the highest levels of Cu (up to 15 $\mu$g Cu/g wet tissue in controls) of any tissue measured, which represents 10 and 22% of the whole body content (0.78 $\mu$g Cu/g) in hard and soft water, respectively. Gill Cu was lower than liver Cu, and was the virtually the same in hard- and softwater fish (1.4 % of the whole body content), despite the fact that background gill Cu in hard water was six fold higher than in soft water (3.0 vs. 0.5 $\mu$g/L).

Gills of fish exposed to 60 $\mu$g/L copper in hard water had significantly elevated Cu at all times starting at day 2 (Fig. 2). In contrast, gill Cu of fish exposed to 2 $\mu$g/L in soft

water were only significantly higher than controls at day 30. In general, gill Cu burdens reflect the exposure Cu concentrations (i.e. four fold higher Cu burden in hard water vs. soft water at the highest Cu exposure level, Fig. 2).

In contrast, the livers did not show this difference between hard and soft water, with ~35 $\mu$g Cu/g wet tissue at the highest Cu exposure concentrations (Fig.3). Nonetheless, the liver burdens did increase over time with Cu exposure within each water chemistry. In hard water, liver Cu of the fish exposed to 60 $\mu$g/L was significantly elevated at day 2, 20 and 30. Livers in the 2 $\mu$g/L soft water treatment showed significantly higher Cu content only at day 20 and 30. Although the gills and livers accumulated Cu, the amounts were small enough to have no detectable effect on whole body Cu above the increase seen with growth. In hard and soft water at Day 30, the whole body Cu burden in all fish averaged 1.1 and 1.7 $\mu$g Cu/g wet tissue, respectively.

*Gill Cu Binding Characteristics*

i) Naïve fish

Hardwater acclimated fish, naïve to Cu, and exposed for 3h to cold copper (30 to 1050 $\mu$g/L, no $^{64}$Cu) showed no detectable accumulation of Cu on their gills (i.e. beyond background levels) with exposures up to 185 $\mu$g Cu/L (Fig. 4A). Only at 1050 $\mu$g Cu/L was there significant accumulation (~six fold *vs.* background of 0.5 $\mu$g/g). The second experiment using $^{64}$Cu (120 to 430 $\mu$g/L, Fig. 4B), was able to detect the accumulation of new Cu at 120 $\mu$g Cu/L and showed that Cu accumulation was approximately linear over the range of Cu employed at each of the exposure times tested (0.5, 1 and 2 h). However,

the amount bound to the gills at each concentration also increased with time (Fig. 4B) suggesting either that the loading of Cu on surface sites was slow, or more likely, that Cu was going beyond surface sites to enter the gill tissue.

Only in the third experiment (2 to 25 $\mu$g Cu/L) did we see clear evidence of saturable binding of copper to the gills in both hard and soft water (Fig. 4C). The binding sites were high affinity and low capacity (but higher capacity in soft water compared to hard water), and saturated at ~15 $\mu$g/L Cu. Indeed, at only slightly higher Cu (~23 $\mu$g/L) in hard water there was a sharp increase in gill Cu suggesting the appearance of a second type of binding on (or in) the gills. In fact, the value at 23 $\mu$g/L fits directly on the curve established in the second experiment for gill binding by 2h (Fig. 4B).

The binding characteristics of the high affinity sites (binding capacity, $B_{max}$ and affinity, $K_{gill=Cu}$) were approximated as follows: first, the $B_{max}$ values in hard and soft water were estimated visually to be 0.038 and 0.12 $\mu$g Cu/g, respectively (i.e. the point on the y-axis where the concentration of Cu on the gill does not increase when the water Cu concentration is increased, see Fig 4C). Secondly, a Langmuir adsorption isotherm:

$$\text{Gill Cu} = \frac{B_{max} \times [Cu]}{(K_{gill=Cu} + [Cu])}$$

was fitted to the data using the estimated values for $B_{max}$ ($\mu$g/g) , [Cu] = total dissolved copper ($\mu$g/L), and gill Cu ($\mu$g/g). Finally, the binding affinity ($K_{gill=Cu}$) was determined from the isotherms as the concentration of total [Cu] needed to produce half-saturation of binding sites. This method yielded $K_{gill=Cu}$ of 2 and 1 $\mu$g/L Cu for hard and soft water, respectively.

ii) Effects of Chronic Cu Exposure

Chronic Cu exposure in hard water had distinct effects on gill Cu dynamics. 'Chronic' gills accumulated more Cu than naïve gills when gills were challenged with Cu concentrations substantially above acutely toxic levels (i.e. an increase in gill Cu binding capacity, Fig. 5A). For example, at an acute Cu of 185 $\mu$g/L, the 'chronic gills' (chronically exposed to 20 and 60 $\mu$g Cu/L) exhibited net Cu accumulation of 0.63 ± 0.18 and 0.86 ±0.26 $\mu$g/g, respectively (i.e. a doubling of Cu over background levels) while the naïve gills showed no net accumulation (Fig. 5A). However, when challenged at near chronic levels this effect was less dramatic (more so at 60, than at 20 $\mu$g/L, Fig. 5B). For example, at 16 $\mu$g/L the 'chronic' gills (60 $\mu$g/L) accumulated about 1.5 times the amount accumulated by naïve gills (i.e. an increase in binding sites, Fig. 5B). In contrast, the 20 $\mu$g/L exposure group accumulated the same amount as naïve gills at 16 $\mu$g/L (i.e. no increase in binding sites, although the data suggest a lower affinity).

In soft water a similar pattern was seen, particularly at the 2 $\mu$g/L chronic level (Fig. 5C). Here, there was a substantial alteration in gill Cu binding properties from saturable to apparently linear ($R^2 = 0.940$) over the range of [Cu] tested. This change is consistent with a lowering of affinity and an increase in capacity (or number of) binding sites on or in the gills. The reduction of affinity is well illustrated at the 7 $\mu$g/L exposure concentration where there was a ~40% reduction in newly accumulated Cu in fish pre-exposed to 2 $\mu$g Cu/L, relative to naïve fish.

## DISCUSSION

Because we measured a suite of different indicators in the same study we can now propose a new rank-order for the chronic effects of copper, one substantially different from that proposed originally by Spear and Pierce (1979). We rank the sensitivity of each indicator by considering the number of statistical differences between Cu exposed and control fish in the four exposure regimes considered (i.e. two Cu concentrations and two water hardness levels). By this criterion the least sensitive indicators are growth, sprint performance, and initial electrolyte loss (i.e. no effect in any treatment group). The others, in order of increasing sensitivity are: acclimation (i.e. increased lethal tolerance, 1 out of 4, difference being statistically significant), increased gill and liver Cu burdens (2 out of 4), and changes in gill-copper binding characteristics (3 out of 4). However, this new rank-order must be considered conditional because of two unusual responses exhibited by our rainbow trout compared to previous studies on the same species: a much greater sensitivity to acutely toxic copper (Fig. 1) but a lower chronic sensitivity to copper.

### *Acute sensitivity to copper*

In their review of the acute toxicity literature extant in 1979, Spear and Pierce (1979) concluded that the Cu sensitivity of most salmonids, including rainbow trout, could be captured in a single equation relating 96h LC50 to water hardness (Fig 1, Line 1). Our results, in contrast, deviated significantly from this line in two respects (Fig. 1, Line 3): a 3 fold greater sensitivity in typical 'hard' waters (120 mg/L as $CaC0_3$) and 20

fold greater sensitivity in very dilute soft waters (20 mg/L). We attribute the higher acute sensitivity in hard water to strain variance (Grande, 1967). In any case, the acute sensitivity that we observed in hard water is at least comparable to that of a second group of salmonids identified by Spear and Pierce (1979; 'sensitive' salmonids, Line 2). The much greater sensitivity of our fish in soft water we attribute mainly to incomplete soft water acclimation, as illustrated (Line 3).

*Sensitivity to soft water*

Previous studies, including our own, have shown that acute exposure of hardwater-acclimated trout to soft water by itself produces physiological disturbances that consist of depression in whole body electrolytes followed by gradual recovery (McDonald and Rogano, 1986). More importantly, acute soft water exposure can substantially amplify the effects of toxicant exposure especially if the toxicant targets ionoregulation (McDonald and Milligan, 1997). For this reason, prior acclimation to soft water is usually recommended in toxicity studies. There seems to be a consensus that a two week period of acclimation is large enough (e.g. Marr *et al.*, 1996; Laurén and McDonald, 1986) but there is still no study that has documented how much time is required for acclimation to be complete. Indeed, Erickson *et al.* (1997) found that two weeks acclimation to soft water had no effect on the relationship between hardness and acute (96h LC50) Cu toxicity in fathead minnows suggesting that either soft water acclimation was unnecessary in this species or had not even begun. In any case, the soft water used in their study had almost six times the hardness of our soft water (~120 vs. 20

mg/L as $CaCO_3$) and we believe that the time for soft water acclimation lengthens with decreasing hardness.

After two weeks soft water acclimation, the initial hardness relationship (Fig. 1, Line 3) was much more pronounced (i.e. slope of Line 3 was 6.1 vs. 2.0 for Line 1), suggesting that soft water acclimation was incomplete in our fish, but was apparently complete by twelve weeks (Line 4, slope was 3.7) by the same criterion. However, in the ten weeks between the initial and final LC50 the fish had grown from 1.5 to 19 g. Although Cu sensitivity is known to decrease with increasing size, this increase in mass can probably only explain about one half of the reduced sensitivity based on the size-toxicity regression of Howarth and Sprague (1978) for juvenile rainbow trout exposed to copper.

Sublethal Cu exposures in soft water started after seven weeks of soft water acclimation. Whether soft water acclimation was complete at this point is uncertain. On the one hand, whole body electrolyte levels at this time were the same as those for hardwater acclimated fish, which is one criterion for acclimation (McDonald and Rogano, 1986). On the other hand, the presence of residual mortality in all soft water groups (including controls) suggests a continuation of soft water stress in this size of rainbow trout. We therefore conclude that rainbow trout (of this size range, <20 g), require a much longer period of acclimation to soft water than two weeks, particularly at very low hardness levels.

*Analyses of chronic effects (from least to most sensitive)*

i) Growth inhibition

Sublethal toxic effects of Cu, and subsequent acclimation (increased tolerance) to Cu are consistent with a 'damage-repair' model (McDonald and Wood, 1993). This model includes an initial damage phase during which the pathophysiological effects of the metal are expressed and reach maximal values, followed by a repair phase with attendant bio-energetic cost during which the effects diminish and may even disappear. The repair phase is commonly accompanied by an increase in lethal tolerance to the metal. One of the strongest supports for this concept is the consistent observation of initial growth reduction followed by recovery in juvenile salmonids exposed to Cu (at least seven studies since 1976: Dixon and Sprague, 1981; Marr, *et al.*, 1996; Lett, *et al.*, 1976; Buckley, *et al.*, 1982; Drummond *et al.*, 1973; Waiwood and Beamish, 1978a; Seim *et al.*, 1984). The same effect has also been noted in perch (Collvin, 1985) and carp (DeBoeck *et al.*, 1997).

In marked contrast to these studies, we found no evidence of an overall reduction, or even an initial reduction in growth in any of our exposures. Similarly, we found no significant initial reduction in whole body $Na^+$ and $Cl^-$. Our previous studies on rainbow trout indicate that under similar exposure conditions (but with unfed fish) we should expect at least 20% loss of sodium and chloride by 48h (Laurén and McDonald, 1986). The key difference between the present and previous studies (Dixon and Sprague, 1981; Marr *et al.*,1996; Lett *et al.*, 1976; Buckley *et al.*, 1982; Drummond *et al.*, 1973; Waiwood and Beamish, 1978a; Seim *et al.*, 1984) is that we fed fish a higher ration (3%

vs. 1 to 2%) that was administered in three rather than one daily feedings. Interestingly, Miller *et al.* (1993), who similarly employed three daily feedings, also reported an absence of Cu effect on growth in rainbow trout. If more frequent feeding is the key variable, then it could be masking the costs of Cu damage-repair either by overcoming appetite suppression (Waiwood and Beamish, 1978a), accelerating repair of the damage responsible for ion losses centered at the gills (Laurén and McDonald, 1985), and/or by providing elevated levels of much-needed electrolytes. Recently, D'Cruz *et al.* (1998) and D'Cruz and Wood (1998) provided direct evidence for these three hypotheses by demonstrating that satiation feeding (twice per day) completely prevented the electrolyte disturbances in rainbow trout exposed to low pH compared to fish exposed to the same low pH but fed a limited ration. Furthermore, they showed that the salt content of the diet was more important than the energy content in preventing the electrolyte effects. Moreover, satiation-fed, acid-exposed fish exhibited greater appetites and growth rates than their comparable satiation-fed controls. The effect of the diet as an important modifier of toxicity has usually been overlooked (see synthesis by Lanno *et al.* (1989)), even though we have observed critical intra-laboratory exposure differences. This would have vast implications on the chronic toxicity to Cu in wild fish, where the quantity and quality of the diet is much more variable.

ii) Swim performance

Another absent effect of Cu exposure was diminished swim performance. Like growth inhibition, swim performance has previously been shown to be a sensitive indicator of Cu exposure (Waiwood and Beamish, 1978b), especially in soft water

(Beaumont *et al.*, 1995), the latter study documenting effects at Cu concentrations as low as 12% of the LC50. However, Waiwood and Beamish (1978b) showed that swim performance fully recovered during chronic exposure suggesting that it was impacted only during the damage phase. Since we did not test swim performance during the damage phase, we do not know whether swim performance was affected while growth was not, but we can confirm that swimming performance was not impaired after 30 days of Cu exposure in any of our treatments.

iii) Acclimation to copper

An increase in lethal tolerance to Cu arising from chronic Cu exposure has been well established, at least for salmonids (Dixon and Sprague, 1981; Buckley *et al.*, 1982). The increase in lethal tolerance is proportional to the exposure concentration (Dixon and Sprague, 1981), is complete in about two weeks, and the maximum response is about a two fold increase in 96h LC50. This is similar to the acclimation response achieved in the present study in the one exposure where it occurred (60 $\mu$g/L Cu in hard water). Although water hardness has not been evaluated as a variable affecting acclimation, by comparing existing studies each done at a single hardness, there is a trend suggesting that the threshold (in terms of relative toxicity) required for acclimation increases with declining hardness. At hardnesses of 374 and 276 mg/L $CaC0_3$ (Dixon and Sprague, 1981; Buckley *et al.*, 1982, respectively) acclimation occurred at 25 to 30% of the Cu LC50 without accompanying mortality, whereas it occurred at 50% of the Cu LC50 at the lower hardness of the present study (120 mg/L as $CaCO_3$) and was accompanied by additional mortality. If this trend holds then the acclimation threshold would be expected

to increase towards the incipient lethal threshold (96h LC50) with declining hardness, i.e. narrowing the window for acclimation. Acclimation to Cu may therefore be a phenomenon confined to relatively hard water, which limits its effectiveness as an indicator of chronic Cu effect.

iv) Tissue accumulation

A number of laboratory studies have examined the relationship between Cu accumulation in tissues and water Cu concentrations. Liver and gill are the tissues most commonly used as indicators of Cu exposure. The general pattern that emerges from these studies is that tissue Cu levels gradually increase in response to exposure at a constant Cu level, until a steady state is reached. This may take from two to six weeks, depending on the Cu exposure level (e.g. Marr *et al.*, 1996). As yet, no comprehensive laboratory model to relate tissue Cu burdens with environmental exposure has been developed, with the exception of Marr *et al.* (1996), who studied whole body Cu only. Nonetheless, a survey of laboratory and field studies reveals an essentially linear relationship between gill Cu concentrations and environmental Cu (Fig. 6A). In contrast, there is no relationship between liver Cu concentrations and water Cu concentrations, despite there being a diversity of fish species in this plot, and differences in exposure duration and water chemistry (particularly hardness, Fig. 6B). Miller *et al.* (1992), came to essentially the same conclusion in their field study, finding the liver and gill of white suckers to be correlated to water Cu concentrations. One of the key conclusions suggested by this analysis is that Cu accumulation may be largely dependent on total

dissolved Cu, whereas toxicity is not only dependent on total dissolved Cu but is reliant on water chemistry as well.

To this framework we can now add two new conclusions. First, Cu concentrations in the water must exceed a certain threshold value before significant tissue accumulation will occur. Only the high Cu concentrations in soft and hard water (2 and 60 $\mu$g/L, respectively) produced elevated gill or liver tissue levels. Since we know from our $^{64}$Cu experiments that Cu was absorbed from the water into the blood in all exposures (L.N. Taylor, unpublished results), the absence of net accumulation in two of the four exposures suggests the fish were precisely regulating Cu balance. The threshold thus represents the point at which Cu homeostasis begins to fail. This, by itself, suggests that tissue accumulation is not likely to be an especially sensitive indicator of low level Cu exposure, which is not surprising since Cu is an essential metal. Secondly, gill Cu appears to be a more sensitive tissue indicator for waterborne Cu exposure than the liver for at least three reasons: the high background Cu levels in the liver (30 times greater than in the gills) means it is more difficult to detect a net increase in liver Cu (Fig. 2 vs. Fig. 3), when the gills accumulate Cu they appear to reach steady state more rapidly than does the liver (Fig. 2 and 3), and lastly, gill Cu tends to more accurately reflect the water Cu concentration, as represented by a four fold higher Cu level in gills between the highest water Cu levels in hard and soft water, but with virtually no difference in liver Cu content.

v) Copper binding by the gills

In this study, we used short-term (3 h) Cu exposures to assay the Cu binding characteristics of the gills in hard and soft water. We found that the only practical way to perform this assay uniformly in hard and soft water was to use $^{64}Cu$. Water Cu concentrations had to be elevated to acutely toxic levels otherwise, at least in hard water (Fig. 4A), in order to detect an increase in gill Cu above background. The use of $^{64}Cu$ permitted the detection of small increases in new Cu on or in the gills within a 3h period. Furthermore, this approach yielded important insights into gill Cu dynamics (relevant to biotic ligand modelling of Cu toxicity, see below) and how binding characteristics change with soft water acclimation and with chronic sublethal Cu exposure. Indeed, these were the measurements in the present study that were able to detect effects resulting from three out of four chronic copper exposures.

vi) Nature of gill Cu accumulation

The radiotracer experiments (Figs. 4B, 4C) identified two different types of binding sites on the gills for copper: low affinity, high capacity sites (non-saturating over the range of Cu levels used, Fig. 4B) and high affinity, low capacity sites saturating at very low Cu concentration (Fig. 4C). The former are, in fact, similar to sites characterized by 5 min Cu exposure *in vitro* (Reid and McDonald, 1991, Table 3) as indicated by the dashed line in Fig. 4B. Reid and McDonald (1991) demonstrated that these sites do, in fact, saturate, but at very high Cu concentrations (>1500 $\mu$g/L). Their low affinity suggests that they are not very specific to copper, and may in fact, reflect a mixture of different binding sites. Furthermore, they are not likely to accumulate Cu at

environmentally realistic exposure levels. Consequently, recent investigations have focussed on the high affinity Cu binding sites for the purpose of modelling the effects of Cu on the gills, specifically the interaction between water chemistry, gill metal burden, and toxicity. This approach (Playle *et al.*, 1993a,b; Bergman *et al.*, 1997; MacRae, *et al.*, 1999), has recently been termed Biotic Ligand Modelling (BLM; Di Toro *et al.*, 1999) and is seen as providing a more mechanistic basis for predicting toxicity of Cu to aquatic biota than the use of only hardness adjusted relationships between toxicity and total dissolved Cu (e.g. Fig. 1). The BLM approach assumes that the gill ligands have their highest affinity for the free metal ion ($Cu^{2+}$ *vs.* $CuOH^{+}$, for example), and that binding is competitive so that cations that compete with $Cu^{2+}$ (e.g. $Ca^{2+}$) or anions that complex $Cu^{2+}$ (e.g. DOC, $OH^{-}$) will reduce the amount of metal bound to the gill ligand. Therefore, once the binding capacity and affinity of all the ligands, including the gill, have been determined, these values can be used to calculate gill metal burden in relation to the concentration of free $Cu^{2+}$. Free $Cu^{2+}$ ion can be calculated with a geochemical modelling program such as MINEQL+ (Schecher and McAvoy, 1994).

Previous estimates for the Cu binding properties of the high affinity ligands are shown in Table 3. To simplify the making of these estimates, previous investigators have used slightly acidic and very soft water (i.e. $Ca^{2+}$ of $\leq$10 mg/L). These conditions ensure that most of the Cu is in the free ion form ($\geq$80% at pH < 7.0) and that competitive and complexing compounds are kept to a minimum. Parenthetically, this approach also ensures that sufficient Cu is accumulated so that it can be detected against background levels in the gills (8-12 nmol/g) without the use of $^{64}Cu$. Note that the binding

characteristics (log $K_{gill=Cu}$ and $B_{max}$) for fathead minnow gill (Playle *et al.*, 1993b) are very similar to those of the rainbow trout and brook trout (MacRae, *et al.*, 1999), although different durations of exposure were employed. In contrast, we estimated a much lower value for $B_{max}$ in the present study; 1.9 *vs* 30 nmol/g. Partly this is because the latter value includes a background Cu of 8-12 nmol/g whereas ours does not, but even then there is a 10 fold difference between the two estimates. In addition, Grosell and co-workers (1997) reported a naïve gill filament saturation level of ~1 $\mu$g Cu/g dry weight in the same hard water used in this study. This value converts to ~3.2 nmol/g wet weight, which is a 5 fold difference from our $B_{max}$ in hard water (0.6 nmol/g). Our log $K_{gill=Cu}$ estimate for rainbow trout is higher than the previous estimate by MacRae *et al.* (1999) (i.e. 7.9 vs. 7.6) but this estimate is subject to the most error. In fact, ours need have been only 1 $\mu$g/L total dissolved copper greater than the estimated value of 1 $\mu$g/L (see Fig. 4C) to yield an identical log *K* to MacRae *et al.* (1999). Furthermore, the acute toxicity of Cu (measured under similar water chemistry conditions) was similar between the two studies. After 24 hours at 20 $\mu$g Cu/L we obtained 50% mortality, *vs.* 75% mortality in the MacRae *et al.* (1999) study. This comparison of toxicity and ligand binding properties suggests that it is log $K_{gill=Cu}$ rather than $B_{max}$ that is the key determinant of toxicity, i.e. the % saturation of binding sites rather than the absolute total number of sites.

To date, no other studies have attempted measurement of Cu binding to the gills in more complex water chemistry to test the validity of the BLM. We have, and show that in hard water the estimated $B_{max}$ is much lower but the affinity is much higher (Fig.

4C, Table 3). The lower $B_{max}$ is not surprising because the binding curve in hard water incorporates the competitive effects of $Ca^{2+}$ and complexation due to DOC. The higher affinity is surprising, especially in the light of the reduced toxicity of Cu in hard water (Fig. 1). One possible explanation is that calcium, which is known to be important for regulating membrane permeability and increasing the stability of membrane proteins, may actually regulate both the number and affinity of binding sites on the gill surface. Whether this effect is actually occurring in the gill epithelium and whether it is acute or chronic is unknown. However, the fact that the acute toxicity to Cu decreased dramatically over ten weeks of acclimation to soft water (Fig. 1) does suggest that chronic exposure to low $Ca^{2+}$ modified gill binding properties for Cu, and that the rate of this change was fairly slow since the first test of toxicity was not conducted until two weeks of soft water exposure. Moreover, if the response to $Ca^{2+}$ reduction was a gradual reduction of the affinity of gill surface ligands for divalent cations, then such a modification would be protective against an exposure to Cu. In any case, the present biotic ligand model lacks any mathematical way of incorporating modulating effects (as opposed to competitive effects), of environmental $Ca^{2+}$ on gill surface ligands and thus, at least implicitly, must assume that such regulation by $Ca^{2+}$ does not occur.

Another way of testing the validity of the BLM approach is to test whether, in fact, any changes in acute Cu tolerance due to chronic Cu exposure are accompanied by changes in ligand binding. We confirmed that there was an alteration in gill binding in the group which showed increased lethal resistance after chronic exposure to 60 $\mu$g Cu/L. The alteration was characterized by an apparent increase in capacity in both the low and

high affinity binding sites (Fig. 5A and B, respectively). However, the most substantial, and arguably most protective, change in gill Cu binding (a large decrease in affinity) was seen in the 2 $\mu$g/L group in soft water (Fig. 5C) for which there was no increase in lethal resistance. Thus, while we show that gill ligand binding properties are sensitive to modification by chronic Cu exposure (i.e. an increase in binding capacity or decrease in affinity), the relationship of these responses to the presence or absence of acclimation remains unclear.

## CONCLUSIONS

The present study leads to four conclusions that are directly relevant to the task of assessing the risk of chronic copper exposure to wild fish populations. Firstly, we confirm that gill Cu burdens are a reliable indicator of chronic Cu exposure (superior in this respect than liver burdens), but other physiological indicators such as growth, ion loss and swim performance may be less reliable because of the important effect of ration quantity (and possibly quality) on their expression. Secondly, the presence of increased resistance to Cu in a wild population may also be a reliable indicator of chronic exposure but only in hard water environments. In soft waters, the combined effect of increasing Cu toxicity and the added stress associated with soft water exposure appear to favor lethality over acclimation. Thirdly, the protracted period of soft water acclimation seen in the present study suggests that, at the very least, laboratory studies of the effects of water hardness on Cu toxicity may need to be re-evaluated (e.g. Fig. 1). It is entirely possible, for example, that the hardness relationship would be quite different if tested on fish

native to soft water environments compared to fish native to hard water environments; i.e. the effects of increasing hardness might be quite different from the effects of decreasing hardness.

Finally, although changes in gill Cu binding characteristics offer the most promise for assessing the effect of chronic copper exposure, there is as yet no clear cut relationship between water chemistry, gill Cu binding, and toxicity. Clearly, more careful and detailed studies are required to resolve these apparently contradictory findings and to derive a compromise set of constants that best predicts both metal accumulation by the gills and resulting toxicity over a wide range of different water chemistries.

## ACKNOWLEDGEMENT

We would like to acknowledge Derek Alsop, Lydia Hollis and Richard Playle for their contribution to this study. We also thank the NSERC Strategic Grants Program, the International Copper Association, Falconbridge Ltd., Cominco Ltd. and the International Lead Zinc Research Organization (ILZRO) for their financial support of this research.

Table 1. Nominal and actual water copper concentrations (mean ± SEM (N)) throughout the 30 day exposure period.

| *Hard Water* | *Nominal (µg/L)* | *Actual (µg/L)* |
|---|---|---|
| Control | 0 | 2.95 ± 1.05 (12) |
| Low copper exposure | 20 | 19.89 ± 1.76 (12) |
| High copper exposure | 60 | 62.60 ± 1.73 (12) |
| *Soft Water* | | |
| Control | 0 | 0.65 ± 0.24 (9) |
| Low copper exposure | 1 | 1.05 ± 0.13 (18) |
| High copper exposure | 2 | 1.73 ± 0.24 (16) |

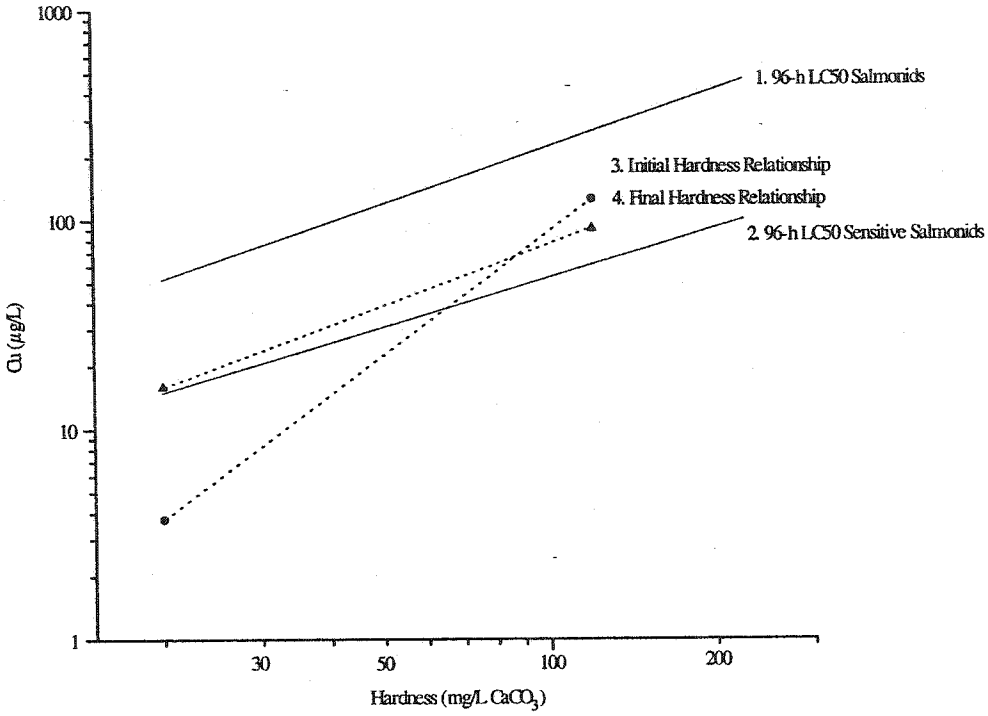
Table 2. Growth (specific growth rate, %/day) and swimming performance (time to 50% fatigue, s) after 30 days exposure to Cu. Specific growth rates (SGR) were calculated from weekly bulk weight measurements (hard water N = 4, soft water N = 7). Sprint performance was measured at 8 and 6 body lengths per second, which corresponded to 57 cm/s in hard water and 60 cm/s in soft water (N=20). Average fish weight and length in soft water was 14.35 ± 0.84 g and 10.68 ± 0.19 cm (Mean ± SEM, N=60). In hard water, average weight and length was 3.73 ± 0.15 g and 6.67 ± 0.09 cm (Mean ± SEM, N=60). Included in parentheses are the 95 % confidence limits. NS= SGR values of Cu exposed fish statistically similar (P>0.05) to control value.

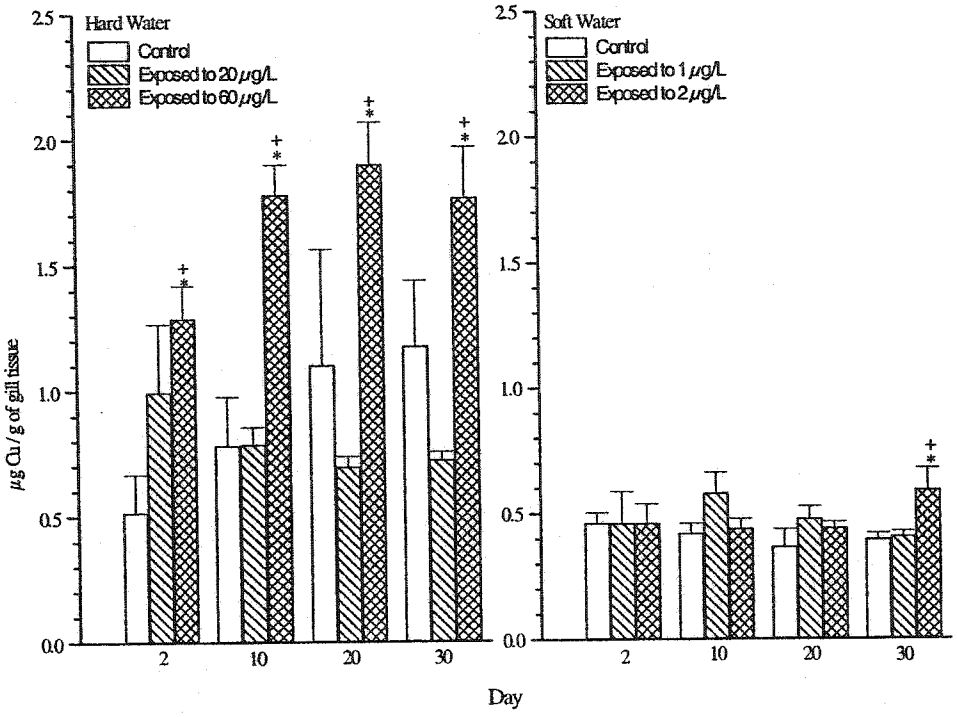
| | *Exposure* | *Specific Growth Rate (%/d)* | *Time to 50% Fatigue (s)* |
|---|---|---|---|
| *In hard water:* | | | |
| | Control | 2.88 (2.4 - 3.4) | 224 (196 - 256) |
| | 20 μg/L | 2.85 (2.4 - 3.3) NS | 282 (236 - 337) |
| | 60 μg/L | 3.07 (2.7 - 3.4) NS | 330 (278 - 392) |
| *In soft water:* | | | |
| | Control | 3.08 (2.5 - 3.6) | 134 (113 - 159) |
| | 1 μg/L | 3.22 (3.0 - 3.5) NS | 201 (169 - 239) |
| | 2 μg/L | 3.36 (3.1 - 3.6) NS | 271 (215 - 340) |

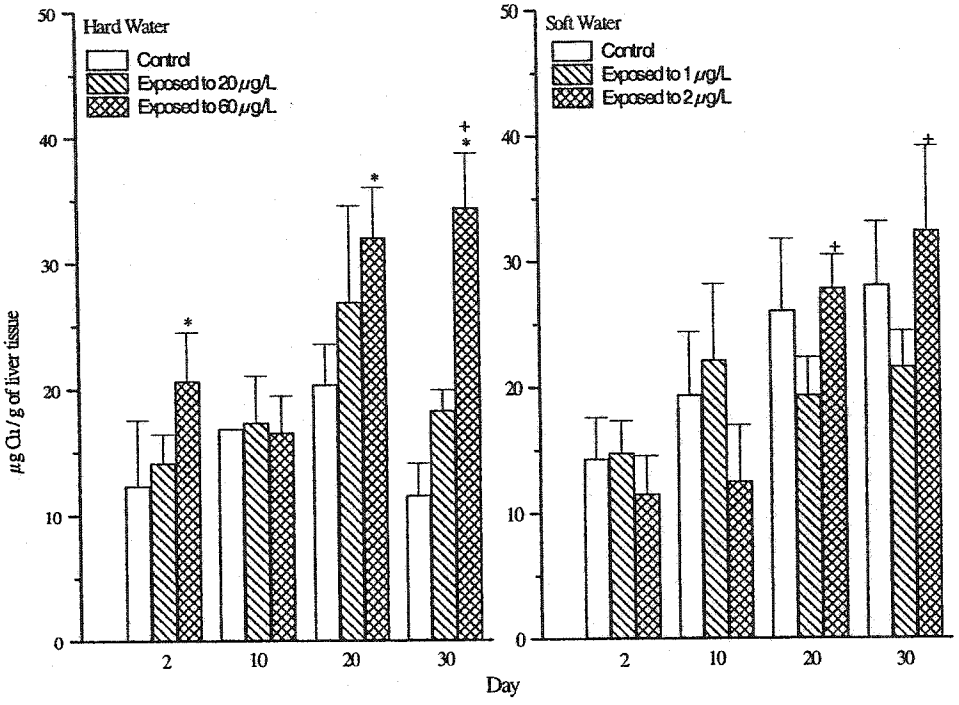
Table 3. Summary of copper binding affinities (log $K_{gill=Cu}$; log of the inverse free Cu ion concentration, in moles/L, required to produce half saturation of binding sites) and capacities ($B_{max}$, nmol/g) determined in soft water ($Ca^{2+}$ ~10 mg/L) in this and other studies using a range of total dissolved Cu from 0 to <30 $\mu$g/L. In the present study, the assumptions for the calculation of binding affinities were that there were 0.05 $\mu$mol binding sites per mg DOC/L (Playle *et al.*, 1993a) and that pH of the gill microenvironment was 7.5 and 6.2 in hard and soft water, respectively (Playle and Wood, 1989).

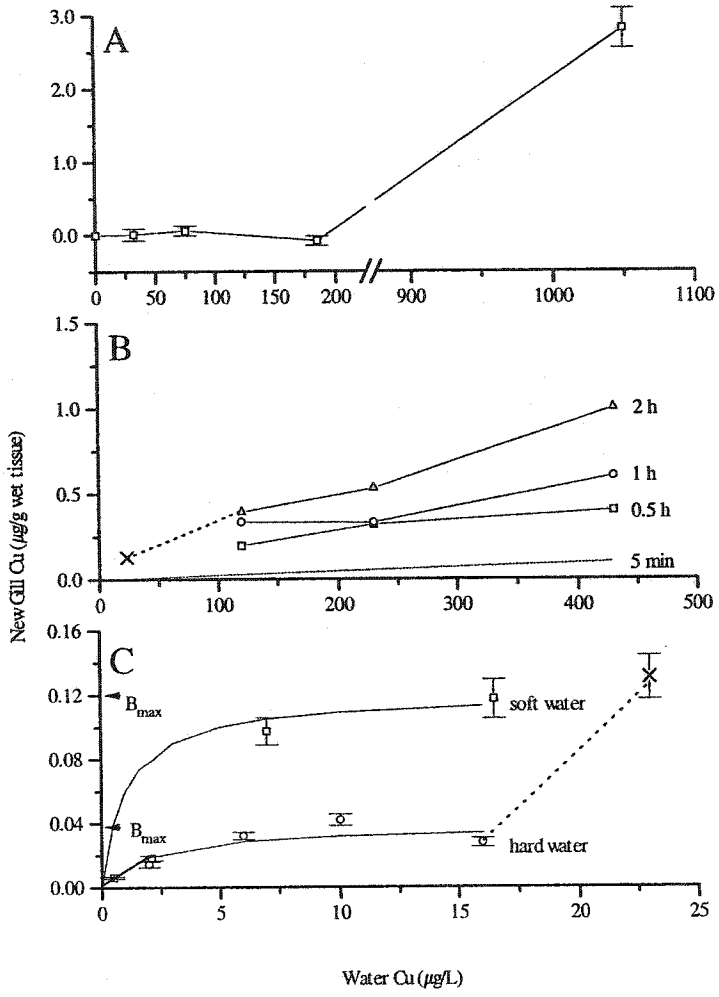
| *Species* | *log $K_{gill=Cu}$* | *$B_{max}$ (nmol/g)* | *Duration (h)* | *Reference* |
|---|---|---|---|---|
| brook trout | 7.1 | 63* | 24 | MacRae *et al.*, 1999. |
| rainbow trout | 7.6 | 30* | 24 | MacRae *et al.*, 1999. |
| fathead minnow | 7.4 | 30* | 3 | Playle *et al.*, 1993b. |
| rainbow trout | 7.9 | 1.9 | 3 | This study (soft water) |
| rainbow trout | 9.2 | 0.6 | 3 | This study (hard water) |
| rainbow trout | 2.4 | 930 | 0.08 | Reid and McDonald, 1991. |

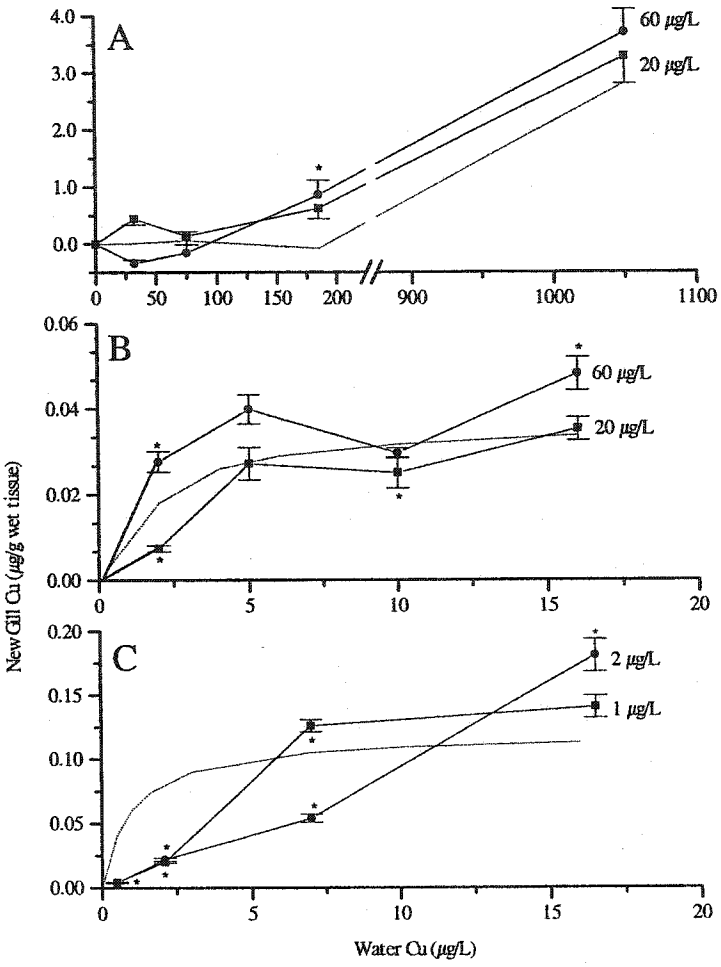
*Gill Cu background levels were 8-12 nmol/g.

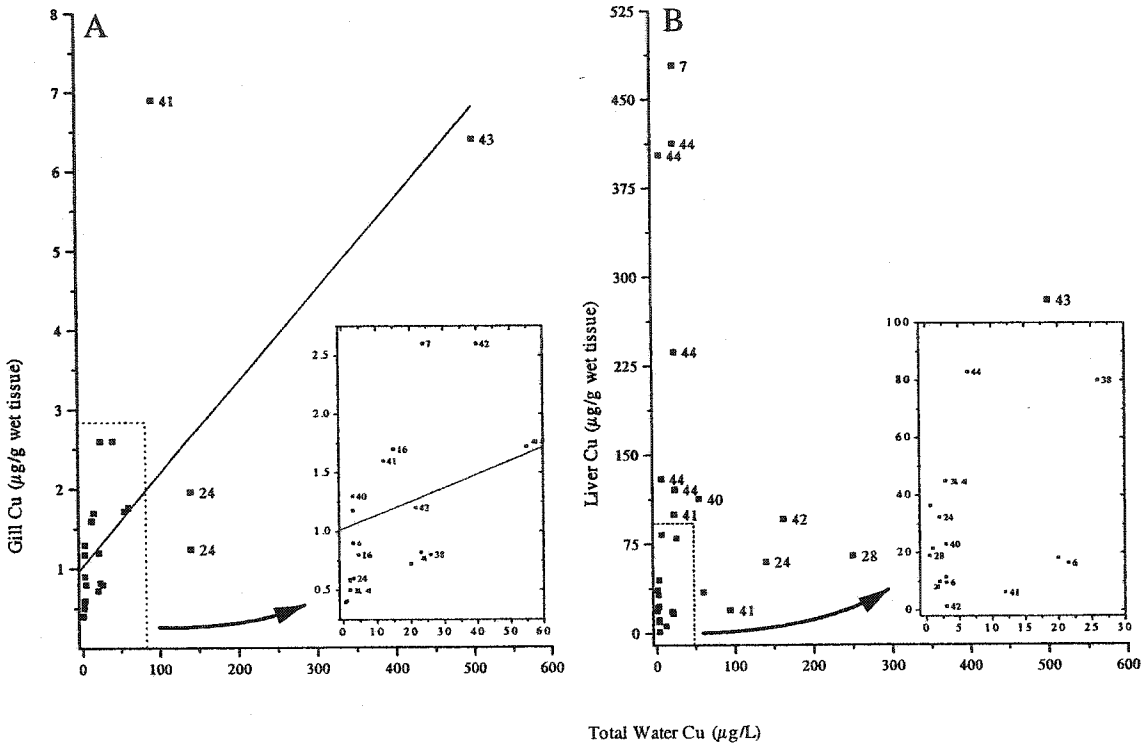

1000
100
10
1
Cu (µg/L)
30
50
100
200
Hardness (mg/L $CaCO_3$)
1. 96-h LC50 Salmonids
2. 96-h LC50 Sensitive Salmonids
3. Initial Hardness Relationship
4. Final Hardness Relationship


Hard Water
Control
Exposed to 20 μg/L
Exposed to 60 μg/L
Soft Water
Control
Exposed to 1 μg/L
Exposed to 2 μg/L
μg Cu / g of gill tissue
0.0
0.5
1.0
1.5
2.0
2.5
2
10
20
30
Day


Hard Water
Control
Exposed to 20 μg/L
Exposed to 60 μg/L
Soft Water
Control
Exposed to 1 μg/L
Exposed to 2 μg/L
μg Cu / g of liver tissue
0
10
20
30
40
50
2
10
20
30
Day
*
+


A
B
C
2 h
1 h
0.5 h
5 min
soft water
hard water
$B_{max}$
$B_{max}$
New Gill Cu (μg/g wet tissue)
Water Cu (μg/L)


A
60 µg/L
20 µg/L
4.0
3.0
2.0
1.0
0.0
0
50
100
150
200
900
1000
1100
B
60 µg/L
20 µg/L
0.06
0.04
0.02
0.00
0
5
10
15
20
C
2 µg/L
1 µg/L
0.20
0.15
0.10
0.05
0.00
0
5
10
15
20
New Gill Cu (µg/g wet tissue)
Water Cu (µg/L)


A
B
Gill Cu (μg/g wet tissue)
Liver Cu (μg/g wet tissue)
Total Water Cu (μg/L)

## CHAPTER 3

# CAN TWO INDEPENDENT INDICATORS OF SUSCEPTIBILITY BE USED TO PREDICT THE SUBLETHAL AND LETHAL EFFECTS OF WATERBORNE COPPER IN TROUT (*Oncorhynchus mykiss*)?


## ABSTRACT

We hypothesized that healthier fish would be less susceptible to the toxic effects of copper. In essence, we set out to use two independent indicators of fish health to predict fish susceptibility to sublethal and lethal effects of waterborne copper. The indicators used to assess a fish's health were growth and sodium permeability (both measured before copper exposure). All fish were implanted with passive integrative transponder (PIT) tags for individual identification. The sublethal study involved exposing juvenile rainbow trout to ~40 $\mu$g/L Cu for 1 week in moderately hard tap water. After 1 week, based on the group response, fish had accumulated significant amounts of copper in the gill, gut and whole body; however, there was no significant impairment of gill $Na^+/K^+$-ATPase activity, and whole body $Na^+$ levels were unaffected by the copper exposure. Individual growth measured before the exposure was correlated with individual growth during the sublethal Cu exposure, so it was concluded that fish which were growing well were less susceptible than fish which were growing poorly. Furthermore, fish that demonstrated a high permeability to sodium before exposure to copper were likely to accumulate more copper at their gills and have a lower growth rate during the sublethal copper exposure. The lethal study was conducted at 400 $\mu$g/L Cu (i.e., ten times the

sublethal exposure) and fish were sampled upon death, which occurred within 25 hours for all fish [LT50 was 1300 min (or 21.7 h; 95%CI: 1239-1360)]. In this lethal study, a fish deemed 'healthy' according to its growth rate was just as vulnerable during a lethal challenge as were 'unhealthy' individuals. However, accumulation of copper on the gills, whole body sodium and final weight explained a large amount of variation associated with time to death (46%). In conclusion, the assessment of fish health was an effective measure of susceptibility to sublethal toxic effects, but not to lethal effects (i.e., its predictive power was largely dependent on the severity of the Cu challenge).

**Key words**: copper, growth, ion regulation, rainbow trout, toxicity, sublethal, lethal

## INTRODUCTION

The idea that the physiological state of an organism can influence its susceptibility to environmental stress has been suggested by several researchers (e.g. Aldrich, 1986, Depledge, 1990, and Depledge and Bjerregaard, 1990). To assess an organism's physiological status the individual response in a health indicator needs to be considered (e.g. Kolok *et al.*, 1998, Gregory *et al.*, 1998, Depledge and Lundebye, 1996). Most researchers ignore individual variation by statistically evaluating the average response of a population with an associated error term (Bennett, 1987). Of interest physiologically; however, is the entire population, since it is likely that upon stress, it will be individuals with extreme responses that will express some physiological trait and survive (Depledge, 1990). Such stress may include the acute and chronic exposure to waterborne metals.

Predicting the impact of copper exposure on the survival of freshwater fish has been the focus of several studies (e.g., Miller *et al.*, 1992, 1993, MacRae *et al.*, 1999, and DiToro *et al.*, 2001). These studies are similar in that they measured indicators resulting from copper exposure, such as gill and other tissue metal burdens. The most prominent effect indicators of chronic copper exposure, documented in the literature on fish, are growth and ionoregulation (e.g. DeBoeck *et al.*, 1997, Waiwood and Beamish, 1978, Marr *et al.*, 1996, Buckley *et al.*, 1982, Laurén and McDonald, 1985, 1987). Fortunately, these two biomarkers of effect can also be measured in the laboratory before metal exposure and can therefore serve as indicators of fish health. Theoretically, we can then

operationally define a 'healthy' freshwater fish as one that exhibits a high growth rate and demonstrates low sodium permeability prior to Cu exposure.

It was the goal of this study to evaluate two indicators of physiological status (growth and ion permeability) measured before Cu exposure to predict the susceptibility of rainbow trout to sublethal and lethal copper exposure. To incorporate the influence of inter-individual variability, all fish were identified through the use of surgically implanted Passive Integrative Transponder (PIT) tags. Initially, both susceptibility measures were evaluated for their independence from each other and the extent of their variability and reproducibility. Sublethal effects of copper were evaluated using $Na^+/K^+$-ATPase activity in the gills, tissue copper concentrations (i.e. gill, liver, gut, carcass and the whole body), whole body sodium concentrations, and the specific growth rate during copper exposure as endpoints. Acute Cu toxicity was measured by time to death and evaluated using the copper content in the gill, carcass and whole body, and the concentration of whole body sodium as endpoints. The two measures of health were then correlated to each indicator or endpoint of copper exposure. It was hypothesized that healthy fish, as defined above, would be less susceptible to the acute and chronic effects of copper.

## MATERIALS AND METHODS

### *Experimental Animals*

Juvenile rainbow trout (RBT, *Oncorhynchus mykiss*, 2 g) were obtained from Rainbow Springs Hatchery (Thamesford, ON, Canada). Fish were held in dechlorinated

Hamilton tap water of moderate hardness (15 °C, pH 8, 0.6 mM $Na^+$, 0.7 mM $Cl^-$, 1.0 mM $Ca^{2+}$, 120 mg/L as $CaCO_3$ hardness, 95 mg/L alkalinity, 3 mg C/L DOM). Photoperiod was set to a light/dark cycle similar to the natural photoperiod for western Lake Ontario. Fish were fed daily a dry ration of commercial trout pellets to satiation before testing. The feed contained ~ 3 $\mu$g Cu/g dry weight.

*Experimental Protocol*

Juvenile rainbow trout (~2g, N=110) were surgically implanted with Passive Integrative Transponder (PIT) tags and left to recover for 3 months. The tags (2 x 12 mm, 134 kHz, Destron Fearing Inc.) and accessories were purchased from Biomark in Idaho, USA. Fish were anesthetized in tricaine methanesulfonate (MS222, Syndel Laboratories, 0.08 g/L buffered with 0.16 g/L $NaHCO_3$) and removed upon loss of righting response. Tags were inserted into their body cavities just posterior to the pectoral fins along the midventral line using a 12 gauge tagging needle. Tag numbers were read using a hand-held scanner (Pocket Reader EX; Destron Fearing Inc.).

i) Sublethal Experiment

Fish (N=65) were monitored for growth every other day over 15 days (a total of 7 measurements) before copper exposure. Fish were not anaesthetized before weighing, but were quickly blotted to remove excess water. The specific growth rate (SGR in %/day) was calculated for each fish. After this time period, fish were sorted into two groups; a control group (N=33) and a copper- treated group (N=30). The sort was based on the SGR for each fish such that each group contained an equal distribution of poor,

medium and high growers and both groups had an equal average weight (7.8 ± 0.3 g (65)). After sorting, fish were allowed to settle for two days before the start of the copper exposure. Fish were fed a dry ration of commercial trout pellets at a rate of 1% wet body weight/day throughout the growth monitoring and test periods.

One day before copper exposure and post-sorting, all fish were analyzed for their sodium leak rate using a flux test. This test involved exposing fish to deionized water (conductivity 18mOhm and with no detectable levels of $Na^+$ or $Ca^{2+}$) for 20 minutes. Individual fish were transferred to static, aerated, 500 mL polyethylene containers holding 200 mL of deionized water. These experimental chambers were placed in a water bath to maintain the experimental temperature (15-16°C). Water samples (5mL) were taken at the beginning and end of the flux period. All water samples taken for later analysis were acidified with 50 $\mu$l concentrated trace metal grade nitric acid (Fisher Scientific, Toronto, ON, Canada). At the end of the flux period, PIT tag numbers were recorded and fish were returned to the holding tank.

Copper stock ($CuSO_4 \cdot 5H_2O$; Fisher Scientific) was metered into diluent water in a mixing head tank, which then delivered the test water to the 50- L exposure tank at a rate of 100 ml/min. The control tank received fresh water with no copper at the same rate. Each exposure and mixing tank was aerated and any feces and organic matter were siphoned from the exposure tanks daily. Copper in the exposure tanks was measured at least twice daily. Actual copper concentrations were 36.2 ± 2.6 $\mu$g/L (N=14) for the copper treated group and 1.3 ± 0.3 $\mu$g/L (N=12) for the control group. After six days of copper exposure, the sodium flux test was repeated on all fish. At Day 7, all fish were

sacrificed by a blow to the head, weighed, and sampled for individual tissues. The gill arches, liver, guts and remaining carcass were frozen for metal analysis. A sub-sample of the gills (filaments only) was reserved for $Na^+/K^+$-ATPase analysis and immediately placed in liquid $N_2$ and then stored at –80 °C.

ii) Lethal Experiment

The remaining tagged fish (7.0 ± 0.3 g, N=44) were monitored for growth before the lethal copper exposure. Fish were weighed a total of seven times over a period of 25 days (i.e. every 3-5 days). Fish weighed 9.4 ± 0.3 g (N=44) at the start of the acute copper exposure. The trout were not sorted into groups before the toxicity trial since all fish were to be exposed to copper. Fish were maintained on the same dry ration of trout pellets as for the sublethal experiment during the growth monitoring phase but were not fed during the lethal challenge to copper. The temperature was 12 °C. The exposure system was identical to the sublethal experiment with the exception of the exposure concentration. All fish were exposed to ten times the sublethal exposure concentration (i.e., 400 $\mu$g/L nominal) where time to death and final weight were recorded upon death. Fish were considered dead when all opercular movements had ceased. Water samples (5.0 mL) were collected every 1-2 hours and acidified with 50 $\mu$L trace metal grade $HNO_3$. The actual exposure concentration was 424.0 ± 15.3 $\mu$g/L (N=20). Fish were sampled upon death for gill arches and the remaining carcasses, which were subsequently frozen for the analysis of copper and sodium concentrations.

*Analytical Techniques*

Tissue samples were weighed and digested in five volumes of 1N trace metal grade nitric acid (Fisher Scientific). Copper was measured after appropriate dilution of the supernatant with reagent-grade deionized water, using flame and graphite furnace atomic absorption spectrophotometry (Varian AA-220, GTA 110, Varian, Walnut Creek, CA, USA) against certified copper standards (Fisher Scientific). Whole body copper and sodium concentrations were calculated by adding the copper or sodium content of the removed tissues to the carcass metal or ion concentration. Sodium was analyzed from tissue digests in the same manner as for Cu. Acidified water samples were measured for copper and sodium using graphite furnace and flame atomic absorption spectrophotometer, as required. Gill filaments were analyzed for $Na^+/K^+$-ATPase activity using the method of McCormick (1993).

*Calculations*

i) Specific Growth Rate

The specific growth rate was calculated based on the exponential curve using Sigmplot® 2000 (SPSS, Chicago, IL), representing weight over time:

$$Wt = ae^{bt}$$

where t= time (days), Wt is the weight in grams, and b is the specific growth rate (in %/day) when multiplied by 100%. In the sublethal experiment during the copper exposure, the SGR was calculated using only two weight measurements (i.e. at the start and end of exposure) and the following equation:

$$\text{Specific Growth Rate (SGR)} = \frac{(\ln wt_{final} - \ln wt_{initial})}{(t_{final} - t_{initial})} \times 100$$

where $wt_{final}$ and $wt_{initial}$ are the fish body weights in grams measured over a time interval. This time interval is represented by $t_{final}$ and $t_{inital}$ in days. This specific growth rate is also expressed as %/day.

ii) Net Sodium Loss

Net losses of $Na^+$ by the fish to the water were calculated from the change in $Na^+$ concentration in the water over the 20 minutes and are expressed in nmol/g· h. The equation is as follows:

$$\text{Net } Na^+ \text{ loss} = \frac{(Na^+_I - Na^+_f) \times V}{Wt \times t}$$

where $Na^+_i$ and $Na^+_f$ are the initial and final sodium concentrations in the water in nmol/L, V is the volume of water in L in which the fish was exposed, Wt is the weight of the fish in grams, and t is the duration of time in hours between the initial and final water samples (i.e. the duration of exposure).

*Statistical Analysis*

Data are expressed as means ± 1 standard error (SEM), sample size (N). Means were compared for differences between treatment groups using the Student's unpaired t-test. In the sublethal experiment, paired Student's t-tests were appropriate for examining significant differences before and after copper exposure (e.g., data such as net sodium

loss and specific growth rates, where repeated measures were made on the same fish). Values of $p<0.05$ were considered significant, unless otherwise indicated. In order to separate the effects of copper from the effects of sorting on growth and sodium loss, the data was analyzed using an ANCOVA. Correlation analysis was completed using the parametric test, Pearson Correlation. Apparent outliers were included in all analyses. In the sub-lethal experiment, multivariate analysis was used to relate the growth and ion loss to each sublethal indicator measured during the copper exposure. In the lethal experiment, a stepwise multiple regression analysis was used to examine the contribution of multiple factors (i.e., gill Cu, whole body Cu, SGR, whole body $Na^+$ and final weight) on the single endpoint, time to death. The time for 50% of the test population to die [i.e., the LT50 and 95% confidence intervals (CI)] was estimated using a log-probit survival analysis relating the time of death in minutes to the percent mortality. All statistical analyses were run using SPSS ® software (SPSS, Chicago, IL, USA).

## RESULTS

i) Susceptibility Biomarkers

Four criteria were used to determine the effectiveness of the two susceptibility biomarkers: variability, reproducibility, independence, and sensitivity. The variability of the growth response in the sublethal experiment was larger than that in the lethal experiment. The average initial growth rates of the fish involved in the sublethal and lethal studies were $1.52 \pm 0.07$ (63) %/day and $1.28 \pm 0.05$ (44) %/day, respectively. However, the range of growth rates was 0.72 – 3.09 %/day in the sublethal study and 0.02

– 1.77 %/day in the lethal study. The range of data for the sodium loss test spanned almost three orders of magnitude, from essentially zero (–53 nEq/g· h) to a maximum of 2852 nEq/g· h. The mean rate of sodium loss for all the fish before Cu exposure was 1052 ± 72 nEq/g· hr (63).

In order to test if the two susceptibility indicators were reproducible, the paired values of all individual fish were analyzed by linear regression to detect if there was a relationship between initial and final measurements (i.e., before and after Cu exposure). The indicator was considered reproducible if a fish with an initially high growth rate or sodium loss rate continued to have a high rate after the exposure (in other words, the initial rate predicts the final rate). Ideally, a reproducible indicator would have a 1:1 relationship between repeated measures. A significant relationship did exist between the initial and final individual growth rates within each group (Fig. 1A, $p<0.001$, and Fig. 1B, $p<0.01$). However, in the control group the initial growth rates explained more of the variation surrounding the final growth rates and was therefore deemed more reproducible or predictive, than in the Cu treated group ($R^2= 0.52$ *vs.* 0.35; see regression analysis, Fig. 1A and B). Upon removing the effect of sorting from the effects of copper, there was no significant different between the control and treated group (i.e., slopes of the regression lines were not significantly different, $F_{(1, 60)} = 0.378$, $p=0.541$). In contrast, the rate of sodium loss was not reproducible in either group (no significant relationship; Fig. 2A and B). Essentially, a fish growing well before sorting and copper exposure remained a high grower after sorting and exposure to copper, but a fish considered leaky by initial rates of sodium loss was not necessarily leaky when tested again one week later. No significant

relationship existed between initial growth and initial sodium loss rates for all fish (Fig. 3). Therefore, it was concluded that the two susceptibility indicators were independent of one another. The sensitivity of each susceptibility indicator was shown by their strength of correlation to each indicator of effect (see below).

ii) Effects of Sublethal Copper Toxicity

The seven indicators measured after the exposure were summarized on a group basis (i.e., mean ± SEM) in Table 1. Of these indicators, only gill, gut and whole body copper concentrations showed a significant increase from control levels as a result of the sublethal exposure. $Na^+/K^+$-ATPase activity, liver Cu, carcass Cu, and whole body sodium concentrations did not show any significant effect of the exposure to 36 $\mu$gCu/L. Gill copper increased by the greatest amount (i.e., 1.6 times increase), followed by the gut and whole body Cu (i.e., 1.4 and 1.2 times increase, respectively). However, gut copper levels were the most variable as a result of copper exposure, where values ranged from 2.8 to 10.6 $\mu$g/g. The range of gill copper concentrations was from 0.6 to 2.0 $\mu$g/g and the whole body copper concentrations were as low as 0.9 and as high as 2.2 $\mu$g/g. The copper burden in the gill, liver, and gut represented ~3, 35 and 27% of the whole body burden, respectively. The balance of Cu was found in the remaining carcass (~35%).

The initial mean control and treated SGR (specific growth rate) was 1.51 ± 0.09 %/day (33) and 1.54 ± 0.10 %/day (30), respectively. After one week of copper exposure the growth rates had dropped to 0.05 ± 0.08 %/day (33) for the control group and to –0.01 ± 0.10 %/day (30) for the fish exposed to copper, and were not significantly different

from each other. Based on the individual growth rates before and after copper exposure, a significant effect of sorting alone was evident in control fish using a paired t-test ($p<0.001$; Fig.1A). Similarly, the individual growth rates of fish sorted and exposed to copper were also significantly different from their initial rates (paired t–test, $p<0.001$; Fig. 1B).

The initial sodium loss rates in deionized water of the two groups of fish (i.e., post-sorting but before copper exposure) were 1195 ± 103 nmol/g· h (N=33; controls) and 895 ± 95 nmol/g· h (N=30; Cu exposed) and were significantly different from each other ($p<0.05$). Following Cu treatment, the control group remained essentially unchanged at 1030 ± 65 nmol/g· h (N=33; paired t-test, $p=0.287$; Fig. 2A). After one week of copper exposure, the rate of Na loss in the treated group had significantly increased to 1388 ± 98 nmol/g· h (N= 30; paired t-test, $p<0.001$; Fig. 2B). Overall, the fish exposed to copper had significantly higher final rates of sodium loss than the control fish after the week exposure (unpaired t-test, $p<0.001$).

iii) Predicting Sublethal Effects of Copper

Correlation analysis was used to evaluate the predictive power of each of the two susceptibility measures. For example, growth before the exposure was tested individually against liver Cu, gill Cu, gut Cu, whole body Cu, whole body Na, $Na^+/K^+$-ATPase activity and the SGR during the exposure (Table 2). The results of this analysis showed that growth (before Cu) was correlated only to the growth rate during the Cu exposure. This analysis was repeated with the rate of sodium loss before the exposure (Table 3). Here, the rate of sodium loss before Cu exposure correlated slightly better

with the amount of Cu in the gill than with the SGR during the Cu exposure. In essence, a fish growing well before the exposure was likely to grow well during the sublethal copper exposure (i.e. a positive correlation). In contrast, a fish having a high sodium loss rate before Cu exposure showed a high amount of copper at the gill (i.e., positive correlation) and a low growth rate during the exposure (i.e., a negative correlation).

iv) Effects of Lethal Copper Toxicity

All fish died within 25 hours when exposed to 424 $\mu$g Cu/L. The first fish died within 885 minutes (~15 h) and the final fish died after 1478 minutes (~25 h). The time required for 50% of the population to die (LT50) was 1300 minutes (95% CI: 1239-1360). At this time fish weighed 9.56 ± 0.03 g (44). At death, the average amount of copper in the gills and whole body was 0.45 ± 0.03 $\mu$g/g (44) and 0.99 ± 0.04 $\mu$g/g (44), respectively. The range of copper concentrations in the gill was 0.25 to 1.14 $\mu$g/g, and on average represented 21% of the whole body copper burden. Average whole body sodium concentrations were 29.89 ± 1.57 $\mu$mol/g (44) upon death, with values ranging from 12.91 to 60.97 $\mu$mol/g.

v) Predicting Lethal Effects of Copper

Growth measured before the lethal challenge was not correlated with the time to death or any indicator (Table 4). Because growth was unable to predict any effect of lethal Cu exposure, regression analysis was performed between the terminal measures and toxicity. The strongest relationship of time to death was with final gill copper (Figure 4). A negative linear fit with this indicator alone revealed an $R^2$ of 0.35 ($p<0.001$, N=44), meaning that a fish with high gill copper tended to die sooner than a

fish with a low gill copper concentration. From this relationship, the lethal copper concentration at the gill, which caused 50% mortality during exposure to 424 $\mu$g Cu/L, was 0.45 $\mu$g/g. Using stepwise multiple regression and all final measures, only whole body sodium and fish weight were able to predict an additional portion of the variation surrounding the time to death ($R^2$= 0.46, $p$<0.001, N=44, Fig. 5). Therefore in this model, gill copper explained 26% of the variation associated with time of death and whole body sodium concentrations and final weight accounted for a further 13 and 7%, respectively. Consequently, approximately 54% of the variation associated with time to death remained unexplained.

## DISCUSSION

In this study, fish health was used to predict the susceptibility to subsequent copper exposures. We selected growth and ion permeability as health indicators because the former integrates homeostatic processes in the whole animal over time (Beyers *et al.*, 1999) and the latter is closely linked to the mechanism of copper toxicity (Laurén and McDonald, 1985). This method is novel in that it uses biomarkers in the non-traditional sense to indicate effect or exposure. It was assumed that a healthy freshwater fish would have a high growth rate and low sodium permeability and hence, be more tolerant to copper. Under chronic exposure conditions (i.e., one week at a sublethal concentration), the growth rate of fish did influence their susceptibility to copper as shown by one traditional biomarker of effect, which was growth during the exposure. In addition, a fish's permeability to sodium was predictive of gill Cu concentration and the growth rate

during Cu exposure. When acutely challenged (i.e., 25 hours at a lethal concentration), a fish exhibiting a high growth rate was no less susceptible to Cu than a poorly growing fish, but fish weight, whole body sodium concentration and accumulation of gill copper during the exposure were better predictors of lethal resistance time. In essence, the severity of challenge had a large influence on the susceptibility measures used in this study.

*Susceptibility Biomarkers*

i) Growth

The ability to identify and track individuals over time using PIT tags allowed for the calculation of individual growth rates. For growth to be an effective biomarker of susceptibility, growth measurement needed to be reproducible, variable (among individuals) and sensitive. To test for 'reproducibility', we fit a regression line to the initial and final growth rates of control fish (Fig.1A). This technique was different from two previous studies (Gregory and Wood, 1998; Kolok *et al.*, 1998), which evaluated 'repeatability' of performance measures using rank correlation analysis. While the analysis of fish rank would satisfy our reproducibility criteria for an effective biomarker, the regression analysis also provided a variability term reflective of the individual response. In control fish, the initial growth rate accounted for 52% of the variability associated with the final growth rate with 48% unexplained. We now believe that this unexplained variation was the result of sorting the fish since no other parameter was altered between the two time intervals.

It has been well established that growth can be affected by appetite, ration, competition and food conversion efficiency (Brett, 1979). By sorting the fish into two groups, we effectively disrupted any dominance hierarchies established when all fish were together in one tank. Specifically, both the number of fish per group and their density were altered upon sorting thereby affecting social interrelations (Brett, 1979). As demonstrated by Sloman *et al.* (2000a, b), the physiological status of fish can be influenced by the social ranking of individuals (i.e. subordinate *vs.* dominant fish). For example, subordinate fish tend to grow more slowly and accumulate more copper in their tissues (Sloman *et al.*, 2002). Essentially, we are suggesting that our fish did not have enough time to re-establish their social structure (i.e. following sorting, we let the fish settle in two tanks for 48 hours before Cu exposure). The SGR of fish treated with copper did not correlate with any tissue copper concentration (i.e., in the gill, liver, gut and whole body), in contrast with the results of the Sloman *et al.* (2002) study, thereby supporting the belief that fish hierarchies were not re-established in our study.

Behavioural interactions also played a role in the variability found among growth rates. Logically, a dominant fish would obtain a higher portion of the daily ration and therefore have a higher growth rate. For this reason, we limited the ration to 1% per day, thereby increasing competition for available food. Before the fish were sorted into groups, the range of individual specific growth rates was four fold (0.72 to 3.09 %/day). Without this large range in growth rates, we would have been unable to classify the physiological status of each fish (i.e., fish growing well and poorly were deemed healthy and less healthy, respectively).

The limited ration also served to improve the sensitivity of growth. Based on previous findings in our laboratory, we discovered that increasing the ration could prevent the growth depression due to copper exposure (i.e., 1% ration distributed over three meals, totaling 3%/day; Taylor *et al.*, 2000). The copper exposure concentrations used in the former study were 20 and 60 $\mu$g/L with nearly zero and 3% mortality occurring at each level, respectively. The Cu exposure concentration of 40 $\mu$g/L and the 1%/day ration in the present study were chosen to produce zero mortality and affect growth. Surprisingly, the effect of sorting was as large as the effect of this sublethal copper exposure.

ii) Ionoregulation

As for the use of growth to predict susceptibility, we required the measure of ion permeability to be reproducible, variable and sensitive. In the present study the sodium leak test was not reproducible in 33 individual control fish from one time to another (see lack of relationship in Fig. 2A). The variability in response among individual fish enabled the classification of 'tight' and 'leaky' fish, which corresponded to healthy and less healthy fish, respectively (i.e., the $Na^+$ loss data ranged from essentially zero to 2852 nmol/g· h for all fish before copper exposure). The sodium leak test also proved to be sensitive such that differences were detectable between control fish and those exposed to copper.

Copper is toxic in rainbow trout by causing a stimulation of sodium efflux and inhibition of sodium uptake (Laurén and McDonald, 1985). For this reason, the sodium loss test was chosen to reflect the individual's pre-disposition to be permeable to ions.

We reasoned that if a fish was more permeable than the average population, it would be more susceptible to copper. During our chronic sublethal copper exposure, the sodium loss rate predicted the accumulation of copper on the gills and the growth rate during the exposure in treated fish (Table 3). However, Edwards (2001) found that the sodium leak test was not predictive of acute toxicity (i.e., not correlated with time to death at 300 $\mu$g Cu/L). Nonetheless, Edwards did find a strong relationship with the rate of sodium loss during Cu exposure and rate of whole body Cu uptake versus the time to death. During our acute exposure to 424 $\mu$g Cu/L and assuming an initial whole body Cu concentration of 1 $\mu$g/g, we were unable to make the same correlation as in the former study. The same lack of relationship with time to death was also evident for rate of whole body $Na^+$ loss, assuming an initial concentration of 55 $\mu$mol/g. The two likely reasons for this discrepancy were the difference in fish size (3 *vs.* 10 g) and the Cu exposure concentrations, both of which created a difference in the severity of challenge [i.e., all the fish in the present study died within 25 hours, whereas it took up to 45 hours in the Edward's (2001) study].

*Predicting Lethal and Sublethal Copper Toxicity*

Predicting mortality is an important element of environmental toxicology. A recent study by Newman and McCloskey (2000) tried to classify death as the result of a unique quality of individuals (i.e., explainable) or as a completely random event (i.e., the chance of death is equal among all individuals receiving the same dose). They concluded that neither hypothesis was the sole determinant for death. The contribution that each

component makes is also likely to change based on acute, chronic or intermittent exposures. For example, the random or chaotic component is likely larger at high toxicant concentrations and the innate quality of individuals is larger at low exposure levels (Newman and McCloskey, 2000). In the present study, the regression model used to predict mortality, which included gill copper, whole body sodium and final weight, could account for 46% of the variation associated with time to death. Therefore, 54% of the variation associated with time to death was either attributable to another indicator which we did not measure, or to the chaotic component of death, or both. According to our hypothesis, growth was an example of an innate quality that could explain a small amount of the variation in chronic toxicity but none for acute toxicity. Sodium permeability was also an important individual quality for this toxicant.

## CONCLUSIONS

Several recommendations can be made about using biomarkers of effect, exposure and susceptibility. In chronic exposures, the measures of whole body sodium, carcass Cu, liver copper and $Na^+/K^+$-ATPase activity were not sensitive indicators of effect and are not likely going to improve a risk assessment for copper toxicity. Growth and sodium loss rates were effective biomarkers of susceptibility to chronic Cu toxicity, and concentrations of copper in the gill, gut and whole body were useful indicators of chronic exposure. In acutely toxic Cu exposures, gill copper, whole body sodium and final weight were most relevant predictors of Cu toxicity, however, the rate of sodium loss and rate of whole body Cu uptake cannot be eliminated as possible biomarkers of exposure.

It was important that gill copper was strongly related to mortality since the Biotic Ligand Model, a model proposed as a regulatory tool for copper water quality criteria, is based on such a relationship (Playle, 1998, DiToro *et al.*, 2001, Santore et al., 2001). In answer to our original hypothesis, the health of an individual fish may influence its susceptibility to the sublethal effects of copper, but not to the lethal effects (i.e., the extent to which fish health was predictive was largely dependent upon the severity of the Cu challenge). Nonetheless, the classification of an individual fish's physiological status was more complicated than expected using our two measures of fish health.

## ACKNOWLEDGEMENTS

We acknowledge D. Baker for his technical assistance during this study. We also thank R. Playle and P. Chow-Fraser for comments on the manuscript. This research was supported by the Metals in the Environment Research Network (MITE-RN), the International Copper Association Inc., Falconbridge Ltd., Cominco Ltd., International Lead Zinc Research Organization, Noranda Inc., Nickel Producers Environmental Research Association, and a Strategic Research Grant from the Natural Sciences and Engineering Research Council of Canada. C.M. Wood is supported by the Canada Research Chair program.

Table 1. The group response as measured by seven terminal indicators after one week of exposure to 36 μg/L copper. Whole body represents the carcass copper concentration plus the other mass-weighted tissue copper levels. * indicates a significant difference between the group exposed to copper and the control group. NS= the group exposed to copper was not significantly different from the control response using a two-tailed, unpaired Student's t-test ($p > 0.05$).

| *Indicator* | *Group* | *Mean* | *Stdev* | *SEM* | *N* | *Sig. (p)* |
|---|---|---|---|---|---|---|
| Na/K-ATPase activity | Control | 0.74 | 0.22 | 0.04 | 33 | NS |
| (μmoles ADP/mg protein/h) | Cu-exposed | 0.74 | 0.28 | 0.05 | 30 | |
| Gill Cu (μg/g) | Control | 0.66 | 0.27 | 0.05 | 33 | <0.001* |
| | Cu exposed | 1.08 | 0.33 | 0.06 | 30 | |
| Liver Cu (μg/g) | Control | 50.59 | 35.52 | 6.18 | 33 | NS |
| | Cu exposed | 55.33 | 28.29 | 5.16 | 30 | |
| Gut Cu (μg/g) | Control | 3.66 | 0.60 | 0.10 | 33 | <0.001* |
| | Cu exposed | 5.13 | 1.86 | 0.34 | 30 | |
| Whole body Cu (μg/g) | Control | 1.15 | 0.30 | 0.05 | 33 | <0.05* |
| | Cu exposed | 1.37 | 0.33 | 0.06 | 30 | |
| Whole body Na (μmol/g) | Control | 34.94 | 5.81 | 1.01 | 33 | NS |
| | Cu exposed | 31.99 | 6.66 | 1.22 | 30 | |
| Carcass Cu (μg/g) | Control | 0.47 | 0.09 | 0.02 | 33 | NS |
| | Cu exposed | 0.51 | 0.07 | 0.01 | 30 | |

Table 2. Correlations of specific growth rate (SGR) before Cu exposure and each indicator measured after one week of exposure to 36 $\mu$g Cu/L (N=30). * indicates a significant relationship.

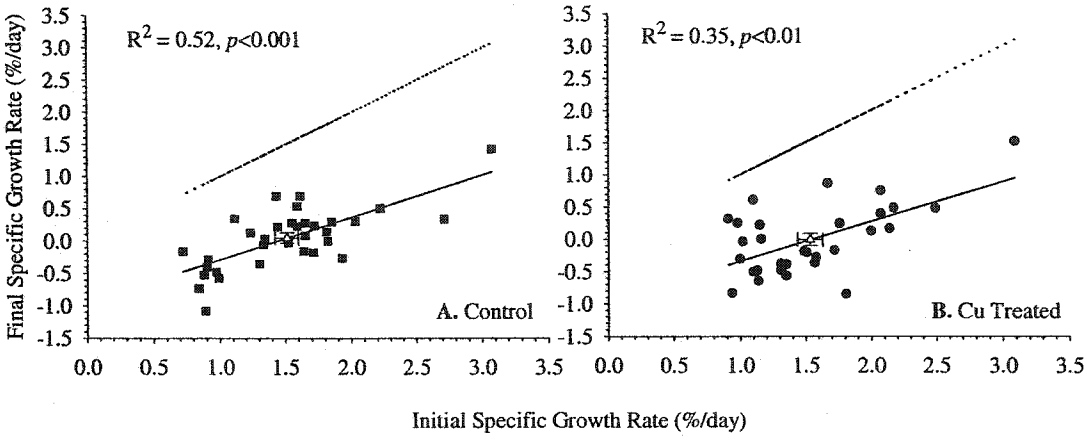
| *Indicator* | *Pearson's Correlation Coefficient (r)* | p *value (2-tailed)* |
|---|---|---|
| SGR during Cu exposure | 0.591 | 0.001* |
| Whole body Na | -0.178 | 0.347 |
| Na loss rate- after Cu exposure | 0.173 | 0.360 |
| Whole body Cu | 0.126 | 0.508 |
| Carcass Cu | 0.096 | 0.615 |
| $Na^+/K^+$- ATPase activity | -0.075 | 0.692 |
| Gill Cu | 0.041 | 0.829 |
| Liver Cu | 0.014 | 0.942 |
| Gut Cu | 0.002 | 0.994 |

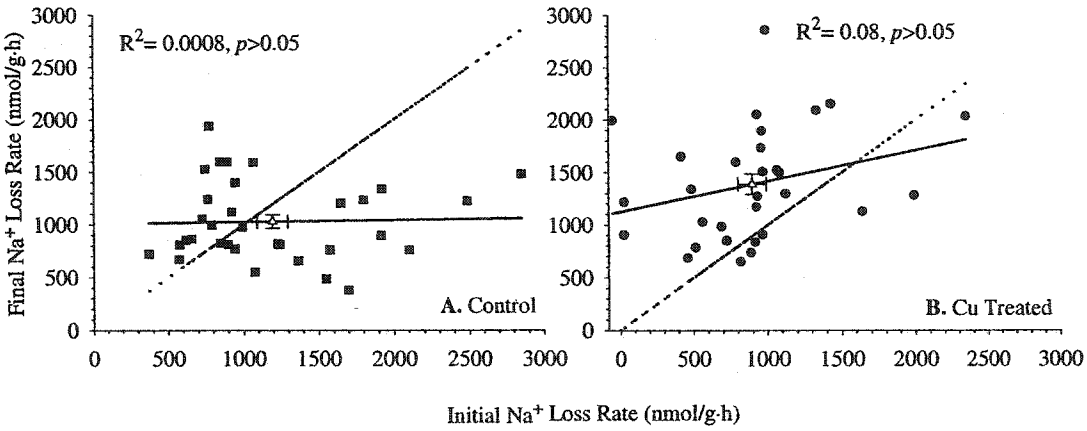
Table 3. Correlations of sodium loss rate before Cu exposure and each indicator measured after one week of exposure to 36 $\mu$g Cu/L (N=30). * indicates a significant relationship.

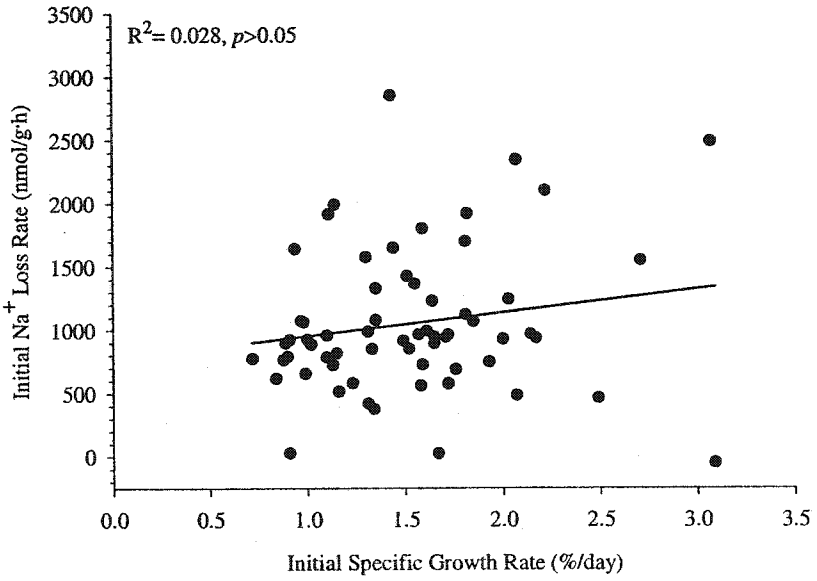
| *Indicator* | *Pearson's Correlation Coefficient (r)* | p *value (2-tailed)* |
|---|---|---|
| Gill Cu | 0.464 | 0.010* |
| SGR during Cu exposure | -0.425 | 0.019* |
| Carcass Cu | 0.323 | 0.082 |
| Na loss rate- after Cu exposure | 0.282 | 0.131 |
| $Na^+/K^+$- ATPase activity | -0.203 | 0.282 |
| Whole body Na | -0.168 | 0.376 |
| Whole body Cu | 0.158 | 0.403 |
| Liver Cu | 0.072 | 0.704 |
| Gut Cu | 0.011 | 0.956 |

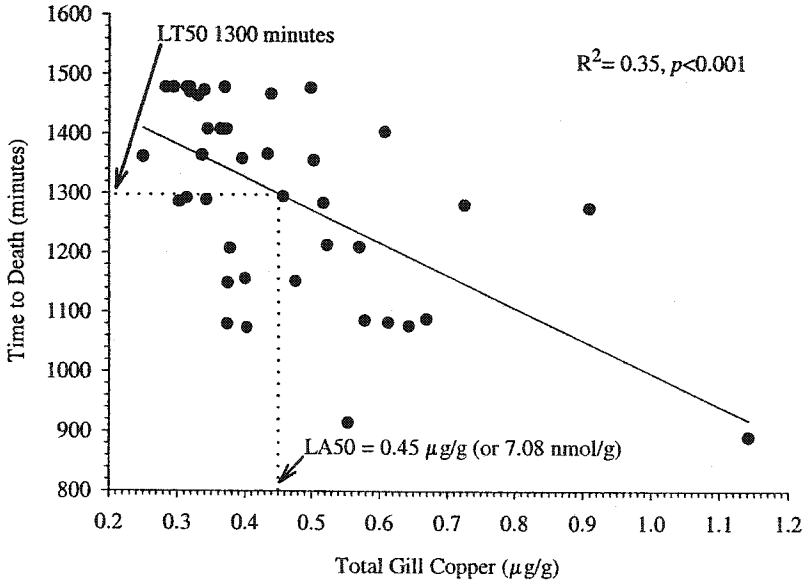
Table 4. Correlations of specific growth rate before a lethal exposure of 424 $\mu$g Cu/L and each indicator measured upon fish death (N=44). All fish died within 25 hours of Cu exposure.

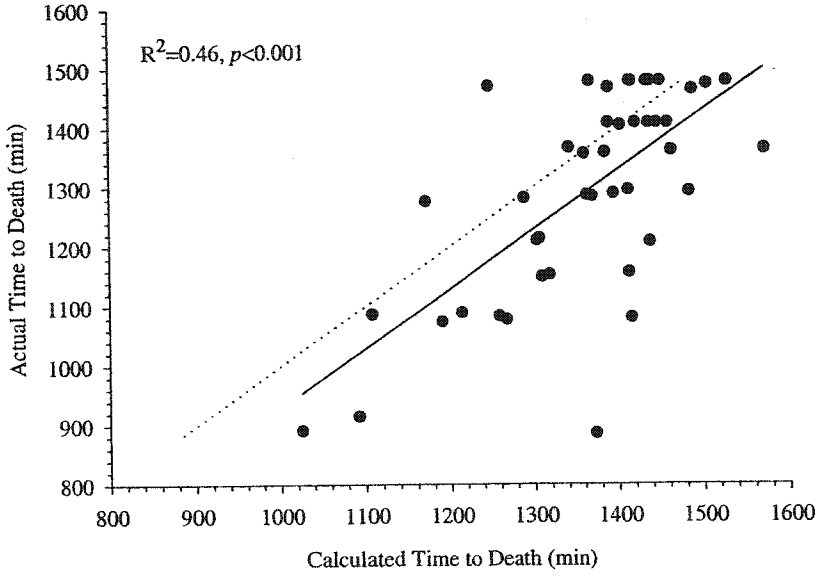
| *Indicator* | *Pearson's Correlation Coefficient (r)* | p *value* |
|---|---|---|
| Whole body Na | -0.223 | 0.145 |
| Time to death | 0.146 | 0.344 |
| Gill Cu | 0.130 | 0.402 |
| Carcass Cu (no gills) | -0.038 | 0.808 |
| Whole body Cu (including gills) | -0.031 | 0.841 |


Final Specific Growth Rate (%/day)
$R^2 = 0.52$, $p<0.001$
A. Control
$R^2 = 0.35$, $p<0.01$
B. Cu Treated
Initial Specific Growth Rate (%/day)


Final Na+ Loss Rate (nmol/g·h)
3000
2500
2000
1500
1000
500
0
R2= 0.0008, p>0.05
A. Control
R2= 0.08, p>0.05
B. Cu Treated
0
500
1000
1500
2000
2500
3000
Initial Na+ Loss Rate (nmol/g·h)


$R^2$= 0.028, $p$>0.05
Initial $Na^+$ Loss Rate (nmol/g·h)
3500
3000
2500
2000
1500
1000
500
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Initial Specific Growth Rate (%/day)


LT50 1300 minutes
$R^2$= 0.35, $p$<0.001
LA50 = 0.45 μg/g (or 7.08 nmol/g)
Time to Death (minutes)
Total Gill Copper (μg/g)
1600
1500
1400
1300
1200
1100
1000
900
800
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2


$R^2$=0.46, $p$<0.001
Actual Time to Death (min)
Calculated Time to Death (min)
800
900
1000
1100
1200
1300
1400
1500
1600

# CHAPTER 4

# AN *IN VITRO* APPROACH FOR MODELLING BRANCHIAL COPPER BINDING IN RAINBOW TROUT


## ABSTRACT

The main objective of this study was to characterize the individual effects of water chemistry ($Ca^{2+}$, $Na^{+}$, dissolved organic matter (DOM), pH, alkalinity) on the rapid binding of copper to the gill surface of rainbow trout using an *in vitro* gill binding assay. In this assay individual gill arches were exposed for 5 min to $^{64}Cu$ labelled copper solutions ranging from 0.02 to 0.16 $\mu$M in water chemistries reflecting the full range of fresh water values for the Great Lakes. The gills displayed saturable Cu binding within this Cu range but gill Cu binding was completely unaffected over the full range of calcium, sodium and alkalinity concentrations used. Only low pH (pH 4.0) and commercial DOM (Aldrich humic acid at $\geq$ 3 mg C/L) altered copper binding to rainbow trout gills *in vitro*. These findings were consistent with the results of geochemical modelling of our water chemistry (using MINEQL+, Version 4.5), which showed that $H^{+}$ and DOM affected the free cupric ion concentration. However, DOM (up to 80 mg C/L) was only able to reduce Cu on the gills by 50%. We hypothesize that in the range of 0.02 to 0.16$\mu$M Cu there are two high affinity Cu binding sites on the gills, one having a substantially higher affinity for copper than DOM. The absence of a calcium effect on gill copper binding was in accord with *in vivo* evidence that calcium primarily acts to alter the physiology of the gill binding sites through acclimatory processes, rather than

through competitive interactions. It was a surprise that water chemistry parameters influence rapid gill-metal binding in a manner different to their influence on acute toxicity and different from the effects on long-term binding reported in other studies. Currently, the biotic ligand model uses the rapid increase of gill copper (believed to reflect binding to the physiologically active receptor sites) to model gill binding characteristics. The distinction between rapid surface binding and metal uptake obviously plays an important role in determining the toxic effects of copper, especially when regulators need to predict the modifying effects of water chemistry.

**Keywords**: Trout, copper, modelling, gill binding, toxicity, water chemistry, biotic ligand model

## INTRODUCTION

Fish gills are the initial target of waterborne copper toxicity (Laurén and McDonald, 1985, McDonald and Wood, 1993, Wood, 2001). Copper binds to physiologically active sites on the gill, which regulate the transport of essential ions across the gill membrane, thus impairing branchial sodium uptake (Laurén and McDonald, 1985, McDonald *et al.*, 1989). The gill surface interaction model (GSIM), originally proposed by Pagenkopf (1983), aimed to explain the variability associated with trace metal toxicity by incorporating the differences in metal speciation, alkalinity, hardness and pH with gill-metal interactions. More recently, Playle and co-workers (Playle *et al.*, 1992,1993a,b) and MacRae *et al.* (1999a), derived conditional stability constants (log K) and binding capacities ($B_{max}$) of the gill for copper ions during three and 24 h exposures, respectively. These studies have been deemed toxicologically relevant for modelling the influence of water chemistry, as seen by their inclusion in the biotic ligand model (BLM discussed below; DiToro *et al.*, 2000, 2001, Paquin *et al.*, 2000), even though a condition of the GSIM was that the surface complexation (adsorption) and absorption of metals should be rapid and reversible (Pagenkopf, 1983). At this point a distinction needs to be made between metal surface binding and that which enters the gill (i.e., metal uptake).

A knowledge of water chemistry, gill metal binding characteristics (from Playle *et al.*, 1993a,b and MacRae *et al.*, 1999a) and acute toxicity provides the necessary framework in which the most recent gill surface interaction model has been developed, termed the biotic ligand model (BLM; DiToro *et al.*, 2000, 2001, Paquin *et al.*, 2000). In

brief, the BLM represents metal interactions with competing cations and complexing ligands found in natural waters and predicts metal accumulation at the gill. The level of accumulation is then used to predict acute toxicity (i.e. lethality). One issue that emerges is whether gill copper accumulations determined in the time frame required for acute mortality are appropriate in understanding the modifying effects of water parameters at the gill surface.

Researchers have documented the influence of water chemistry parameters such as hardness, pH, alkalinity and dissolved organic matter (DOM) on copper toxicity (for example, Zitko *et al.*, 1973, Zitko and Carson, 1976, Howarth and Sprague, 1978, Chakoumakos, 1979, Miller and Mackay, 1979, Laurén and McDonald, 1986, Erickson *et al.*, 1996). Generally, increases in hardness, pH, alkalinity and DOM provide protection against the toxic effects of copper and also protect against copper accumulation at the gill. Water chemistry influences accumulation by either competition or complexation (Playle *et al.*, 1992). Competition for biotic ligands occurs between Cu and other cations found in natural waters such as $Ca^{2+}$, $Na^{+}$ and $H^{+}$, whereas carbonates ($CO_3^{2-}$), hydroxides ($OH^{-}$) and DOM complex copper thus rendering it less bioavailable. The amount of copper accumulation at the gill is limited by the number of binding sites on the gill and the affinity of the sites for copper (Reid and McDonald, 1991, Playle *et al.*, 1993a, b, MacRae *et al.*, 1999a, also see review by Playle *et al.*, 1998). These constants were derived on the basis of there being one type of binding site on the gill, however Taylor *et al.* (2000) showed that there were at least two types of sites within the

toxicological range of copper (i.e. 96h –LC50 in soft water was 16 $\mu$g/L or 0.25 $\mu$M and the sites were identified between 1-25 $\mu$g/L or 0.016-0.4 $\mu$M).

The main objective of this study was to characterize the individual effects of $Ca^{2+}$, $Na^{+}$, pH, alkalinity, and DOM on the initial binding of copper to the gill surface. In order to focus primarily on surface reactions of metal to tissue, a rapid five minute *in vitro* gill binding assay was used. The use of isolated gill arches eliminated any changes in water chemistry caused by the organism itself (i.e. through excretion, mucus sloughing, general body surface/skin binding). In addition, gentle aeration allowed mixing of the arch with the exposure water, hence breaking down the gill boundary layer. Our method adapted from that of Reid and McDonald (1991), involved using radiolabelled copper ($^{64}Cu$) at environmentally realistic and toxicologically relevant concentrations (i.e.$\mu$M) in order to determine the 'newly accumulated' gill copper. Copper exposure concentrations were chosen based on our previous finding that high affinity sites saturated at < 0.24 $\mu$M total copper (Taylor *et al.*, 2000). The validity of the *in vitro* method was evaluated through binding site characterization and included: 1) changes in gill Cu binding with time, 2) the relationship between gill-Cu binding and copper concentration, 3) the displacement of copper from the gill surface, 4) the resemblance to *in vivo* gill binding and lastly, 5) the influence of fish size on gill copper binding.

## MATERIALS AND METHODS

*Experimental animals*

Adult (147.6 $\pm$ 2.6 g, N=90) and juvenile (2-50 g, N=30) rainbow trout were obtained from a commercial supplier (Humber Springs, Orangeville, ON, Canada) and maintained in dechlorinated Hamilton tap water (14°C, pH 8, 1.0 mM $Ca^{2+}$, 0.6 mM $Na^{+}$, 0.7 mM $Cl^{-}$, hardness 120 mg/L as $CaCO_3$, alkalinity 95 mg/L, 3.1 mg C/L DOM) for at least two weeks prior to experimentation. Trout were fed commercial trout pellets (Martins Feed Mill, Elmira, ON, Canada) to satiation three meals per week. The feed contained natural traces of ~ 3 $\mu$g Cu/g dry weight. Photoperiod was set to a light/dark cycle similar to the natural photoperiod for Western Lake Ontario.

*Binding Site Characterization*

A gill binding method, modified from Reid and McDonald (1991), was employed. Adult rainbow trout were killed with a single blow to the head. The three most anterior arches on both sides of the gill basket were removed from each fish (for a total of six arches per fish). The last posterior arches were not used in order to maintain a similar average gill arch weight. Only one arch was placed in each exposure cup, for a total of six cups running at any time. A standard soft water was prepared for all exposures by adding reagent-grade chemicals to deionized water (18 megaOhm; U.S. EPA, 1991). Added salts were 0.57 mM $NaHCO_3$, 0.22 mM $CaSO_4$, 0.19 mM $MgSO_4.7H_20$ and 0.03 mM KCl (analytical grade, BDH Inc., Toronto, ON, Canada). Other parameters of the synthetic soft water included, 40-48 mg/L as $CaCO_3$ hardness, 30 mg/L as $CaCO_3$

alkalinity and a pH of 7.7-7.8. The exposure solutions (50 mL) included radiolabelled $^{64}Cu$ (as copper nitrate, half-life 12.8h), which was obtained from the McMaster Nuclear Reactor (Hamilton, ON, Canada). Each exposure cup was fitted with an individual airline in order to provide constant mixing. Arches were exposed for 5 minutes followed by a 10s rinse with Cu-free water in order to release all loosely bound copper.

*Specific Features of Cu Binding In Vitro*

Characterization of the copper binding sites included evaluating several key features which were: (1) Cu accumulation over time, (2) binding *vs.* [Cu], (3) the fraction of Cu that is surface bound (i.e. reversible), (4) the relationship between *in vitro* and *in vivo* binding and (5) the influence of fish size on copper binding. The time dependency experiment involved exposing individual arches (N=6 per time) to a nominal concentration of 0.16 $\mu$M Cu (specific activity (SA) was 2.42-3.76 mBq/$\mu$g) for varying amounts of time ranging from as short as 10s up to 1 h. To determine the affinity of the gill for copper (log K) and the maximum number of binding sites ($B_{max}$), gills were exposed to varying copper concentrations (0 to 0.5$\mu$M, SA was 0.1-37.66 mBq/$\mu$g) using a fixed time (5 min.) selected on the basis of the preceding series. The concentrations $\leq$ 0.16 $\mu$M total copper (i.e. within the range of saturability) were used to estimate free cupric ion concentrations (0-110 nM; using MINEQL+ Version 4.5, Schecher and McAvoy, 2001, see analytical techniques for details) in order to accurately determine the log K. To distinguish the amount of copper that was truly surface bound from the amount that had actually entered the gill cells (i.e. copper uptake), a 'pulse-chase'

experiment was conducted. This test involved the standard five minute exposure to 0.16$\mu$M (i.e. the 'pulse', SA was 3.64–6.06 mBq/$\mu$g) but replaced the 10s rinse in Cu-free water with a rinse containing 100 times unlabelled 'cold' copper (i.e. the 'chase', 16 *vs.*0.16 $\mu$M nominal Cu concentrations). This concentrated copper rinse was for 30s, 1, 2.5 or 5 minutes and was intended to displace any surface bound $^{64}$Cu. Any radioactivity that remained was assumed to be copper inside the cells. The *in vitro* technique was compared to *in vivo* by exposing whole adult rainbow trout for five minutes to 0.16 $\mu$M Cu (SA was 0.9-1.71 mBq/$\mu$g). The exposures were carried out in 3L of the synthetic soft water contained in black acrylic boxes (one fish per box). At the end of the five minutes, fish were killed by a single blow to the head and the entire gill basket removed. The same arches were isolated, as mentioned above for the *in vitro* assay, and counted for radioactivity. Lastly, an experiment was conducted to determine if the amount of copper bound per gram of gill tissue *in vitro* was dependent upon fish size. An assortment of fish was utilized ranging from 3 to 215 g. Arches were isolated from these fish and exposed to 0.16 $\mu$M Cu (SA was 2.33-3.15 mBq/$\mu$g) for five minutes.

*Effects of Water Chemistry Variables*

Water parameters were varied one at a time including: calcium, sodium, alkalinity, pH and dissolved organic matter (Table 1). These five variables were chosen based on their type of effect on copper binding: cation competition with $Na^+$, $Ca^{2+}$ and $H^+$ ions and modifiers of copper speciation or complexing ligands including alkalinity, pH and DOM. All experiments were conducted for five minutes at the nominal copper

concentration of 0.16 $\mu$M (specific activity (SA) ranged from 0.5-5.76 mBq/$\mu$g). Water samples (5.0 mL) from each exposure cup were collected and acidified with 50 $\mu$L concentrated trace metal grade nitric acid (Fisher Scientific, Toronto, ON, Canada). The effect of added calcium on copper binding was evaluated over the concentration range of 0 to 1.2 mM $Ca^{2+}$, whereas the range of sodium tested was much larger at 1 $\mu$M to 30 mM. Alkalinity was altered in the test water by replacing $NaHCO_3$ with NaCl and adding $KHCO_3$, in order to maintain constant sodium levels. The range of alkalinity tested was as low as 0.2 mEq/L increasing to 2.3 mEq/L as $CaCO_3$ equivalents. Water pH was evaluated by testing in acidic, neutral and alkaline conditions [i.e. pH 4.0, 7.0, 7.7 (unadjusted) and 9.0]. Lastly, commercial dissolved organic matter (Aldrich humic acid, Sigma Aldrich, Oakville, ON, Canada) was evaluated at environmentally realistic levels up to 20 mg carbon/L and one extreme level of 80 mg carbon/L. Solutions for the DOM experiment were allowed to age for 3 h in order to allow interaction time with the copper (as recommended by Ma *et al.*, 1999).

In order to test if acclimation time to low calcium water influenced gill copper binding, one *in vivo* experiment was also conducted. Juvenile rainbow trout (~10 g, N=10) were exposed to 0.27 $\mu$M radiolabelled copper (SA was 578 kBq/$\mu$g) for 3 h. The gills were excised and counted for radioactivity. These fish had been acclimated for varying amounts of time to soft water ($Ca^{2+}$ 0.02 mM) prior to the 3 h gill binding exposure. Specifically, the first group of hard water acclimated fish ($Ca^{2+}$ 1 mM) were also exposed to copper in hard water (i.e. 0 time in soft water), the second group of hard water acclimated trout were acutely transferred to soft water only for the 3 h exposure to

copper, and lastly, the third group of hard water acclimated trout were transferred to soft water for 21 h prior to the 3 h exposure of copper in soft water (i.e. a total of 24 h in soft water).

*Analytical techniques*

The radioactivity in gill tissues and water samples was measured in a well-type gamma counter with a 7.62 cm NaI crystal (Packard Minaxi Auto-Gamma 5000 Series, Packard Instruments, Meridan, CT, USA). Total copper, calcium and sodium concentrations in water samples were measured using flame or graphite furnace atomic absorption spectrophotometry (Varian SpectrAA-220FS and GTA 110, Walnut Creek, CA, USA) against certified standards (Fisher Scientific). Alkalinity was measured in the bulk solutions by titrating 5.0 mL to pH 4.0 with 0.02 M HCl prepared from a 2.0 N HCl standard (Sigma-Aldrich, Oakville, ON, Canada) and aerated before and after the exposure time. To prepare solutions containing dissolved organic matter at desired levels we assumed that 1.0 mg of Aldrich humic acid contained 0.5 mg carbon (i.e. 50%). Actual total carbon and inorganic carbon concentrations were measured on the bulk test solutions using a total organic carbon analyzer (Shimadzu TOC- 5050A, Tokyo, Japan). The DOM concentrations were calculated automatically by subtracting inorganic carbon from total carbon, and are reported as dissolved organic carbon (in mg carbon/L). The pH in individual exposure cups was measured directly using a PHM-84 meter with a GK2401C combination electrode (Radiometer, Copenhagen, Denmark). Free cupric ion concentrations were calculated using a geochemical modelling program (MINEQL+,

Version 4.5, Schecher and McAvoy, 2001) and the above measured aqueous chemistries. All estimations were conducted in a system in equilibrium with the atmosphere with the exception of variable alkalinity, where a closed system simulation was assumed.

*Calculations*

Newly accumulated gill copper concentrations (nmol/g) in the gill were calculated based on the accretion of radioactivity in the gill:

$$Cu_{gill} = \frac{^{64}Cu_{gill}}{SA}$$

where $^{64}Cu_{gill}$ = radioactivity of the gill in counts per minute (cpm) per gram of wet gill tissue and SA = specific activity of the water in cpm/nmol.

The binding characteristics of the saturable sites on the gill (binding capacity, $B_{max}$ and affinity, log K) were calculated using non-linear regression. The fit of newly accumulated gill copper (nmol/g) against the calculated free cupric ion concentration ($Cu^{2+}$; nM) is given by the equation:

$$\text{Newly accumulated gill Cu} = \frac{B_{max} \times [Cu^{2+}]}{(K_{gill\text{-}Cu} + [Cu^{2+}])}$$

where Bmax is in nmol/g and $K_{gill\text{-}Cu}$ is in nmoles/L.

*Statistics*

All data presented are means ± 1 standard error (N). A Student's *t* test (two-tailed, unpaired) was used to test for significant differences between two treatments. In cases where treatments were compared to a control group, an analysis of variance was conducted followed by a Dunnett's test to isolate the significant differences. Significance was set at $p < 0.05$. Non-linear regressions were applied using Sigma Plot 2000 allowing for the calculation of saturation kinetic parameters (i.e. maximum number of binding sites on the gill ($B_{max}$), the affinity of the gill for copper (log $K_{gill\text{-}Cu}$) and the time to half saturation).

## RESULTS

*Site Characterization*

Initial experiments determined the time course for metal saturation on the isolated gill arches (Fig. 1A). A plateau of newly accumulated gill copper appeared at two and four minutes of waterborne copper exposure. Copper binding became increasingly variable at times greater than eight minutes. To determine whether the gills were in fact saturating in five minutes, a second more detailed time course evaluated copper binding at times < 300 seconds (Fig.1B). The fit of non-linear regression to all the data points revealed a time to half saturation ($t_{½}$) of 171 s. Unexpectedly, a second plateau may also be present at exposure times less than 50 seconds, with a $t_{½}$ of less than 10 seconds. We concluded that five minutes was an appropriate exposure time to employ for the remainder of the experiments due to the technical difficulties associated with < 10 second

exposures and the fact that saturation was occurring and the response variability was considered minimal.

Isolated arches were exposed to a range of copper concentrations for five minutes in order to characterize the binding sites for copper (Fig.2). At least two types of sites were identified: 1) saturable high affinity, low capacity sites found below 0.16 $\mu$M copper, and 2) lower affinity, higher capacity sites which bound copper in a linear fashion up to 0.5 $\mu$M copper. Using the estimated free cupric ion concentration, the affinity or log K for the high affinity saturable sites was 8.1 and the $B_{max}$ was 0.0165 nmol/g.

The displacement of radiolabelled copper with unlabelled copper allowed for the distinction between surface bound copper and that which had entered the gill cells (Fig.3). The removal of loosely bound copper was found to increase with longer rinse times. The maximum rinse time of five minutes could only displace up to half of the bound copper. Therefore, the remaining 50% was assumed to have entered the gill cells (i.e. copper uptake).

The *in vitro* preparation used in this study may be comparable to *in vivo* gill binding also conducted for five minutes on large fish. Intact whole fish bound 0.117 $\pm$ 0.018 (N=3) nmol Cu/g of gill tissue, whereas the isolated gill arches bound 0.071 $\pm$ 0.012 (N=6 from 6 fish) nmol Cu/g. Both were exposed to 0.30 $\mu$M total copper and the amounts accumulated were not statistically different from each other (P= 0.099).

The size of the fish greatly influenced *in vitro* gill copper binding (Fig. 4), an effect which was especially evident in small fish weighing less than 50 g. The relationship was best described by a power function ($y = 0.2394x^{-0.4685}$, $R^2 = 0.72$,

P<0.001). Based on this significant relationship, the size range used in this study (110 to 220 g) was not likely a factor adding to the variability associated with newly accumulated gill copper binding.

*Effects of Water Chemistry Variables*

*Cation Competition*

Over the range of calcium tested, there was no significant effect on the amount of newly accumulated copper by isolated gills at 0.17 $\mu$M total copper (Fig. 5A). The average gill copper concentration, regardless of [$Ca^{2+}$], was 0.046 $\pm$ 0.003 (33) nmol/g. Despite the absence of a direct calcium effect, there was an impact of varying calcium acclimation times *in vivo* on the amount of copper bound to the gills (Fig. 5A-inset). Increasing the exposure time to low calcium water from 0 to 24h increased the amount of gill copper by ~six times. However, after 12 weeks of soft water acclimation newly accumulated gill copper levels returned to levels in fish acclimated to high calcium water (i.e. 1.85 and 2.75 nmol Cu/g of gill tissue, respectively).

Gill copper binding at 0.18 $\mu$M copper was also independent of the sodium concentration (Fig. 5B). The average newly accumulated gill copper amount was 0.027 $\pm$ 0.002 (65) nmol/g, regardless of sodium concentration. The absence of an effect was evident over the whole range of sodium (1$\mu$M to 30mM).

*Copper Speciation and Complexation*

The effect of varying alkalinity was tested over the range of 0.2 to 2.3 mEq/L at 0.18 $\mu$M total copper and pH 7.7 (Fig. 6A). There was no significant effect on newly accumulated gill copper binding with all values averaging 0.0369 $\pm$ 0.004 (34arches from 6 fish) nmol/g. According to MINEQL+ estimations in a system open to the atmosphere, the free cupric ion does not vary with changes in alkalinity at pH 7.7 and the $CuCO_3$ species accounted for ~55% of the total copper species. However, in a closed system (i.e. where carbon dioxide cannot be released into the air) the free copper concentration was estimated between 20 and 4% of the total copper species and the copper carbonate species ranged from 47 to 89% at the lowest and highest alkalinities tested, respectively.

Acidic conditions were able to affect newly accumulated gill copper. Gill copper more than doubled at pH 4.0 compared to neutral and pH 9.0 (i.e. 0.0410 vs. 0.0155 and 0.0141 nmol/g, respectively; Fig. 6B). The calculated speciation of copper at 0.20 $\mu$M was dramatically different at these three pHs. At pH 4.0, 100% of the copper species was free $Cu^{2+}$, whereas at pH 7.0, 7.7 (unadjusted) and 9.0 it had declined to 70, 19 and 0%, respectively. At the unadjusted pH of 7.7 the only other species of copper (other than $Cu^{2+}$) that was positively charged was $CuOH^+$ (26% of the copper species present). However at the more alkaline pH of 9.0, $CuOH^+$ was the only positively charged species [1.4% $CuOH^+$, 2.5 % $Cu(OH)_2$, 53.1 % $CuCO_3$, and 42.9 % $Cu(CO_3)^{-2}$].

The concentrations of dissolved organic matter employed in this study were able to complex 0.18 $\mu$M copper to varying degrees (from 78 to 100% Cu-humate; Fig.7). Geochemical modelling of the test water required the use of Playle *et al.*'s (1993b) Cu-

DOC binding constant (log K) of 9.1. The resulting change in free copper (from ~20 to 0%) was reflected by a decrease in the amount of copper bound to the gills, however no dose-response was evident. Approximately half of the newly accumulated gill copper was kept off the gills over the concentration range of DOM used (~0.04 *vs.* 0.02 nmol Cu/g of gill tissue).

## DISCUSSION

The gill surface interaction model (GSIM), conceived by Pagenkopf (1983), provided the foundation on which more recent gill binding models have been formulated. One key aspect of the GSIM, on which the present study was based, states that for surface metal-gill interactions the exchange must be rapid and reversible (Pagenkopf, 1983). Indeed our *in vitro* model was successful based on these prerequisites because this is the first study to identify the fraction of exchangeable sites on the gill.

### *Characterization of Binding Sites In Vitro*

The *in vitro* technique employed in this study was adapted from one previously developed by Reid and McDonald (1991). The most important modifications were working at $\mu$M (rather than mM) concentrations of radiolabelled copper and eliminating the preparatory rinse of the isolated arches with 5 mM ethylenediaminetetraacetic acid (EDTA), which was formerly used to remove $Ca^{2+}$ and $Mg^{2+}$ from the gills. We found that our method allowed for consistent determinations of newly accumulated gill copper (i.e., all five minute assays conducted at 0.16-0.3 $\mu$M Cu ranged from 0.016 to 0.06 ng

Cu/g of gill tissue). The initial time course study revealed a less variable response of newly accumulated gill copper at exposure times less than 8 minutes. Beyond this, binding increased greatly and the variability around the mean response also increased, presumably reflecting variability in the viability of the tissue and the increased penetration of the preparation by Cu associated with the deterioration of viability. The second time course which focused on exposures under 300 seconds, displayed the gill surface saturating rapidly with copper ($t_{½}$ = 171s) at a water concentration of 0.16 $\mu$M. Beyond this concentration, another set of sites appear to be accessed by copper and are identified as lower affinity, higher capacity sites. This result, of a second set of sites, confirms previous findings conducted *in vivo* using juvenile rainbow trout exposed to copper radiolabelled with $^{64}$Cu for three hours, which were also tested within the same range of water copper (Taylor *et al.*, 2000). In the present study using isolated arches, we have verified the same breakthrough phenomenon to lower affinity sites within the range of ~0.2-0.3 $\mu$M total copper.

*In vivo* gill copper binding has been traditionally evaluated in soft water at pH < 6.5 for three or 24 hours (Playle *et al.*, 1992, 1993a, b, MacRae *et al.*, 1999a, also see review by Playle, 1998). Because these studies were conducted without radiolabelled copper, a low pH and long exposure time were employed in order to maximize the amount of free cupric ion (> 98% of the copper species present) and to detect differences against the background levels of copper on the gills, respectively. We know that our brief exposure time of five minutes reflects at least 50% of surface binding based on the pulse-chase experiment. Because only 50% of the bound copper could be stripped off of

the gill, we can conclude that a portion of the radiolabelled copper had indeed entered the gill tissues in five minutes. The portions of surface-complexed metal and metal uptake across the gill surface in previous traditional gill binding studies is unknown. However, measurable copper accumulation did occur within 20 minutes in the study by Playle and co-workers (1992). Indeed, studies on algae (Crist *et al.*, 1990) and yeast (Huang *et al.*, 1990) indicate copper binding to cell surfaces in 5 and 50 seconds, respectively. Whether surface binding or metal uptake or some combination of the two most accurately predicts metal toxicity is also unknown. Nonetheless, we believe true surface binding, over a shorter time interval, simplifies the task of modelling the influence of competitive and complexing agents in exposure water. We provide evidence for the key assumption originally made by Pagenkopf (1983), which stated that the rates of metal exchange between the gill surface and exposures are rapid when compared to the time required for a bioassay test. The *in vitro* technique of isolating gill arches also simplifies some of the confounding factors involved in whole animal gill binding assays.

The gill microenvironment established in a whole fish has been one factor known to influence gill copper binding (Playle *et al.*, 1992). Transfers of carbon dioxide and ammonia can acidify or alkalinize expired water, depending on the water pH. Inspired water would be rendered more acidic at the pH and buffering capacity used in the present study (Playle and Wood, 1989, Lin and Randall, 1990, Playle *et al.*, 1992), a shift which strongly affects the speciation of copper. For example, the amount of the free cupric ion calculated using MINEQL+ changes from 15% at pH 7.7 to 71% at pH 7.0 at 0.16$\mu$M total copper. This magnitude of pH change, (i.e. 0.7 pH units), is reasonable for live trout

in our water (Playle and Wood, 1989, Playle *et al.*, 1992). The *in vitro* protocol of the present study eliminates this effect plus any differences in ventilation frequency and volume between individual fish and any non-specific binding to the skin surface or sloughed off mucus (Reid and McDonald, 1991).

Another confounding factor known to influence copper toxicity is fish size (Howarth and Sprague, 1978). Fish size did indeed have a profound effect on gill copper binding in our study, however this effect was largely seen in fish less than ~25 g. We conclude that the gills of smaller fish bind more copper per unit gill mass, therefore in using larger fish (110-216 g) we have reduced any associated variability on the amount of copper bound by the gills. The magnitude of our size effect is consistent with Howarth and Sprague's (1978) conclusion that smaller fish were more sensitive to copper toxicity, presumably because they bind more copper at the site of copper's toxic action. According to Howarth and Sprague (1978) a 10 g trout is 2.5 times more resistant to copper than a 0.7 g fish. Our fish size - gill copper binding relationship predicts a 0.7 g fish to have 3.5 times more newly accumulated copper (per unit weight of gill), than a 10 g fish. Kamunde *et al.* (2001) described a negative exponential relationship between fish mass and gill copper uptake rates *in vivo* also using radiolabelled copper. By comparison, a theoretical 10 g fish would likely bind ~ two times more Cu in five minutes than the same size fish in one hour from Kamunde *et al.* (2001), indicating that significant copper regulation may have occurred during this time frame. In other words, in one hour a fish could begin to detoxify, store and eliminate copper. MacRae *et al.* (1999a) used juvenile rainbow trout ranging 15-40 g and Playle *et al.* (1992, 1993a, b)

used 0.5 to 4g fathead minnows to model gill copper binding yet neither evaluated the influence of size. A partial explanation for our size effect may be due to differences in surface area per unit volume of gill tissue in actively growing fish (Hughes, 1972).

*Effects of Water Chemistry*

In the present *in vitro* study, we have clearly defined the initial response of gill tissue to copper exposure, as influenced by calcium, sodium, alkalinity, pH and DOM. *In vivo*, it has been firmly established that all of these water chemistry parameters play a role in mitigating copper binding (Zitko and Carson, 1976, Pagenkopf, 1983, Playle *et al.*, 1992, 1993a, b, MacRae *et al.*, 1999a, Taylor *et al.*, 2000) and copper toxicity (Chakoumakos, 1979, Miller and Mackay, 1979, Spear and Pierce, 1979, Laurén and McDonald, 1986, Erickson *et al.*, 1996, Taylor *et al.*, 2000).

i) Alkalinity and pH

It is generally accepted that calcium (reported as water hardness) competes with copper for binding sites on the gills and that the accompanying bicarbonate/carbonate concentrations (usually present as $CaCO_3$ alkalinity) complex copper, thereby reducing the amount of free or available cupric ion. By contrast, we found no effect of calcium or alkalinity on newly accumulated gill copper binding over the range found in the Great Lakes. Playle *et al.* (1992) also found no effect of calcium on copper binding at pH 6.3 but found gill copper accumulation was eliminated at pH 4.8 by $Ca^{2+} \geq 2100$ $\mu$Eq/L. It was believed that the increase in hydrogen ion concentration at the lower pH may have added to the competition by calcium. In contrast, we found a decrease in pH (at constant

[$Ca^{2+}$]) caused an increase in gill copper binding. Apparently, the increase in free cupric ion concentration at pH 4 outweighed the competitive effect of $H^+$ ions for these high affinity sites. At pH 9.0 and 7.0, there was no difference in newly accumulated gill copper, thus confirming the availability of copper hydroxide species to the gill (Chakoumakos, 1979). The lack of an alkalinity effect on the rapid binding of copper to the gill is somewhat supported by the previous finding which showed that alkalinity did not alter copper uptake over 24 hours (Laurén and McDonald, 1986). This is despite the fact that the free cupric ion concentration in our study (as estimated by MINEQL+ in a closed system), varied ~20% over the range of alkalinities tested. We assumed that a system closed to the atmosphere was appropriate for modelling changes in alkalinity because of the short exposure time. Realistically, it would likely take more than five minutes for the release of carbon dioxide from the water, whereas the geochemical model assumes equilibrium conditions. Also in support of this assumption, the alkalinity and pH were measured before and after the exposure time and both remained unchanged despite the gentle aeration required for mixing. The copper complex present in the largest amount was $CuCO_3$. However, it has been reported that copper carbonate complexes are not available for binding/uptake and hence are considered non-toxic copper species (Chakoumakos, 1979).

ii) Calcium

Although our data did not exhibit a competitive effect of calcium at the gill, our *in vivo* experiment did show that calcium may influence the gill through acclimatory processes. This result is in agreement with hypotheses previously stated by Taylor *et al.*

(2000), where the role of calcium in regulating membrane permeability and stabilizing membrane proteins may also include regulating the number and affinity of binding sites on the gill surface. Fish which were fully acclimated to hard water or soft water bound similar amounts of newly accumulated copper, likely as a result of their ionoregulatory homeostatic stability. In striking contrast, hardwater acclimated trout acutely transferred to soft water for 3 or 24 hours bound increasingly larger amounts of copper, a result likely due to their ionoregulatory homeostatic upset. The effect of ion poor water on the morphology of the gills has been reported (see review by Laurent and Perry, 1991) and therefore it is reasonable to suggest calcium may affect the physiology of gill metal interactions. Gunderson and Curtis (using an isolated arch technique; 1995), reported the effect of calcium on gill permeability and identified different calcium binding sites responsible for the membrane permeability function.

iii) Sodium

We also found no effect of sodium on the initial response of the gill to copper. This effect was absent over the large range of sodium concentrations tested (1$\mu$M to 30 mM). The rationale for the extended test range (i.e. outside the range of North American freshwaters) was to encompass the sodium sensitive (< 200 $\mu$M $Na^+$) and sodium in-sensitive copper uptake pathways over 2 h, proposed by Grosell and Wood (2002). In addition, the mechanism of acute copper toxicity is to impair sodium balance, and trout have been shown to compensate by altering sodium and copper uptake from the diet (Pyle *et al.*, submitted). We agree with Pyle *et al.* (submitted), that sodium plays an important

role in modifying copper uptake and toxicity, nonetheless our results indicate that it does not affect the rapid high affinity binding of copper to the gills.

iv) Humic Acid

Commercial dissolved organic matter reduced copper binding to isolated arches but over the range of DOM tested (3 to 82 mg carbon/L) a dose-response was not exhibited. Previous studies have shown that the presence of DOM at $\geq$ 5 mg/L reduced copper to background levels on the gills (Playle *et al.*, 1993a,b, Hollis *et al.*, 1997, Richards *et al.*, 2001). One reason we were not able to completely keep Cu off of the gills may be due to the increased level of detection or sensitivity of our assay (i.e. radiolabelled Cu and 5 minute exposures). In addition there has been a range of reported values for the affinity of DOM for copper, presumably because many types of sites exist on organic acids. Morel (1983) found that copper-DOM binding constants vary with log Ks ranging from 6 to 11 in waters with pH >6. Playle *et al.* (1993a,b) specifically estimated stability constants for the high affinity copper binding sites on DOM to be log $K_{Cu\text{-}DOM}$ of 9.1 and on the gill to be log $K_{Cu\text{-}gill}$ of 7.4. Accordingly, copper would likely bind to DOM rather than the gill surface. MacRae *et al.* (1999b) reported two types of binding sites for Cu on Aldrich humic acid; low and high affinity sites with log Ks of 6.15 and 8.14, respectively. The higher affinity sites characterized by Playle *et al.* (1993a,b) are most relevant at the low copper concentrations employed in our study. For modelling free copper concentrations in the present study, we employed Playle and co-workers (1993b) log $K_{Cu\text{-}DOM}$ of 9.1. Therefore, in comparison to our calculated affinity constant for copper to the gill, log $K_{Cu=gill}$ of 8.1, Cu would certainly be bound by DOM

rather than the gill. In contrast, at any DOM concentration used in our experiment, copper accumulation was reduced by only 50%. We must conclude then the possible existence of another set of high affinity sites on the gills for Cu. These sites would have a stronger affinity for copper than does DOM. The only other possible explanation would be that the Cu-DOM complex itself was able to bind to the gill surface. Wilkinson *et al.* (1993) suggested this possibility with aluminum as a ternary complex, where the metal plays a bridging role [i.e. $(H_2O)_x$-(DOM)-Al-Ligand-gill]. Moreover, fulvic acids (a major component of DOM) have been shown to bind to isolated Atlantic salmon gill cells (Campbell *et al.*, 1997). However, these studies also report the necessity for acidic conditions in order for this to occur and at our pH of 7.7 the binding of a Cu-DOM complex at the gill surface would be unlikely.

## CONCLUSIONS

The *in vitro* technique used in this study was successful in modelling the initial or rapid response of copper binding to the gill surface and in understanding the influence of water chemistry, one variable at a time. The use of isolated arches simplified the gill micro-environment and ultimately research will be necessary to combine acute toxicity, water quality and gill binding in whole fish. It was a surprise that water chemistry parameters influence gill-metal binding in a manner different to their influence on acute toxicity and different from the effects on long-term binding reported in other studies. This was evident by the absence of a protective effect from the competing ions calcium, sodium and hydrogen, no effect at all of alkalinity or high pH and the fact that

commercial DOM did not prevent copper from binding to the gills. Currently, the biotic ligand model uses the rapid increase of gill copper (believed to reflect binding to the physiologically active receptor sites) to model gill binding characteristics (DiToro *et al.*, 2000). The distinction between rapid surface binding and metal uptake obviously plays an important role in determining the toxic effects of copper, especially when regulators need to predict the modifying effects of water chemistry.

## ACKNOWLEDGMENTS

We would like to acknowledge J.G. Richards at McMaster University for his contribution to this study. We also thank M. Schwartz and Dr. R. Playle at Wilfrid Laurier University for the DOM analysis. This research was supported by the Metals in the Environment Research Network (MITE-RN), the International Copper Association Inc., Falconbridge Ltd., Cominco Ltd., International Lead Zinc Research Organization, Noranda Inc., Nickel Producers Environmental Research Association and a Strategic Research Grant from the Natural Sciences and Engineering Research Council of Canada. C.M. Wood is supported by the Canada Research Chair program.

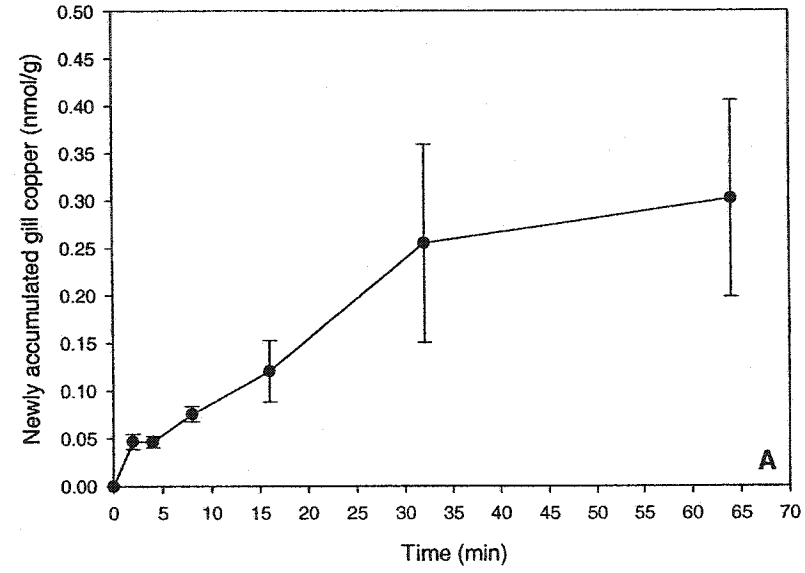
Table 1. Experimental and typical ranges for each water constituent tested using the *in vitro* gill binding method. A standard synthetic soft water was used in all experiments (see methods, U.S. EPA, 1991) and the exposure temperature was 20°C. Each parameter was adjusted individually while all others were held constant.

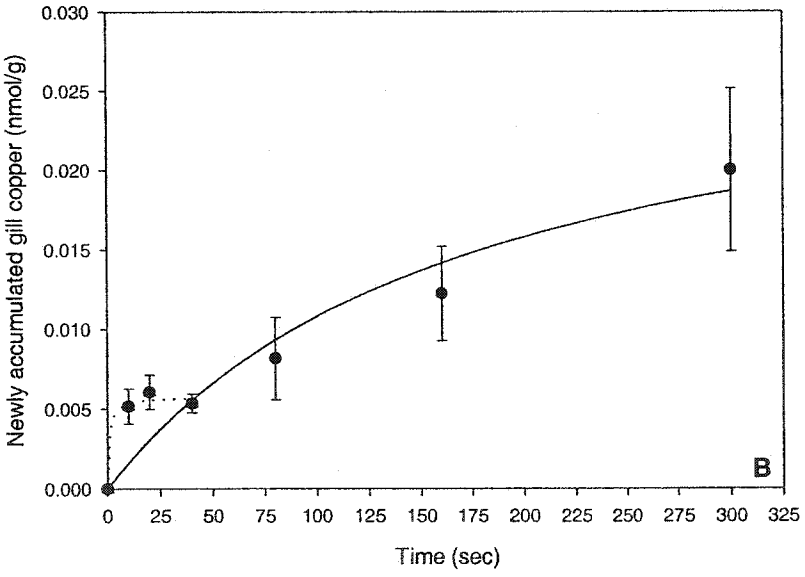
| | *Test Parameter Range* | *Typical Range in Freshwater* | *Method of Adjustment* |
|---|---|---|---|
| $Na^+$ | 0.001 – 30 mM | 0.043 – 0.52 mM[a] | NaCl |
| $Ca^{2+}$ | 0.2 – 1.1 mM | 0.3 – 1 mM[a] | $CaSO_4$ |
| Carbonate alkalinity | 0.2 – 2.3 mEq/L | 1 – 2.6 mEq/L[a] | $KHC0_3$ ($NaHCO_3$ replaced with NaCl) |
| $Cu^{2+}$ | 0 – 0.5 $\mu$M (total) | 3 – 78 nM (dissolved)[b] | $CuN0_3$ |
| DOM | 2 – 82 mg C/L | 1 – 12 mg C/L[c] | Aldrich humic acid |
| pH | 4.0, 7.0, 7.7, 9.0 | 7.8 – 8.1[a] | HCl or KOH |

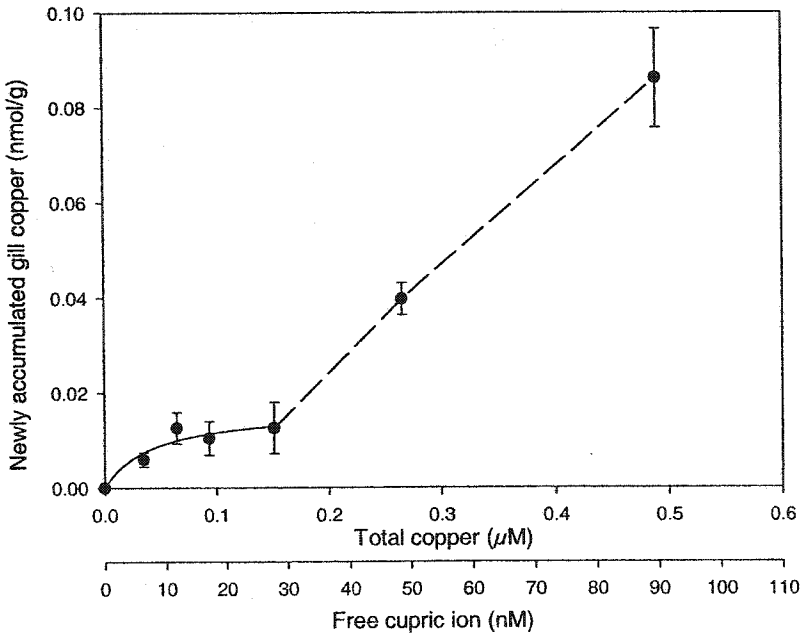
[a] Beeton *et al.*, 1999.

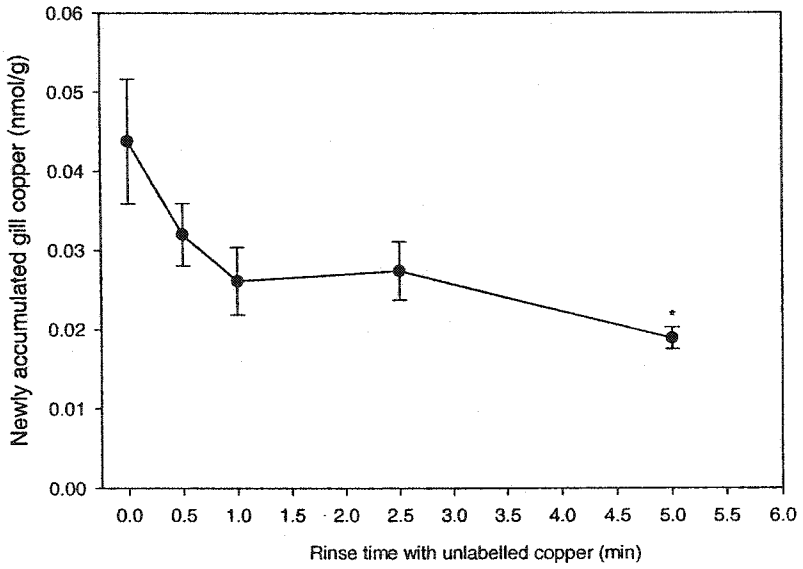
[b] Batley *et al.*, 1999.

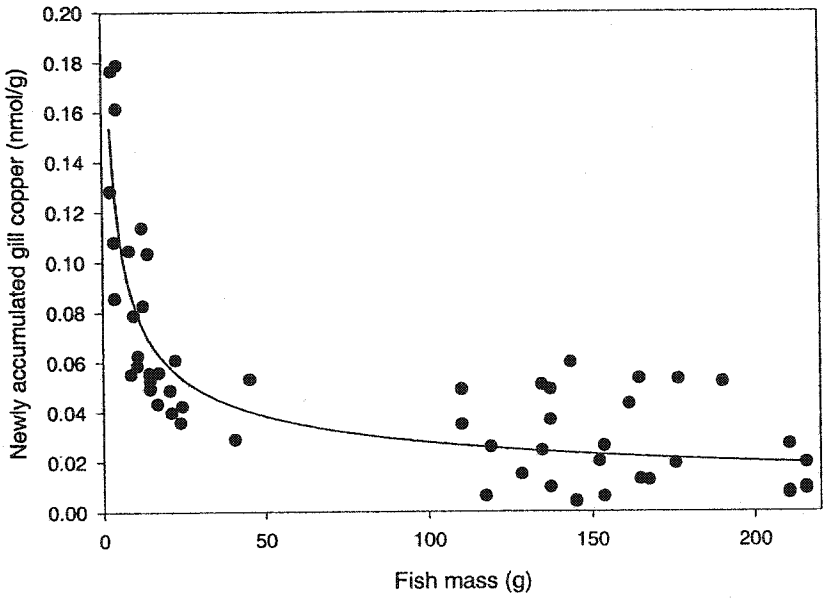
[c] Morel, 1983.

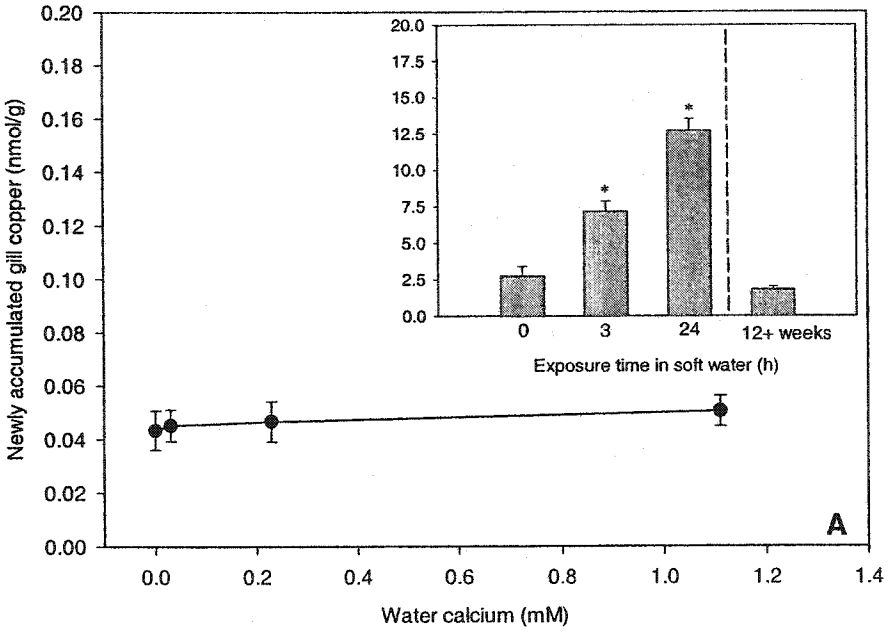

Newly accumulated gill copper (nmol/g)
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
Time (min)
A

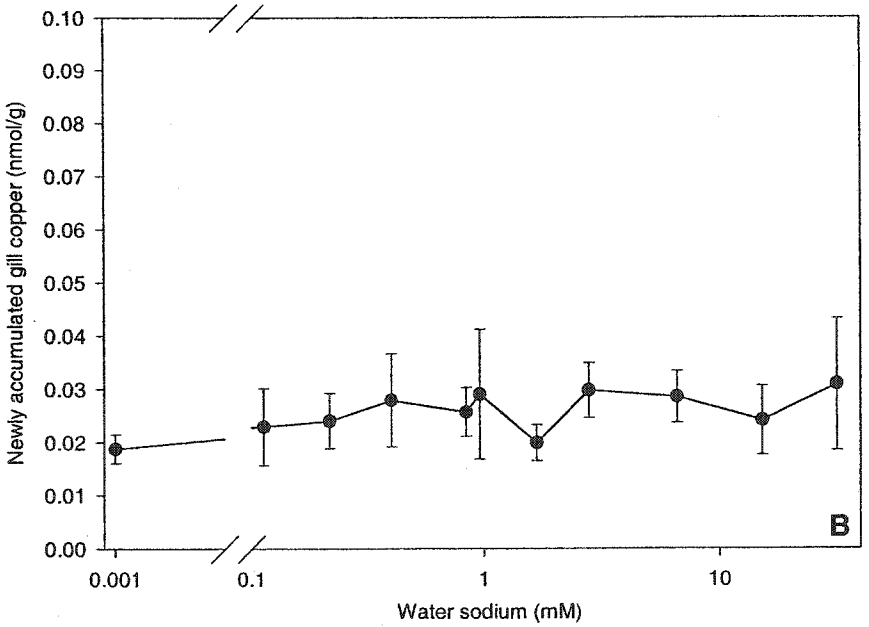

Newly accumulated gill copper (nmol/g)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
0 25 50 75 100 125 150 175 200 225 250 275 300 325
Time (sec)
B

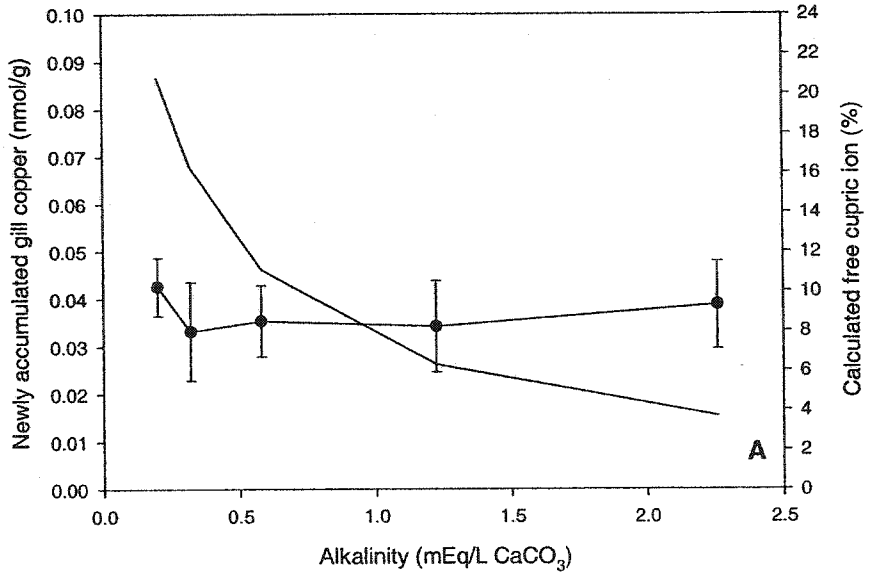

Newly accumulated gill copper (nmol/g)
0.10
0.08
0.06
0.04
0.02
0.00
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Total copper (μM)
0
10
20
30
40
50
60
70
80
90
100
110
Free cupric ion (nM)

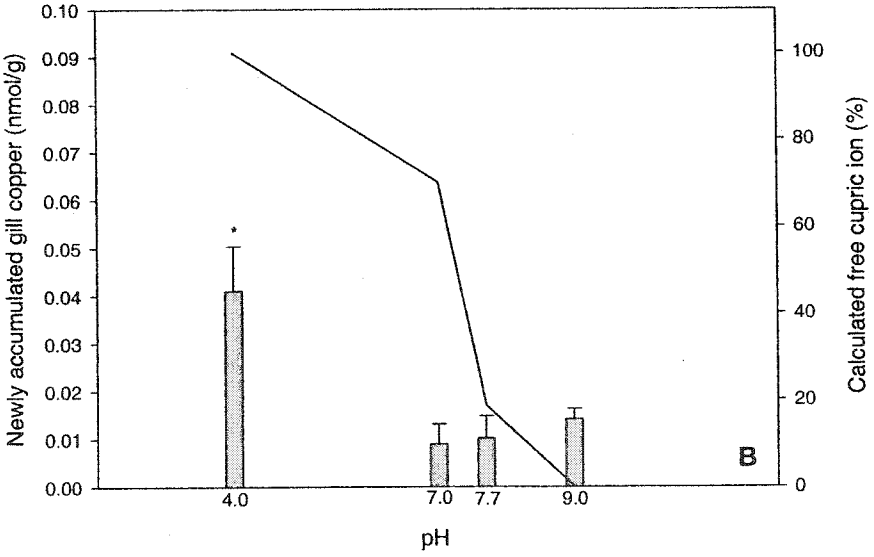

Newly accumulated gill copper (nmol/g)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
Rinse time with unlabelled copper (min)

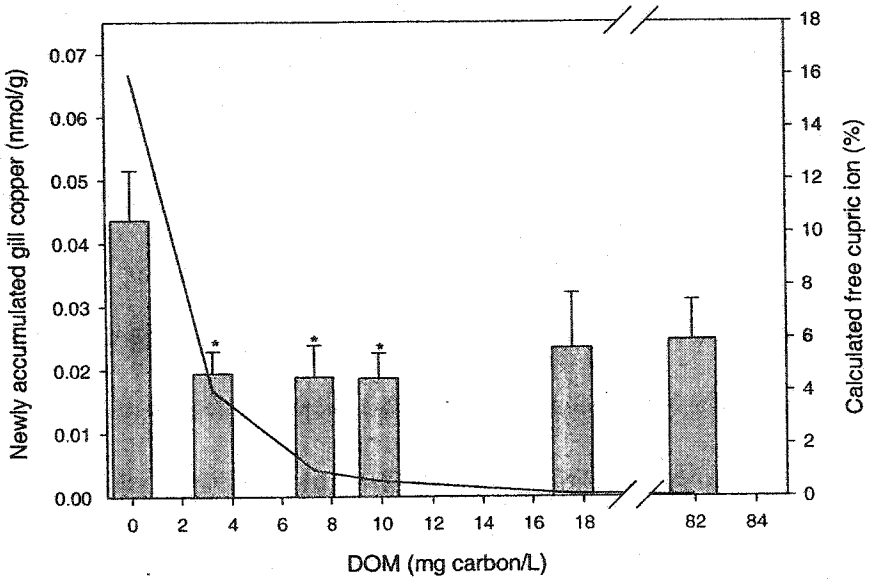

Newly accumulated gill copper (nmol/g)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
50
100
150
200
Fish mass (g)


Newly accumulated gill copper (nmol/g)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Water calcium (mM)
A
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0
3
24
12+ weeks
Exposure time in soft water (h)
*
*


Newly accumulated gill copper (nmol/g)
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0.001
0.1
1
10
Water sodium (mM)
B


Newly accumulated gill copper (nmol/g)
Calculated free cupric ion (%)
Alkalinity (mEq/L $CaCO_3$)
A
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.10
0.0
0.5
1.0
1.5
2.0
2.5
0
2
4
6
8
10
12
14
16
18
20
22
24


Newly accumulated gill copper (nmol/g)
Calculated free cupric ion (%)
pH
B
*
4.0
7.0
7.7
9.0
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.10
0
20
40
60
80
100


Newly accumulated gill copper (nmol/g)
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
Calculated free cupric ion (%)
0
2
4
6
8
10
12
14
16
18
0
2
4
6
8
10
12
14
16
18
82
84
DOM (mg carbon/L)

# CHAPTER 5

# AN EVALUATION OF SODIUM LOSS AND GILL METAL BINDING PROPERTIESTO EXPLAIN SPECIES DIFFERENCES IN COPPER TOLERANCE


## ABSTRACT

It was hypothesized that the strength of copper binding (affinity, log K) and maximum number of binding sites (saturation, $B_{max}$) for copper at the gill surface vary among different species of fish and as a result, may predict sensitivity differences to waterborne copper. Two species that are different in their copper sensitivity are the rainbow trout (RBT- *Oncorhynchus mykiss*) and yellow perch (YP-*Perca flavescens*). We explicitly compared acute toxicity (via 96-h LC50s) and $Na^+$ loss in both organisms in two distinct water chemistries [i.e. hard (HW) and soft water (SW)]. For both species, the Cu binding sites at the gill surface were characterized for their affinity and saturability. The binding properties of the gill were quite similar between the two species in each water chemistry. Based on estimations of the free cupric ion concentration, the affinity or log K was 8.4 for both species in SW, whereas in HW the affinity was higher (~9.7). The $B_{max}$ value in SW was 1.88 nmol/g for RBT and YP, while in HW saturation occurred at 3.63 nmol/g for RBT and 9.01 nmol/g for YP. More importantly, the amount of copper bound to the gills at 50% mortality (i.e. the LA50) was different between the two species (YP LA50s were nine times higher than RBT in SW and HW). According to 96-h LC50s, YP were less sensitive to copper than RBT, however, the difference between

the two species was similar in HW (1.05 vs. 4.16 $\mu$M) and SW (~0.10 vs. 0.44 $\mu$M). Perch were more tolerant because they lost less sodium upon exposure to copper, yet this mechanism of tolerance was not reflected by the amount of copper at the gill surface. The influence of water chemistry on the binding properties of the gill demonstrates the dynamic nature of the gill in maintaining ionoregulatory homeostasis, a key issue in the future development of the chronic biotic ligand model.

**Key words**: copper, gill binding, sodium loss, yellow perch, rainbow trout, toxicity

## INTRODUCTION

It has long been recognized that different fish species can vary in their tolerance to waterborne copper. Two families that are different in their copper sensitivity are Salmonidae and Percidae. According to Spear and Pierce (1979), salmonids can be almost two orders of magnitude more sensitive than Perciformes to waterborne copper. In rainbow trout (a salmonid; *Oncorhynchus mykiss*), the mechanism of acute copper toxicity is clearly understood as an ionoregulatory disturbance of sodium levels that exceed homeostatic control (Laurén and McDonald, 1985, Wilson and Taylor, 1993, Pilgaard *et al.*, 1994). This mechanism is believed to result from copper causing a chemical disruption (i.e. an inhibition of $Na^+/K^+$-ATPase) and a physical disruption (i.e. weakening of cell junctions) of the gill surface. The yellow perch (*Perca flavescens*), a member of the Percidae family but one not included in the review by Spear and Pierce (1979), is endemic to metal contaminated areas such as the Sudbury region in Northern Ontario and the Rouyn-Noranda region in Northern Quebec (two historical metal mining/smelting locations). In yellow perch, the mechanism of toxicity may be qualitatively or quantitatively different from rainbow trout, which may explain the documented difference in copper tolerance between these two families.

Currently only water hardness and the total dissolved metal concentration are used in copper water quality standards, even though it is now well known that pH, dissolved organic matter and alkalinity also play important roles in modifying the toxic effect of copper (e.g., Miller and Mackay, 1979, Cusimano *et al.*, 1986, Laurén and McDonald, 1986, Playle *et al.*, 1993a, b, Welsh *et al.*, 1993, Pilgaard *et al.*, 1994,

Erickson *et al.*, 1996). In an attempt to revise existing water quality standards, the acute toxicity of copper has been characterized by the accumulation of copper on the gill surface in a limited number of fish species [fathead minnow (Playle *et al.*, 1993a, b), rainbow and brook trout (MacRae *et al.*, 1999), and rainbow trout (Taylor *et al.*, 2000)]. This new technique, termed the Biotic Ligand Model (BLM; DiToro *et al.*, 2001), is the first to take into account the influence on toxicity of both the abiotic and biotic ligands present in natural waters. The abiotic ligands (e.g. -$CO_3$, -DOM, -OH) and the biotic ligands of the fish gill surface epithelium compete for copper in natural waters. Geochemical modelling programs can adequately model the state of the abiotic ligands, however modelling the behaviour of the biotic ligand is more difficult since only a few species have been characterized under simplified conditions. Ideally, the binding properties of the gill surface might explain the presence or absence of certain species of fish in metal contaminated lakes.

The main objective of the present study was to use a species comparison approach in order to understand sensitivity differences to copper. We explicitly compared: (1) acute copper toxicity (via 96-h LC50s) to confirm the difference in tolerance, (2) the pattern of ion loss ($Na^+$) to understand the mechanism of toxicity and mechanism of copper tolerance, (3) gill-Cu binding relationships and (4) the accumulation of copper at the gill at 50% mortality (LA50s). In order to fully assess copper bioavailability, the comparison was completed in two distinct water types, hard and soft water, thereby incorporating the influence of abiotic ligands. In both species, the biotic ligand was characterized (i.e. affinity and saturability of the gill) and it was hypothesized that species

differences in toxicity would be reflected by copper binding to high affinity sites on the gill which are the target ligand in the biotic ligand model.

## METHODS AND MATERIALS

### *Experimental Animals*

Juvenile rainbow trout (RBT, *Oncorhynchus mykiss*, 1-2 g) and yellow perch (YP, *Perca flavescens*, 1-2 g) were obtained from Rainbow Springs Hatchery (Thamesford, ON, Canada) and Kinmount Fish Farm (Kinmount, ON, Canada), respectively. Fish were maintained for at least two months in de-chlorinated Hamilton tap water (HW) of moderate hardness (18°C, pH 8, 0.6 mM $Na^+$, 0.7 mM $Cl^-$, 1.0 mM $Ca^{2+}$, 120 mg/L as $CaCO_3$ hardness, 95 mg/L alkalinity, 3 mg C/L DOM) prior to testing. For experiments using soft water (16°C, pH 7.2, 0.13 mM $Na^+$, 0.10 mM $Cl^-$, 0.13 mM $Ca^{2+}$, 20 mg/L as $CaCO_3$ hardness, 15 mg/L alkalinity, 0.4 mg C/L DOM), fish were acclimated for a minimum of nine weeks. Soft water (SW) was synthesized by mixing one part HW to six parts ion-reduced water, the latter produced by reverse osmosis (Anderson Water Systems, Dundas, ON, Canada). Photoperiod was set to a light/dark cycle similar to the natural photoperiod for Western Lake Ontario from January through to June. Fish were fed daily to satiation a dry ration of commercial trout pellets (Martins Feed Mill, Elmira, ON, Canada). The feed contained ~3 $\mu$g Cu/g dry weight.

*Experimental Protocol*

i) Acute Toxicity of Copper in Hard and Soft Water

The concentrations of copper, which were lethal to 50% of the animals (i.e. 96-h LC50s), were determined in hard and soft water for both species. Rainbow trout [5.68 ± 0.28 g (N=60) for HW and 10.58 ± 0.46 g (N=99) for SW] and yellow perch [2.73 ± 0.18 g (N=60) for HW and 5.27 ± 0.27 g (N=97) for SW] were exposed at the same time and in the same tanks to each concentration in order to ensure the exact environmental conditions for both species. Fish (N=10 per species and per concentration) were exposed in a flow-through system to a minimum of five Cu concentrations plus a control. In hard and soft water, the copper concentrations ranged from 0.38 to 15.35 $\mu$M and 0.11 to 0.77 $\mu$M, respectively. Background Cu concentrations were 46.4 ± 16.5 (N=12) and 10.2 ± 3.8 (N=9) nM in control hard and soft water controls, respectively. Copper stocks ($CuSO_4 \cdot 5H_2O$, Fisher Scientific, Toronto, ON, Canada) were metered into diluent water in mixing head tanks. These tanks then fed 20 L polyethylene tanks at a rate of 300 ml/min each. Tanks were individually covered, aerated and checked for mortality daily. Mortality checks were made every ~1 to 2 hours during the initial period of exposure (<12 h). Upon death, fish were removed and their whole bodies placed in 1 N trace metal grade nitric acid (Fisher Scientific) for digestion. Whole bodies were analyzed for sodium content measured by flame atomic absorption spectrophotometry (AAS, Varian AA-1275). Water samples (15 mL) were acidified with 50 $\mu$L concentrated trace metal grade nitric acid and analyzed for Cu by either flame or graphite furnace AAS (Varian AA-1275), against Fisher Scientific certified copper standards.

ii) Gill Copper Binding Characteristics in Hard and Soft Water

In order to determine inter-specific differences in copper tolerance we characterized the high affinity gill-Cu binding sites, using the 3-h radiolabelled $^{64}Cu$ technique of Taylor *et al.* (2000). In HW, RBT and YP were exposed for three hours to five different nominal Cu concentrations of 0.08, 0.16, 0.24, 0.32, 0.48 $\mu$M radiolabelled with $^{64}Cu$ (specific activity 92.77 to 148.05 mBq/$\mu$mol). Mean weight was 4.49 ± 0.25 g (N=50) and 3.73 ± 0.28 g (N=50) for RBT and YP in hard water, respectively. In SW, the six nominal exposure concentrations were 0.08, 0.16, 0.24, 0.32, 0.48 and 0.80 $\mu$M radiolabelled with $^{64}Cu$ (specific activity 65.45 to 120.73 mBq/$\mu$mol). The mean weight was 12.72 ± 0.75 g (N=60) and 5.82 ± 0.29 g (N=60) for RBT and YP in soft water, respectively. The McMaster Nuclear Reactor (Hamilton, ON, Canada) supplied the $^{64}Cu$ (as $CuNO_3$). Exposures were conducted under static conditions in Ziploc® bags containing 5 L of water. Five RBT and five YP were placed in each bag with two bags at each concentration. Bags were individually aerated and placed in black plastic boxes. Water samples (5 mL) were taken at the beginning and end of the exposure and acidified (50 $\mu$L concentrated trace metal grade nitric acid). After three hours, fish were sacrificed with a blow to the head. Gill arches were removed and rinsed for 10 s in control water and the radioactivity determined in both the tissue and water samples using a well-type gamma counter with a 3" NaI crystal (Packard Minaxi Auto-Gamma 5000 Series). The appearance of radioactivity in the gills allowed for the calculation of "newly accumulated" gill copper (i.e. with no background). Water samples were further analyzed for total copper by flame or furnace AAS, as appropriate. The geochemical modelling

program WHAM (Windermere Humic Aqueous Model; Tipping, 1994) and our water chemistry constituents were used to convert total copper concentrations into the bioavailable free cupric ion concentrations ($Cu^{2+}$). Input variables included all major cations and anions ($Ca^{2+}$, $Na^{+}$, $Cl^{-}$, $Mg^{2+}$, $HCO_3^{-}$, $CO_3^{-2}$, $K^{+}$, $Cu^{2+}$, $SO_4^{-2}$), plus dissolved organic matter and pH.

This same technique (i.e., 3-h gill binding) was also used to calculate the lethal accumulation on the gill surface at 3-h at the copper concentration that caused 50% mortality at 96-h in HW (i.e. the 3-h LA50 at the corresponding 96-h LC50 for each fish species). The actual exposure concentrations in hard water were 1.17 $\mu$M for RBT and 4.31 $\mu$M for YP, both of which were radiolabelled with $^{64}Cu$ (specific activity was 43.21 and 47.66 mBq/$\mu$mol, respectively). In soft water, the LC50s for both species fell within the range used to characterize the high affinity binding sites, therefore, we were able to calculate the LA50s from the equation of the gill-Cu binding curve at the corresponding LC50 (~0.1 and 0.44 $\mu$M for RBT and YP, respectively).

*Calculations*

i) Net Sodium Flux

The rate (Jnet) of sodium loss in nmol/g· h during Cu exposure was calculated based on the concentration of whole body sodium for both species in soft and hard water:

$$Jnet = \frac{WB\ Na_{exposed} - WB\ Na_{control}}{time}$$

where WB $Na_{exposed}$ is the whole body concentration of sodium at the end of the Cu exposure in nmol/g, WB $Na_{control}$ is the mean whole body sodium concentration of control (unexposed) rainbow trout or yellow perch in nmol/g, and time is the amount of time over which the fish was exposed to copper in hours (i.e. until death or until sacrificed at the end of 96-h).

ii) Gill Cu Binding

Newly accumulated copper concentrations in the gill (nmol/g) were calculated based on the appearance of radioactivity in the gill:

$$Cu_{gill} = \frac{^{64}Cu_{gill}}{SA}$$

where $^{64}Cu_{gill}$ is the radioactivity of the gill arches in counts per minute per gram of wet gill tissue and SA is the mean measured specific activity of the water in cpm/nmol.

*Statistics*

All data presented are means $\pm$ 1 SE (N) except for the LC50 data where means and 95% confidence limits are given. LC50 values were determined by linear functions relating the log concentration of copper to probit transformation of percent mortality (TOXSTAT, Version 3.5 Western EcoSystems Technology Inc., Cheyenne, WY, U.S.A.). All other data were analyzed for statistical significance by a Student's *t*-test (two tailed, unpaired). Significance was set at $p < 0.05$ unless otherwise stated. Percent data were transformed (arcsin) in order to attain a normal distribution, producing

asymmetrical error bars. Gill binding saturation curves were fitted by non-linear regressions using Sigma Plot 2000® (SPSS, Chicago, IL, U.S.A.).

## RESULTS

### *Acute Toxicity of Copper*

In comparing the 96-h LC50 values for both species, copper was approximately four times more toxic to juvenile rainbow trout than to yellow perch in hard water (Table 1). We were unable to accurately calculate a 96-h LC50 in SW for rainbow trout in the present study due to high mortality at the low copper concentrations, but it was approximately 0.1 $\mu$M. We were, however, able to calculate 48-h LC50 values in soft water for both species, which revealed under this circumstance that copper was four times more toxic to RBT.

### *Sodium Loss Due to Copper Exposure*

Regardless of water chemistry, the whole body sodium concentrations of rainbow trout were significantly higher than in yellow perch ($P<0.05$). Whole body sodium concentrations from control rainbow trout and yellow perch in hard water were 61.56 $\mu$mol/g ± 7.63 (7) and 46.73 $\mu$mol/g ± 0.98 (9), respectively, and were significantly lower for YP ($p$ <0.001). Control RBT and YP whole body sodium concentrations were 55.90 $\mu$mol/g ± 0.64 (10) and 40.10 $\mu$mol/g ± 0.73 (10) in soft water, respectively.

In both species upon exposure to copper, whole body sodium decreased with increasing copper concentrations. In those circumstances where there was 100%

mortality, YP and RBT lost ~60% of their initial whole body sodium concentration. However, the Cu concentration at which this level was reached was different between the two species (Fig.1). In soft water, this occurred at water Cu concentrations of 0.11 and 0.91 $\mu$M for RBT and YP, respectively. In contrast, the Cu concentrations were 8.39 $\mu$M for RBT and 15.34 $\mu$M for YP in hard water. Rainbow trout had higher mortality and hence greater sodium loss at lower Cu concentrations than yellow perch. The sodium loss threshold for toxicity was defined as the percent loss occurring at the concentrations which produced 50% mortality (i.e. the LC50). In SW, based on the 48-h LC50 values for RBT, the sodium loss threshold was ~30%. For YP in soft water at their 96-h LC50, the threshold was ~40%. These thresholds were the same in hard water, where RBT and YP lost ~30 and ~40% of their whole body sodium levels at their respective 96-h LC50 concentrations.

Overall, the rate of sodium loss was much greater in soft water than in hard water and was dependent on Cu concentration in both species (i.e. larger rates of loss at higher Cu concentrations; Fig.2). In soft and hard water, over the full range of Cu concentrations tested, YP had significantly lower rates of sodium loss than RBT. For example, in soft water the maximum rate of loss in yellow perch was 3000 nmol $Na^+$/g· h, whereas it was 5000 nmol $Na^+$/g· h for rainbow trout. In hard water this maximum was 1500 and 2300 nmol $Na^+$/g· h for perch and trout, respectively. At the threshold for toxicity (i.e. the rate at their LC50), the rate of sodium loss in soft water was lower for YP than RBT, however, this was not the case in hard water. The toxicity threshold rate of sodium loss in soft water was ~250 and ~750 nmol/g· h for YP and RBT, respectively.

In hard water, the rate for perch was ~400 $Na^+/g \cdot h$ and only ~250 nmol $Na^+/g \cdot h$ in rainbow trout.

*Gill Copper Binding Characteristics*

The binding of copper to the gills tended to increase with increasing copper exposure concentrations in both hard and soft water (Fig. 3). In soft water, there was no significant difference in gill copper binding between the two species at any concentration [Fig.3 (A)]. However in hard water, there was a trend towards lower copper binding in RBT than YP that was significant at 0.33 $\mu$M [Fig. 3(B)]. In soft water, gill-copper reached an apparent 'plateau' between 0.28-0.42 $\mu$M total Cu, but then sharply increased with gill-copper concentrations reaching three times the plateau value at ~0.61 $\mu$M total Cu. For rainbow trout in hard water the pattern was similar with the 'plateau' around 0.24-0.33 $\mu$M and the increase at 0.47 $\mu$M total water Cu. This was not the case for yellow perch in hard water, where the gills tended to bind Cu in a linear fashion. The calculated LA50 values for the two species in soft water were 0.2 and 1.7 nmol/g for RBT and YP, respectively. These values were calculated from the equation of the saturation curve since the 96-h LC50 values were within the range of copper concentrations used in the 3-h gill binding assays. In striking contrast, the measured LA50 concentrations in hard water were 3.1 and 27.8 nmol/g for RBT and YP at their 96-h LC50 concentrations, respectively.

## DISCUSSION

The present study explicitly tested and answered three important questions. First, is the mechanism of copper toxicity different between rainbow trout and yellow perch? Second, what is the basis for the difference in tolerance between the two species? Third, does gill copper binding predict the difference in tolerance? Our results show that the mechanism of copper toxicity is not different between rainbow trout and yellow perch. Specifically, the threshold for toxicity was reached when both species lost 30-40% of their whole body $Na^+$ and at 60% loss there was complete mortality. As for the mechanism of tolerance, clearly yellow perch possess an ability to resist $Na^+$ loss, however, this resistance was not a result of binding less copper at their gills. Interestingly, we found that the gills of yellow perch were able bind the same amount of Cu as RBT in softwater and slightly more copper than RBT in hard water. In summary, there was only a very small difference in gill copper binding despite a very large difference in toxicity.

### *Species Differences in Acute Copper Toxicity*

We demonstrated that yellow perch are more tolerant to waterborne copper, however, not nearly as tolerant as predicted from the 96-h LC50 equation developed by Spear and Pierce (1979) for perch-like fishes belonging to the order Perciformes (which is represented by Line 1 in Fig.4). This equation was based on 11 studies using bluegill (*Lepomis marchochirus*), striped bass (*Roccus saxatilis*) and pumpkinseed (*Lepomis gibbosus*) over a range of water hardness (i.e., 10 to 300 mg/L as $CaCO_3$; Line 1 in

Fig.4), but did not include data for YP. Also not consistent with the general Perciformes data was the reported difference in toxicity between hard and soft water. In our study, yellow perch were about nine times more sensitive in soft water than in hard water, whereas the Perciformes equation predicts only a three fold difference (as represented by our steeper slope, Line 1 vs. 4 in Fig.4). In fact, our yellow perch toxicity data were more comparable to the Salmonidae values summarized in the same report (Line 2 in Fig. 4; Spear and Pierce, 1979).

The toxicity of copper to rainbow trout in the present study confirms previous findings in our laboratory (Taylor *et al.*, 2000), which found our rainbow trout to be comparable to the Spear and Pierce (1979) sensitive Salmonidae data derived from two species of Pacific salmon (see Lines 3 and 5 in Fig.4). The toxicity equations of Spear and Pierce (1979) predict a larger species difference in soft water than in hard water (Perciformes 11 times more tolerant in soft water and five times more tolerant in hard water). In contrast, we found a similar difference in hard water and soft water (YP were about four times more tolerant than RBT). A possible reason for this difference may be due to the historical soft water data upon which the toxicity equations were based, where it was generally accepted that two weeks was sufficient acclimation time (e.g., Shaw and Brown, 1974; Howarth and Sprague, 1978). Previously, we argued against this concept, noting that two weeks was too short to fully acclimate (Taylor *et al.*, 2000). We defined 'fully acclimated' to be when whole body sodium concentrations in soft water were equal to the hard water values (Taylor *et al.*, 2000). In the present study, this definition applied to rainbow trout, however yellow perch sodium concentrations were still different after a

minimum of nine weeks of soft water acclimation. This may explain why the difference in toxicity was smaller in SW than in HW between the two species. The importance of soft water acclimation becomes extremely relevant when evaluating a toxicant, such as copper, which disrupts ionoregulation.

*Mechanism of Copper Toxicity*

The mechanism of acute copper toxicity in rainbow trout involves a concentration-dependent stimulation of $Na^+$ efflux and inhibition of $Na^+$ uptake at the gill (Laurén and McDonald, 1985). The present study demonstrates that the same mechanism applies for YP. The biomarker used in this study to reflect this toxic action was whole body sodium. The loss of whole body sodium (expressed per gram of wet mass, rather than dry mass) has been reported as a sensitive indicator of ionoregulatory disruption resulting from exposure to mine polluted waters (Grippo and Dunson, 1991, 1996). Indeed, Croke and McDonald (2002) compared sodium loss due to copper exposure at two different water calcium concentrations in rainbow trout and fathead minnow and found that a 20-30% loss of whole body sodium was the threshold for lethality. In that study, fathead minnow were the more sensitive species to waterborne copper toxicity and showed greater rates of sodium loss, which was even more pronounced in the low calcium water (Croke and McDonald, 2002). Similarly, we found a 20-40% loss in whole body sodium at the toxicity threshold concentrations for RBT and YP, and the finding of an increased rate of sodium loss in the more sensitive species was duplicated in the present study.

*Mechanism of Copper Tolerance*

There are two possibilities, based on the result of whole body sodium loss, by which greater copper tolerance may exist in different fish species: (1) by resisting sodium loss (i.e., loss occurs but at a higher Cu concentration), or (2) by tolerating sodium loss (i.e., the loss still occurs but with no mortality). Based on our findings, the first possibility applies to yellow perch. Yellow perch were able to resist sodium loss since the same threshold for mortality (i.e., the loss of 20-40% whole body sodium) occurred at a higher copper concentration than for RBT. The question of how yellow perch are able to resist sodium loss due to copper exposure is an area that requires future research.

Clearly, yellow perch have a higher threshold for the damage which leads to sodium loss. Freda and McDonald (1988) and McDonald *et al.* (1991) characterized morphological differences between yellow perch and rainbow trout gills in the context of low pH ($H^+$ is a toxicant which is often compared to copper due to their similar mechanisms of toxicity). They attributed the ability of YP to resist net ion loss to the larger depth of tight junctions in the gills and to a reduced chloride cell proliferation. It is likely the yellow perch may also use these physical differences to resist ion loss due to copper. Aside from morphological differences, perch also maintain lower whole body sodium concentrations than rainbow trout, thereby reducing the $Na^+$ concentration gradient between the internal and external environment. The ability of the gills to prevent ion loss, thus providing tolerance, may also be the result of copper binding to the gills.

*Gill Copper Binding*

Copper binding to the gills has been directly correlated with toxicity (MacRae *et al.*, 1999). Therefore, it was not unreasonable to hypothesize that fish differing in their sensitivity to copper may have different gill-metal binding properties. At present, the Biotic Ligand Model predicts toxicity based on the rapid binding of copper on the gill (DiToro *et al.*, 2001). The evaluation of gill copper binding characteristics traditionally requires the conversion of total copper to free copper (i.e., $Cu^{2+}$ ions). This was accomplished using the geochemical modelling program WHAM (Tipping, 1994), which effectively models the interactions of metals with natural DOC (P.G.C. Campbell, personal communication). Previously in our laboratory, we identified at least two types of binding sites in rainbow trout in hard and soft water (Taylor *et al.*, 2000). Based on this result, we can characterize the binding of free copper to sites on the gill in the same manner [Fig.5 (data from Fig.3)]. The fact that these two types of sites were also present in yellow perch at the same copper concentrations was a novel finding. The saturable gill-Cu binding sites can be described as high affinity and low capacity, beyond these, at 0.4 - 0.5$\mu$M total copper, a second set of sites may exist (see data points beyond the saturation curves in Fig.5). In SW, there was no statistical difference between RBT and YP newly accumulated gill copper, therefore only one curve was fitted to the data. The maximum number of high affinity binding sites ($B_{max}$) for both species was calculated at 1.88 nmol/g wet gill tissue. In contrast, YP saturated at marginally higher gill copper concentrations than RBT in hard water. The $B_{max}$ values for high affinity binding sites in RBT and YP were 3.63 and 9.01 nmol/g of wet gill tissue, respectively. The affinity of

these sites for free $Cu^{2+}$ ions was distinctly different between the two water chemistries. In soft water the log K was 8.4 for both species, whereas in hard water the affinity had increased to log K values of 9.9 and 9.5 for trout and perch, respectively.

As in the case of ion loss there are at least three explanations for differential copper tolerance, based on gill-Cu binding characteristics, they are: (1) the gill may bind Cu less strongly [i.e. a decrease in affinity (log K)], (2) the gill may saturate at a lower $Cu^{2+}$ ion concentration [i.e. a decrease in capacity ($B_{max}$)], or (3) copper can accumulate in the gill but is not 'biologically reactive' (e.g. through non-specific binding and increased storage and elimination processes). Based on our findings, the first explanation may not apply. The affinity of the perch gill surface for cupric ions was lower than trout in hard water, however in soft water the affinity was equal between the two species. This result does not agree qualitatively with the toxicity data, where the difference in toxicity was similar in hard and soft water. The second reason explaining tolerance involves the saturation concentration of the gill. In soft water the binding capacity was equal in both species and in hard water the perch gill Cu capacity was only somewhat higher than rainbow trout, an argument against the second hypothesis. It is this last result, which leads us to believe the third option may also apply to yellow perch in hard water. The copper was binding to the gill but must not have been 'biologically reactive' so it would not have had a negative impact on survival (i.e. the toxic range is far beyond the characterized sites in hard water). MacRae *et al.* (1999) also used a species comparison approach to evaluate gill copper binding characteristics between rainbow trout and brook trout (*Salvelinus fontinalis*) in soft water. Brook trout were more tolerant to copper

toxicity than rainbow trout and the reduced sensitivity may have been related to a lower affinity for copper (log Ks were 7.14 and 7.56, also based on non-linear regression). The soft water log K for rainbow trout in MacRae *et al.* (1999) was lower than our log K (7.56 vs. 8.4) and was likely the result of three factors. The first factor is the finer resolution in our study using radiolabelled $^{64}Cu$ (and consequently starting at a zero background), secondly, our ability to work at lower total Cu concentrations (<0.16 $\mu$M) thereby reaching higher affinity sites and thirdly, the MacRae *et al.* (1999) study reported 24 h gill binding compared to the present 3 h gill binding time interval. The evaluation of binding capacity in the MacRae *et al.* (1999) study revealed that brook trout had a significantly higher $B_{max}$ than the rainbow trout (63 *vs.* 29 nmol/g including background), supporting the notion that copper can bind to the gill but may not necessarily be toxic. The possibility that a surplus of copper can bind to the gill but exhibit no toxic effect is one not currently taken into consideration by the Biotic Ligand Model and an area for future research.

The accumulation of 'new' copper on the gills at the LC50 concentration (defined as the 3-h LA50) provided toxicologically-relevant information in which to compare the two species. In other words, the amount of Cu bound to the gill in three hours would be predictive of the percent mortality at 96 hours. The calculated LA50 values for the two species in soft water were 0.2 and 1.7 nmol/g for RBT and YP, respectively. These values were calculated from the equation of the saturation curve since the LC50 values fell within the tested range. In striking contrast, the measured LA50 concentrations in hard water were 3.1 and 27.8 nmol/g for RBT and YP at their LC50 concentrations,

respectively. In comparison to their $B_{max}$ values, the LA50 in soft water occurred when ~11 and 90% of the sites were filled for RBT and YP, respectively. For YP in hard water, the LA50 occurred beyond the characterized set of saturable sites. In other words, 100% of the high affinity, low capacity sites were filled plus an unknown percentage of the lower affinity, higher capacity sites were also filled.

MacRae *et al.* (1999) measured gill copper binding at 24 hours during a 120 h (five day) toxicity test. For brook trout and rainbow trout in soft water the 24-h LA50 was 22 nmol/g, which included the background level of copper. If the background concentration of 12 nmol/g (MacRae *et al.*, 1999 and Playle *et al.*, 1992) is subtracted from this value we can compare it to our 'newly' accumulated 3-h LA50 for rainbow trout and yellow perch (i.e. 22-12 = 10 nmol/g). For both species in soft water, our 3-h LA50 was ~0.2-2 nmol/g, which is approximately 2 and 20% of the MacRae *et al.* (1999) 24-h LA50 of 10 nmol/g. In hard water, the rainbow trout 3-h LA50 was a third of 10 nmol/g whereas the yellow perch 3-h LA50 was three times higher at 28 nmol/g. According to the Biotic Ligand Model, the gill LA50 for a particular species should occur at the same concentration independent of dissolved organic matter (DOM), $Ca^{2+}$ concentrations or pH (DiToro *et al.*, 2001). In summary, the gill binding characteristics were the same in soft water between RBT and YP and the difference in copper toxicity was large. In hard water the gill binding characteristics were only slightly different and the toxicity difference was considerable. More importantly, the LA50s and LC50s were consistently different between the two species (i.e., YP LA50s and LC50s were nine and four times higher than RBT, respectively, in SW and HW).

## CONCLUSIONS

The species comparison approach used in the present study was an effective way to distinguish possible mechanisms of copper tolerance. The resistance of sodium loss in yellow perch may be due to differences in gill morphology (i.e., permeability) and reduced sodium gradients between the fish and its environment. The binding of copper to the gill did explain toxicity qualitatively but not quantitatively, which may have important implications for the acute Biotic Ligand Model. In our study, we show that Cu burden does not necessarily translate into toxicity, but more likely the surplus Cu is dealt with by effective detoxification, storage and elimination mechanisms. The influence of water chemistry, particularly soft water acclimation, on the binding properties of the gill demonstrates the dynamic nature of the gill in maintaining ionoregulatory homeostasis. This type of gill behaviour will become a key issue in the future development of the chronic Biotic Ligand Model, especially since the process of soft water acclimation may only be relevant to fish kept in the laboratory rather than in their natural environment.

## ACKNOWLEDGMENTS

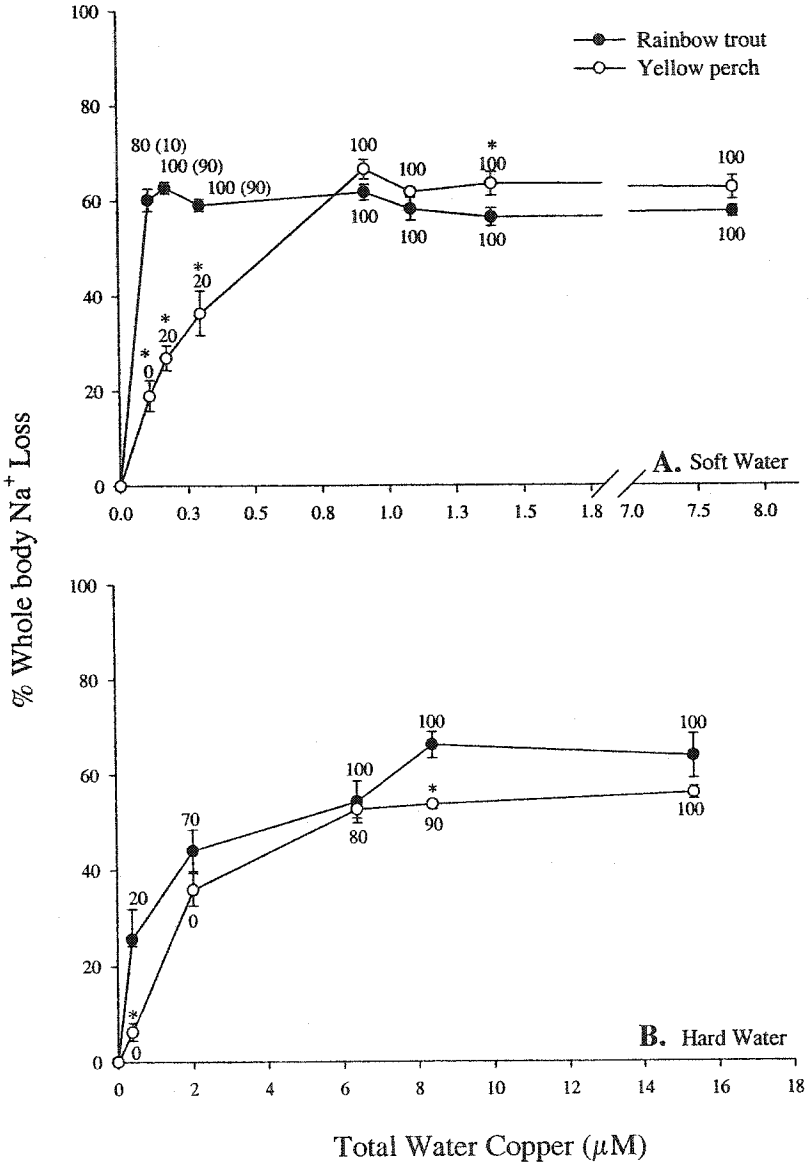
We would like to acknowledge J.G. Richards at McMaster University for his contribution to this study. We also thank P.G.C. Campbell for assistance with the geochemical modelling program (WHAM). This research was supported by the Metals in the Environment Research Network (MITE-RN), the International Copper Association Inc., Falconbridge Ltd., Cominco Ltd., International Lead Zinc Research Organization, Noranda Inc., Nickel Producers Environmental Research Association and a Strategic

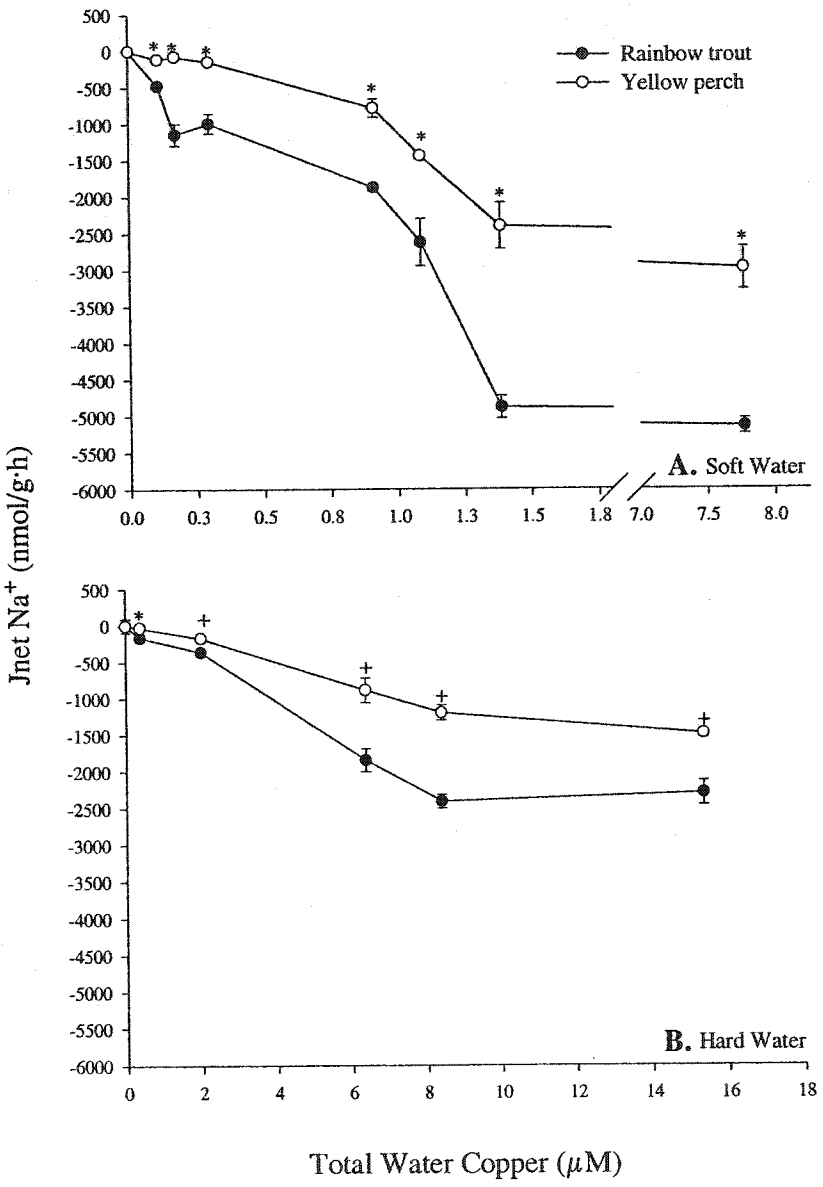
Research Grant from the Natural Sciences and Engineering Research Council of Canada.

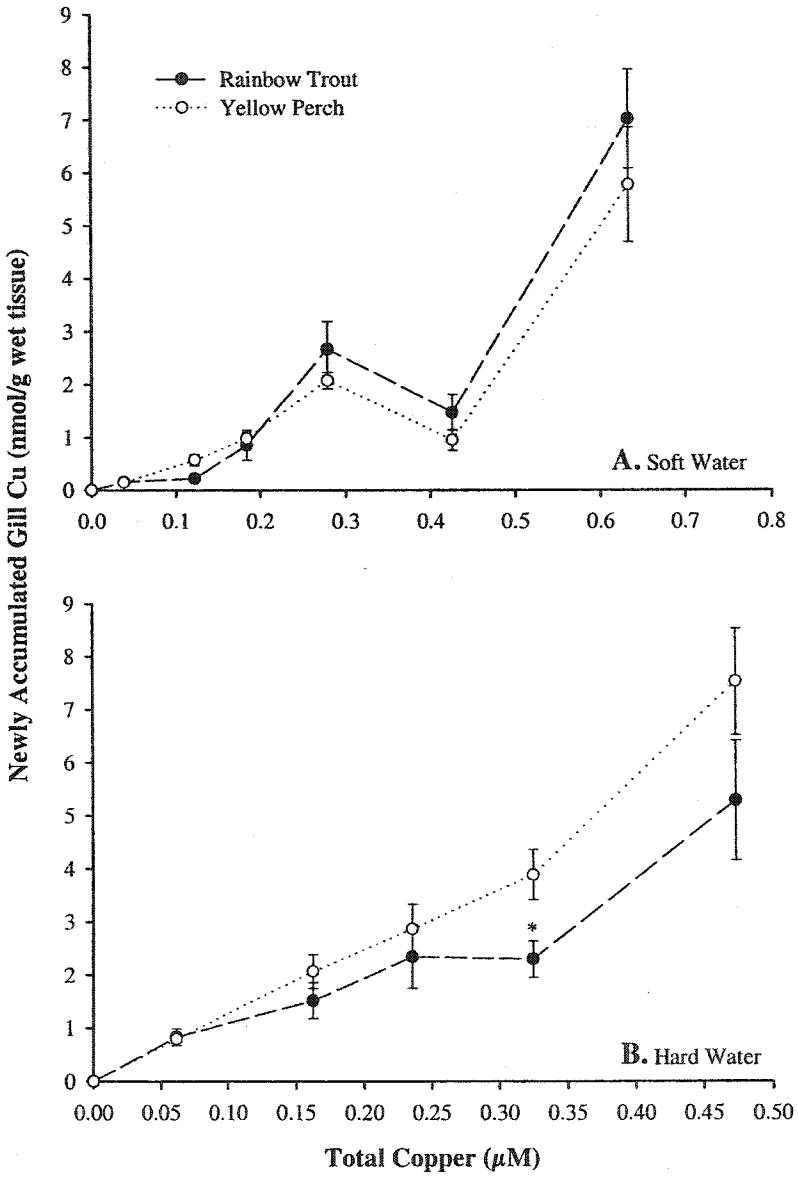
C.M. Wood is supported by the Canada Research Chair program.

Table 1. Acute toxicity of copper to rainbow trout (RBT) and yellow perch (YP) in hard water (HW, 18°C, pH 8, 0.6 mM $Na^+$, 0.7 mM $Cl^-$, 1.0 mM $Ca^{2+}$, 120 mg/L as $CaCO_3$ hardness, 95 mg/L alkalinity, 3 mg C/L DOM) and soft water (SW, 16°C, pH 7.2, 0.13 mM $Na^+$, 0.10 mM $Cl^-$, 0.13 mM $Ca^{2+}$, 20 mg/L as $CaCO_3$ hardness, 15 mg/L alkalinity, 0.4 mg C/L DOM). Fish were acclimated to SW for a minimum of nine weeks prior to experimentation.

| *Water* | *Toxicity Test* | *Species* | *Lethal Concentration (µmol/L)* | *95% Confidence Intervals* | *Mean wet weight (g)* | *SEM* | *N* |
|---|---|---|---|---|---|---|---|
| HW | 96h-LC50 | RBT | 1.05 | 0.68-1.58 | 5.6 | 0.3 | 60 |
| | | YP | 4.16 | 3.54-4.88 | 2.7 | 0.2 | 60 |
| SW | 96h-LC50 | RBT | ~0.10 | - | 10.6 | 0.5 | 99 |
| | | YP | 0.44 | 0.37-0.53 | 5.3 | 0.3 | 97 |
| | 48h-LC50 | RBT | 0.14 | 0.13-0.15 | 10.6 | 0.5 | 99 |
| | | YP | 0.53 | 0.48-0.60 | 5.3 | 0.3 | 97 |


Rainbow trout
Yellow perch
% Whole body $Na^+$ Loss
A. Soft Water
B. Hard Water
Total Water Copper ($\mu$M)


Rainbow trout
Yellow perch
A. Soft Water
B. Hard Water
Jnet Na+ (nmol/g·h)
Total Water Copper (μM)


Rainbow Trout
Yellow Perch
A. Soft Water
B. Hard Water
Newly Accumulated Gill Cu (nmol/g wet tissue)
Total Copper (μM)
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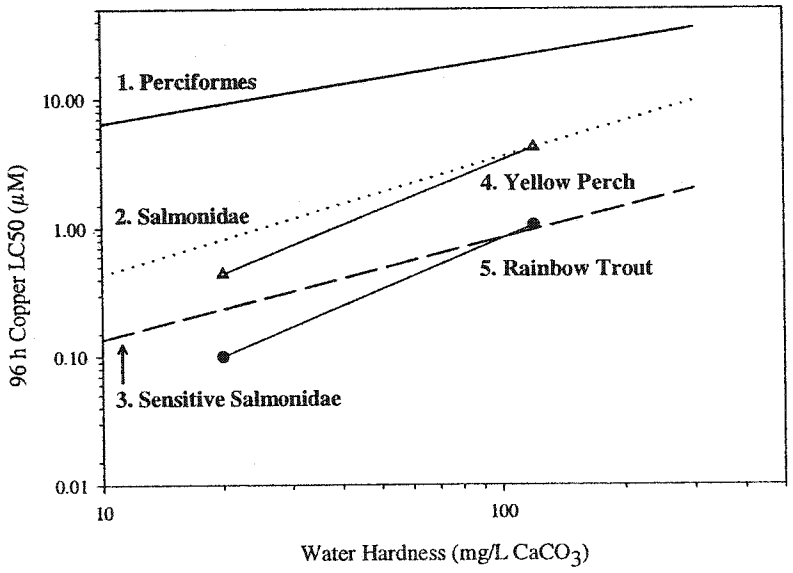
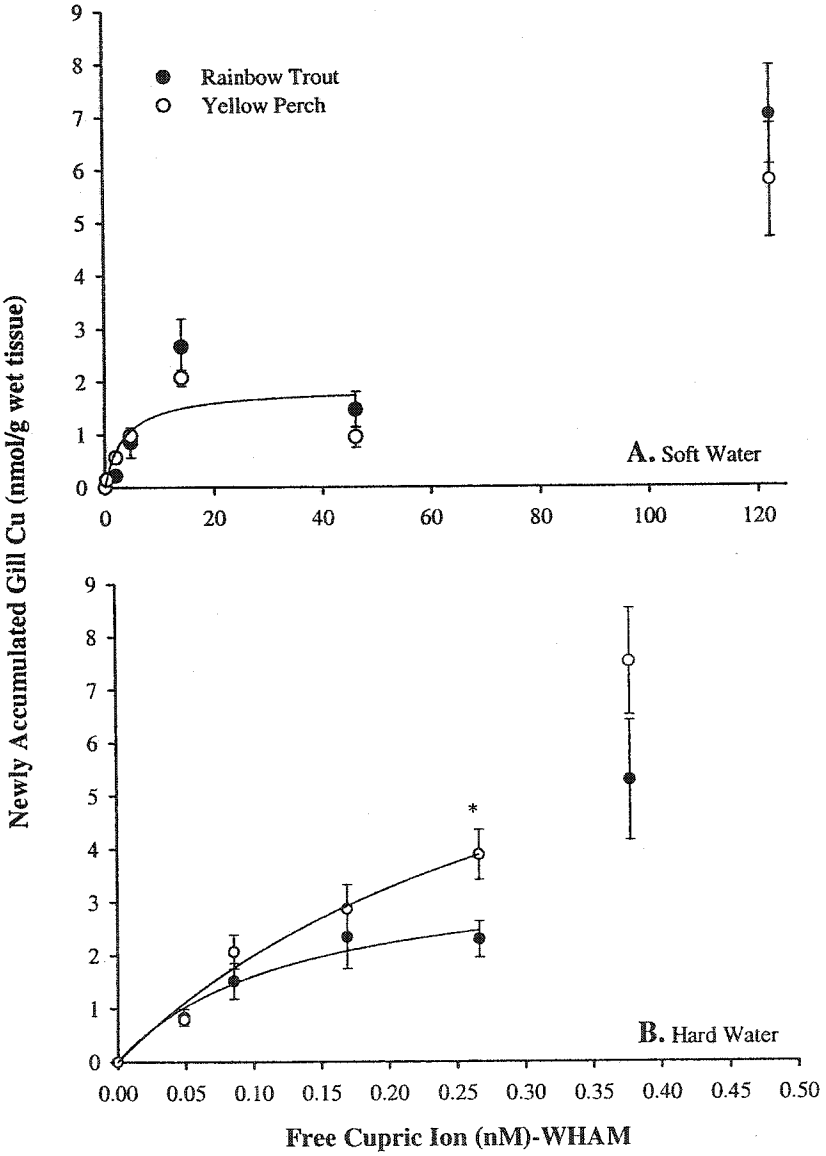

1. Perciformes
2. Salmonidae
3. Sensitive Salmonidae
4. Yellow Perch
5. Rainbow Trout
10.00
1.00
0.10
0.01
10
100
96 h Copper LC50 (μM)
Water Hardness (mg/L CaCO3)


Rainbow Trout
Yellow Perch
A. Soft Water
B. Hard Water
*
Newly Accumulated Gill Cu (nmol/g wet tissue)
Free Cupric Ion (nM)-WHAM
0
1
2
3
4
5
6
7
8
9
0
20
40
60
80
100
120
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50

## CHAPTER 6

# SWIM PERFORMANCE AND GILL BINDING CHARACTERISTICS FOR MEASURING THE EFFECTSOF CHRONIC CU EXPOSURE IN WILD YELLOW PERCH (*PERCA FLAVESCENS*).


## ABSTRACT

The objective of this study was to evaluate the effects of copper exposure on swimming performance and gill binding characteristics of wild yellow perch (*Perca flavescens*), a species endemic to metal-contaminated lakes of the Sudbury Region in northern Ontario. Perch were collected from lakes varying in the degree of metal contamination (Cu = 1 - 21 $\mu$g/L), on two separate occasions for the investigation of swim performance and analysis of gill binding characteristics. Swim performance tests indicated that perch from the contaminated lake had slightly greater endurance in a fixed velocity sprint test than fish from reference lakes, although the analysis of critical swimming speeds ($U_{crit}$) did not reveal this same distinction between the groups. Differential sprint performance was in part due to differences in fish size within contaminated and reference lakes. Yellow perch from the contaminated lake also had higher resting levels of muscle glycogen and greater lactate production during high intensity exercise compared to YP from the reference site. Acclimation occurred in the metal contaminated yellow perch, as seen by the significantly elevated time to death (LT50) during an acutely lethal challenge to 600 $\mu$g Cu/L. However, gills from perch from the contaminated lake bound about three times more copper at death. In contrast, at

a lower range of water Cu (10-400 $\mu$g/L), the gills of fish from the contaminated lake tended to saturate with copper at lower concentrations than gills of fish from the reference lake (~8 vs. 23 $\mu$g Cu/g of gill tissue). Consequently, perch from the contaminated lake exhibited a lower rate of sodium loss during the copper exposure (~10-600 $\mu$g Cu/L). This study suggests that the amount of copper bound to the gills may not be diagnostic of acute toxicity for wild yellow perch from metal contaminated lakes.

**Key words**: swimming performance, copper, yellow perch, gill binding, chronic toxicity

## INTRODUCTION

Laboratory studies, using trout as a model species, have evaluated the impact of copper exposure on swimming performance and gill binding characteristics. Specifically, copper can impair maximum sustainable swimming performance (Waiwood and Beamish, 1978, Beaumont *et al.*, 1995). For example, critical swimming speeds ($U_{crit}$) have been shown to decrease by 35% at copper concentrations as low as 12% of the 96 h LC50 (i.e., at the concentration which caused half of the population to die in a 96 h exposure; Waiwood and Beamish, 1978). On the other hand, earlier findings in our laboratory have shown that chronic exposure to non-lethal copper concentrations (20 and 60 $\mu$g/L in hard water, 1 and 2 $\mu$g/L in soft water), led to increased sprint performance when fish were swum in the absence of copper (Taylor *et al.*, 2000). At least three factors could have contributed to the differences between these results: 1) the type of swim test used to assess copper toxicity [$U_{crit}$ or endurance at a fixed velocity (sprint)], 2) the acute or chronic exposure to copper prior to the swim test, and 3) the presence or absence of metal during the swim test.

Of the three other studies which evaluated locomotor activity (using brook trout, *Salvelinus fontinalis*; Drummond *et al.*, 1973) and swim performance (using brown trout *Salmo trutta*; Beaumont *et al.*, 1995, or rainbow trout; McGeer *et al.*, 2000a) in relation to copper, all but one tested the acute effect of fish swimming in copper contaminated water. McGeer *et al.* (2000a) evaluated rainbow trout (*Oncorhynchus mykiss*) critical swimming speeds in water containing no metal after fish were chronically exposed to copper and found that a reduction in $U_{crit}$ was only evident when fish were fed a high

ration. As far as we are aware, there has been only one other study by Rajotte and Couture (2002), which evaluated swimming performance of wild yellow perch, *Perca flavescens*, naturally exposed to elevated waterborne metals in their environment.

While swimming performance integrates the whole organism response, the accumulation of copper by the gill reflects the target tissue response to the effects of chronic metal exposure. Chronic copper exposure seems to cause moderate changes in metal binding properties of the gill (i.e., the affinity and saturability; Taylor *et al.*, 2000, McGeer *et al.*, 2000b, Kamunde *et al.*, 2002). A predictive model for acute toxicity has been developed (the'biotic ligand model'- BLM; Paquin *et al.*, 2000, DiToro *et al.*, 2001, Santore *et al.*, 2001) that uses such gill binding characteristics. The principles of the BLM, based on physiology, geochemical modelling and toxicology, have important implications for environmental risk assessment. However, this model was developed under controlled laboratory conditions with a limited number of fish species: fathead minnow (*Pimephales promelas*; Playle *et al.*, 1993a,b, Playle, 1998), and rainbow trout and brook trout (*Salvelinus fontinalis*; MacRae *et al.*, 1999). Consequently, the current BLM may be somewhat limited in its ability to predict metal toxicity to fish inhabiting natural, metal-contaminated environments. To our knowledge, no studies have attempted to advance the BLM by applying our existing knowledge of gill-metal binding to wild fish naturally exposed to elevated waterborne metal concentrations.

The objective of this study was to evaluate the effects of chronic metal exposure on swim performance and gill-copper binding characteristics in a wild fish population. Different types of swim performance were evaluated using two different tests, namely the

$U_{crit}$ and the fixed velocity sprint test. Gill-copper binding was characterized using the short-term (3-h) metal-binding technique of Playle *et al.* (1993a,b). Yellow perch was chosen as the test organism due to its ubiquity in metal contaminated, soft water lakes in the Sudbury region of northern Ontario (a historical metal mining and smelting area). Perch collected from these metal-contaminated lakes were tested in relation to perch populations from lakes containing low concentrations of metals (i.e., reference lakes). The comparison of populations from a contaminated and reference site is considered preferable to comparing species of varying tolerance (e.g., rainbow trout *vs.* yellow perch), because the former maximizes the probability that any observed differences are caused by the environmental gradient (Huey and Bennett, 1986). We hypothesized that the acclimation processes that permit yellow perch to live in metal contaminated lakes would lead to improved swim performance and altered gill-copper binding characteristics, compared to yellow perch inhabiting a reference lake.

## MATERIALS AND METHODS

### *Experimental Animals*

Juvenile yellow perch were collected on two separate occasions from lakes in the Sudbury region of northern Ontario, Canada. The first collection was used to evaluate swim performance (August 1998) and fish were collected from Vermilion Lake (VL; 46° 31' N, 81° 24' W), a reference site, and Whitson Lake (WL; 46° 35' N, 81° 01' W), a metal contaminated site. The second collection was to characterize gill copper binding in relation to Cu exposure (June 2000) and fish were collected from Halfway Lake (HL; 46°

54' N, 81° 38' W), a reference site, and Hannah Lake (HNL; 46° 27' N, 81° 03' W), a metal contaminated site. Water quality characteristics for these lakes are provided in Table 1. All experiments with wild perch were conducted at Laurentian University on the same day fish were collected. Fish were held in their respective lake water before testing. Swim performance was evaluated at 21°C and gill-metal binding experiments were conducted at 18°C. These temperatures were within ± 2°C of lake water temperature from which fish were collected. Fish were not fed at anytime.

A separate test group of yellow perch were purchased from a local hatchery (Caledonia Farms, Caledonia, ON). These fish were held at McMaster University in moderately hard (i.e., 120 mg/L as $CaCO_3$) dechlorinated Hamilton, Ontario tap water and fed daily to satiation with commercial trout chow (Martins Feed Mill, Elmira, ON, Canada). Fish were acclimated to laboratory water for a minimum of one month before testing and were not fed the day of testing. Photoperiod was set to a light/dark cycle similar to the natural photoperiod for western Lake Ontario. These hatchery-reared yellow perch served as a laboratory control and a benchmark for comparison with the swimming tests conducted on wild yellow perch.

i) Swim Protocol

Three separate swim protocols were conducted using yellow perch from WL and VL in de-chlorinated Sudbury, Ontario tap water at Laurentian University, and hatchery yellow perch in Hamilton de-chlorinated tap water at McMaster University. The sprint test protocol was as described by McDonald *et al.* (1998) and the swim flume used to exercise yellow perch was the same as detailed in McFarlane *et al.* (2001). Vermilion

Lake fish were swum in three batches of five or six individuals per group. Whitson Lake fish were swum in three batches of seven to nine individuals per group and the hatchery yellow perch were swum in six batches with three fish per group. For the first 5 minutes fish were allowed to orient themselves to the current (~ 5-10 cm/s). Over the next 2 min, water velocity was steadily increased to the test velocity of 40 cm/s. Fish were removed from the flume upon fatigue, defined as when the fish became impinged on the back screen after three attempts to manually re-orient them to the current. Fatigue time, fish length and fish weight were recorded. Mean time to fatigue (in seconds) was calculated as the geometric mean (i.e., mean of the log sprint time) as recommended by Brett (1964). This calculation produces asymmetric errors of the mean, but since the upper and lower errors were rarely very different, they have been averaged and expressed as a single error (c.f. McFarlane *et al.*, 2001).

The critical velocity test ($U_{crit}$) employed stepwise increases in water velocity as outlined by Brett (1964). The orientation velocity was ~5-10 cm/sec and the subsequent velocity increments were ~5 cm/sec every 30 minutes until fatigue. The same criteria for fatigue were used as in the sprint test. The critical velocity was then calculated and was assumed to be a speed that was sustainable for at least 200 minutes (Beamish, 1978).

The forced exercise test involved chasing fish for 8 min through continuous manual stimulation. Following the 8 min, fish were typically unresponsive to further manual stimulation and exhibited a loss of equilibrium, both used as indicators of exhaustion (Parkhouse *et al.*, 1988). The endpoint for the chase test is different than in sprint and $U_{crit}$ tests: the chase test swims fish until exhaustion, meaning the fish would

not have swum at any speed, whereas in the other two tests fish swim until they fatigued at the test speed. The distinction between chase *versus* sprint or $U_{crit}$ tests is that in the latter two types of tests the fatigued fish would have likely swum again, but at a lower speed.

ii) Acute Copper Toxicity and Gill Copper Binding Protocol

Fish from Halfway and Hannah Lakes were challenged to a range of six nominal copper concentrations (0, 50, 100, 200, 400, 600 $\mu$g/L) in a combined toxicity-, gill binding-, and sodium flux- test. This streamlined test was designed to use a minimal number of wild fish. To compare fish between lakes, we used distilled water (pH 6.8, background Cu 10 $\mu$g/L) for the exposures so all fish were treated identically. Fish were placed in aerated plastic Ziploc® bags containing 3 L of distilled water at the appropriate copper concentration (as $CuSO_4 \cdot 5H_2O$; Fisher Scientific, Toronto, ON). Each bag contained five fish with two bags per concentration (N=10 per concentration). The highest concentration was chosen to produce 100% mortality in order to estimate the time required for 50% mortality (LT50) and compare the acute toxicity in yellow perch between reference (HL) and metals contaminated (HNL) lakes . Every hour water samples were collected (5 mL) and acidified with 50 $\mu$L of trace metal grade nitric acid ($HNO_3$; Fisher Scientific), to establish the rate of sodium appearance in the water and to verify copper concentrations. At the end of 3 h the remaining live fish were sacrificed. The gills were excised, rinsed briefly (10 sec) in clean distilled water to remove any loosely bound copper, and frozen for subsequent metal analysis. The remaining carcass was also frozen for the same analysis.

*Tissue Sampling and Analysis*

White muscle samples were taken from both resting and experimentally-exhausted and fatigued fish after being anaesthetized in an overdose of tricaine methane sulphonate (2 g/L MS-222, pH 7.5; Syndel Laboratories, Vancouver, BC, Canada). Muscle sampling followed two standard methods. For smaller fish (<5 g), the entire fish was freeze-clamped between two aluminum blocks pre-cooled with liquid nitrogen. White muscle samples were excised from the frozen fish, and ground to a fine powder using a mortar and pestle. For larger fish (>5 g), white muscle was rapidly excised from the lateral surface posterior to the dorsal fin, dorsal to the lateral line, and immediately freeze clamped. The entire sampling procedure took less than 15 seconds. All tissues were stored in liquid nitrogen until metabolite analysis for glycogen and lactate. For glycogen, 100 mg of frozen tissue were digested according to methods of Hassid and Abraham (1957), and glucose was enzymatically analyzed following the protocol outlined in Bergmeyer (1983). For analysis of lactate, 100 mg of tissue were homogenized with 1 mL of 8% perchloric acid, and the enzymatic analysis of the supernatant was carried out by procedures detailed in Bergmeyer (1983).

Blood samples were also taken from fish either before or after each swim challenge. Blood was collected via caudal puncture, and plasma was analyzed after appropriate dilution for sodium, by flame atomic absorption spectrophotometry (AAS; Varian AA-220, Varian, Walnut Creek, CA, USA) and chloride, by titration with a Radiometer-Copenhagen CMT-10 chloridometer (Radiometer-Copenhagen, Copenhagen,

Denmark). Gill and liver samples of control fish (not exercised) from WL and VL were also collected and analyzed for copper.

Tissue samples (gill, liver and carcass) were weighed and digested in five volumes of 1 N trace metal grade $HNO_3$ (Fisher Scientific). Copper was measured, after appropriate dilution of the supernatant with reagent-grade deionized water (18 mOhm), using flame and graphite furnace AAS against certified copper standards (Fisher Scientific). Acidified water samples were also measured for copper and sodium using flame or graphite furnace AAS (GTA 110), as required.

*Calculations*

i) Condition Factor

Condition factor ($K_n$; Le Cren, 1951) describes the weight to length relationship by the equation:

$$K_n = \frac{wt}{l^b} \times 100$$

where wt is the weight of the fish in grams, l is the length of the fish in centimeters. The scaling exponent (b) was determined from the power fit of length *vs.* weight in the control groups of yellow perch (i.e., Caledonia Hatchery, Vermilion Lake and Halfway Lake).

ii) Critical Swimming Speed

The critical swimming speed ($U_{crit}$) was calculated according to the expression from Brett (1964):

$$U_{crit} = V_p + [(t_f / t_i) \times V_i]$$

where $V_i$ is the velocity increment (cm/sec), $V_p$ is the penultimate velocity at which the fish swam before fatigue, $t_f$ is the endurance at the final velocity increment, and $t_i$ is the time between velocity increments. $U_{crit}$ is expressed in cm/s and body lengths/s (BL/s; using the average fork length of fish swum from each source).

iii) Sodium Loss

Net losses of $Na^+$ by the fish to the water were calculated from the change in sodium concentration in the water over the first hour of exposure and are expressed in $\mu$mol/g· h. The following equation was used:

$$\text{Net } Na^+ \text{ loss} = \frac{(Na^+_i - Na^+_f) \times V}{Wt \times t}$$

where $Na^+_i$ and $Na^+_f$ are the initial and final sodium concentrations in the water in $\mu$mol/L, V is the volume of water in L in which the fish was exposed, Wt is the total weight of the fish per bag in grams, and t is the duration of time in hours between the initial and final water samples.

*Statistics*

Values are reported as means ± 1 SEM (N). Means were considered significantly different from one another using one way analysis of variance (ANOVA) followed by a

Tukey Honestly Significant Difference test for multiple comparisons. For the comparison of two means, an independent samples Student's *t*-test was used. Significance ($\alpha$) was set at $p<0.05$. LT50 values are expressed as regression estimates (in minutes) with standard deviations in parentheses (i.e., upper and lower deviations were averaged and expressed as a single standard deviation), which were generated by a log-probit survival analysis (JMP ® 4.0 software; SAS Institute). Gill-copper saturation curves were fitted by non-linear regression using Sigma Plot 2000 ® (SPSS, Chicago, IL, USA).

## RESULTS

### *Measures of Physiological Status*

In total, ten different measures were used to describe the physiological state of the groups of yellow perch before any test. Beginning with fish size, the hatchery yellow perch were significantly larger (in mass and length), than the two groups of wild fish used in the swimming experiments (Table 2). There was no difference in fish size between VL and WL, however ,there were significant differences between the two groups of wild perch used for the acute toxicity/gill binding experiment (i.e., in mass and length). To calculate condition factor, the scaling coefficient was derived from the length *vs.* weight power relationship using reference fish only (i.e., CH, VL, HL). The scaling coefficient of 3.24, obtained from this relationship, was used to determine the condition factor for perch from the contaminated lakes. From the first field collection, perch from WL had significantly lower condition factors than fish from the reference site and hatchery. In

contrast, in the second field collection, perch from HNL had slightly higher condition factors than fish from the reference lake.

Significant differences were also found among background tissue copper concentrations (Table 2). The liver consistently had a higher copper concentration than the gill among all groups of perch. Yellow perch from contaminated lakes had approximately two to three times higher background gill copper concentrations and three to four times higher liver copper concentrations, than their respective reference fish. The ratio of gill to liver copper ranged from 1:2.7 (VL), 1:3.6 (HL), 1:4.1 (HNL), 1:6.2 (WL) and 1:7.6 (hatchery). Overall, hatchery yellow perch held in Lake Ontario water had the lowest gill and liver copper concentrations. In relation to water copper concentration, the livers of yellow perch from WL had four times the background copper concentration found in VL fish livers, which roughly corresponds to the 3.6 fold difference in water copper concentrations (i.e., 18 *vs.* 5 $\mu$g/L, Fig.1). However, hatchery fish had 20 times less copper in their livers than WL fish but only six times less copper in the water (i.e., 18 *vs.* 3 $\mu$g/L). The gills show a similar trend, with fish from WL having twice the copper concentration of VL fish, again roughly corresponding to a three- fold difference among water copper levels. However, WL fish had 33 times the gill copper concentration of hatchery fish with only a six- fold difference in water Cu levels. In contrast, gill copper levels in wild perch from the gill binding experiment did not reflect the magnitude of change in water copper concentrations (i.e., 3 and 20 times difference between the gill and water, respectively). In addition, fish from the contaminated lake on the second field collection had significantly higher (>two times) carcass copper concentrations (i.e., the

carcass was the whole body minus the gills). Carcass sodium concentrations were also determined, but were not significantly different between HL and HNL fish.

Sodium and chloride concentrations were also determined in plasma, but only revealed differences between hatchery and wild perch. For perch from the swimming experiment, there were no differences in plasma ion levels measured on resting, and fatigued, or exhausted, fish; therefore, the pre- and post- exercise values were pooled (Table 2). Hatchery yellow perch had consistently lower plasma sodium levels than VL and WL yellow perch; however, mean chloride levels in hatchery fish fell between the means for the two wild fish groups.

*Measures of Performance*

i) Sprint Performance

Wild yellow perch would not swim unless a dark cover was placed over the swim flume. A sprint speed of 40 cm/s was chosen for all fish to fatigue within 30 min. For wild perch, fish from WL had a greater endurance as compared to VL fish: these fish from the contaminated lake took twice as long to fatigue as fish from the reference site (i.e., 141 ± 14 (23) vs. 74 ± 13 (17) s, Fig.2). However, both groups of wild perch had much lower sprint times in comparison to the laboratory held hatchery perch [i.e., time for them to fatigue was 618 ± 88 (20) s]. Both length and weight were analyzed for a relationship to sprint time to determine whether size was responsible for these large differences. There were no significant relationships between fish size and endurance when each source of yellow perch was evaluated individually, a result of the small size

range within each group. However, when all fish were analyzed together, fish length (and weight to a lesser extent) related strongly to fatigue time (Fig. 2A). In essence, length accounted for ~65% of the variation associated with sprint time.

ii) Critical Swimming Speed

In contrast to sprint performance, the $U_{crit}$ swim test did not detect significant differences between the groups of perch. Hatchery perch had the highest $U_{crit}$, although not significantly, at 39.6 ± 6.3 (20) cm/s (or 3.09 ± 0.08 BL/s), whereas the two groups of wild yellow perch from VL and WL were lower at 26.4 ± 3.2 (6) cm/s (or 2.60 ± 0.30 BL/s) and 29.7 ± 2.4 (8) cm/s (or 2.80 ± 0.20 BL/s), respectively (Fig.3B). As with sprint performance, the critical swimming speed was not dependent upon the length or weight of the fish when each fish source was evaluated individually. However, when all the groups were analyzed, a significant relationship existed between fish length (also with weight, to a lesser extent) and critical swimming speed (Fig. 3B). Fish length accounted for 50% of the variation among $U_{crit}$ values.

iii) Muscle Fuel Use

Chronic metal exposure may have affected resting muscle glycogen levels. Significant differences in resting muscle glycogen concentrations were found among all three groups of perch used in the swim performance experiment (Table 2). Wild perch had 2 to 2.5 fold higher glycogen concentrations than hatchery fish.

After each swim trial the change in lactate (Δ lactate) was calculated as the concentration difference between resting and fatigued or exhausted yellow perch (Fig. 3). In all groups of yellow perch, the Δ lactate was largest after the chase test, where fish

were swum until exhaustion. For the two wild groups of perch, the Δ lactate after the sprint test was greater than after the $U_{crit}$ test, however this relationship was only significant for WL fish. There was no significant difference in Δ lactate between these two tests in the hatchery perch.

*Acute Copper Toxicity*

The time for 50% of the exposure group to die (LT50) was calculated at the highest Cu exposure concentration (624 μg/L). The LT50 for perch from the reference site (HL) was 110.0 (±1.6) min. In contrast, for perch from the metal contaminated lake (HNL) the LT50 was significantly higher at 186.2 (±1.3) min. On average, the larger fish from the contaminated lake were able to live 40% longer than the smaller perch from the reference site during the lethal challenge.

*Tissue Copper Accumulation*

Copper concentrations in the gill and remaining carcass were not significantly different between fish sampled directly from reference lakes and fish exposed for 3-h to distilled water with no copper (i.e., distilled water controls). For this reason, control and reference values were pooled (Table 2 and Fig.4A and B). Carcass sodium was also not significantly different between the two groups of controls so these values were also pooled as a single control level (Table 2 and Fig. 4C). Upon comparison of these pooled controls, carcass copper concentrations increased two fold in the reference site perch, whereas the fish from the contaminated lake were unchanged upon death (Fig. 4A). In

contrast, gills from both lakes were significantly elevated beyond control levels at this Cu concentration (Fig. 4B). Gill copper concentrations at death were significantly higher in fish from HNL. Carcass sodium levels were significantly depressed below control concentrations in fish from HL, but not from HNL.

*Sodium Loss*

The rate of sodium loss increased with increasing water copper concentrations, and perch from HL (reference site) lost sodium at a higher rate than the fish from HNL (contaminated site) at each copper concentration (Fig. 5). The rate of sodium loss increased by the same amount at each water copper level for fish from both lakes, as indicated by the nearly identical slopes of the regression lines. Differences in sodium loss rates were in part due to differences in body size (i.e., approximately 50% of the variation in sodium loss was due to the total mass of fish per bag), with Hannah Lake perch over twice as big as Halfway Lake perch.

*Gill Copper Binding*

Below ~300 $\mu$g/L total copper, there was no difference in gill-Cu binding between the gills from either lake (Fig. 6). Beyond this concentration, gills from HL fish bound significantly more copper than gills in HNL fish. That is, yellow perch gills tended to saturate with copper at 8 $\mu$g/g from the contaminated lake, and were estimated to saturate at 23 $\mu$g/g in fish from the reference lake.

## DISCUSSION

This study is the first to report the effects of chronic metal exposure on swim performance and gill binding properties of wild yellow perch. A simplified study design was crucial to extend our framework of laboratory effects to the field. Certain tests were combined, thereby attaining the largest amount of information from the smallest number of fish. In brief, wild yellow perch were distinctly different from hatchery yellow perch based on swim performance and glycolytic fuel use during exercise. In addition, yellow perch from a metal contaminated lake were able to survive an acute Cu exposure longer, lost sodium at a lower rate, and accumulated more copper at their gills by death than perch from the reference lake.

### *Measures of Physiological Status*

Of the ten indicators chosen to represent the physiological status of the test organisms, tissue copper concentrations were the most effective in distinguishing among the wild groups of yellow perch. The gill, liver and carcass Cu concentrations were consistently elevated in response to increasing water copper concentrations in the lakes. In contrast, condition factor, carcass Na concentration and plasma ion levels were either not sensitive enough or were not consistent in their response among the contaminated and reference site yellow perch. Our reported condition factors for VL (reference site) and WL (contaminated) agreed well with Eastwood and Couture (2002), who also found lower condition factors in fish from WL than VL. However, our condition factors from HL (reference site) were lower than for fish from HNL (contaminated), hence, an

opposite relationship with copper contamination. It is important to note, however, that these three indicators we considered inadequate for identifying metal effects are those factors that can vary with diet, which can be highly variable between lakes especially where metals have direct and indirect effects on invertebrate (or other) prey items. Muscle glycogen levels were also significantly different between the groups of wild and hatchery yellow perch; however, the relevance of this may be limited to a relatively small effect on swim performance.

*Measures of Performance*

i) Swim Performance and Fuel Use

The swimming behaviour of yellow perch has been evaluated in relation to temperature change (Otto and Rice, 1974), seasonal variation (Hergenrader and Hasler, 1967), acidity and size (Nelson, 1989,1990), and metal contamination (Rajotte and Couture, 2002). From the present study, we can now add a few insights into the exercise physiology of this organism. One insight was in relation to their behaviour; yellow perch in our experiments would not swim unless a dark cover was placed over the swim flume. Yellow perch tend to avoid high light intensities (Rudstam and Magnuson, 1985) and, therefore, their activity has often been described as bimodal. That is, they feed at dawn and dusk (e.g., Keast and Welsh, 1968), and are largely inactive at night (Jansen and Mackay, 1992). Their swimming behaviour in the laboratory is also likely a reflection of this life strategy involved in feeding, escaping and social interactions.

Our estimates of critical swimming speeds in wild yellow perch agree well with those reported in the literature. Otto and Rice (1974) report $U_{crit}$ values of 33cm/s at 20 °C and values from Nelson (1989) ranged from 30 to 40 cm/s: our critical swimming speeds were 26 to 40 cm/s (approximately 3 BL/s). The absence of a Cu effect in the present study is somewhat contradictory to the results reported by Rajotte and Couture (2002), in which metal contamination significantly reduced $U_{crit}$. Nonetheless, their study did report higher $U_{crit}$ values in a lake with intermediate metal contamination, thus $U_{crit}$ did not consistently decrease with increasing metal levels in the environment.

To our knowledge, only one other study has reported fatigue times at a fixed velocity for yellow perch; therefore, comparisons can be made with the Rajotte and Couture (2002) report, the wild and hatchery fish in the present study, and literature values for salmonids. Fortunately, the Rajotte and Couture (2002) study also evaluated yellow perch of a similar size range from Vermillion and Whitson Lakes. However, in contrast to our study, their fish in the contaminated lake (WL) had lower endurance than fish from the reference lake (VL). This discrepancy may be the result of different sampling seasons (fall *vs.* summer in the present study) and sampling years (1997 *vs.* 1998 in the present study). Our hatchery-raised, laboratory-maintained population of yellow perch were able to swim for much longer than wild yellow perch and even longer than juvenile rainbow trout (reported times of 50% to fatigue for rainbow trout was ~180 s at 40 cm/s and 13 °C; McFarlane *et al.*, 2001). This result is in direct contrast to juvenile Atlantic salmon (*Salmo salar*), where wild yearlings had superior swim performance over the hatchery-reared population (two to three times higher fatigue times;

McDonald *et al.*, 1998). However, their results were largely dependent upon size scaling relationships.

McDonald *et al.* (1998) also reported a higher anaerobic capacity and lower glycogen content of white muscle in wild fish in comparison to hatchery-reared fish. In contrast, we found a higher anaerobic capacity (as indicated by the change in muscle lactate during exercise, Fig.3) in only one group of wild yellow perch (i.e., from the contaminated lake) and significantly lower glycogen contents in the hatchery perch. Our glycogen levels from the wild perch were somewhat higher than those reported for other wild yellow perch (~23 *vs.* 17 $\mu$mol/g wet weight; Nelson, 1990). Notably, and unlike rainbow trout (McDonald *et al.*, 1998b), yellow perch size (i.e., weight or length) did not correlate with sprint performance or critical swimming speeds when the individual groups were analyzed. This lack of correlation was likely due to the narrow size range tested from each group. When yellow perch from all three groups were pooled (VL, WL and CH), a significant relationship existed between fish length and fatigue time (Fig. 2A) and fish length and $U_{crit}$ (Fig. 2B). However, since there were no allometric relationships for these parameters within groups, the effect of size on Ucrit cannot be discriminated from metal effects. Nevertheless, Nelson (1989) reported only a small size effect on yellow perch critical swimming speed, with critical swimming speed being positively related to body mass but only explaining 4% of the variation in $U_{crit}$ (Nelson, 1989). Since differences in fish size could only explain part of the variation in performance, chronic metal exposure may have had a small effect on swim performance. While the analysis of critical swimming speed revealed no difference in swim performance, there

was a slight improvement in sprint performance from the metal contaminated lake fish, compared to the reference lake perch. A difference in anaerobic capacity may be an explanation for this result.

The regulation of plasma ion levels ($Na^+$ and $Cl^-$) was not impaired after any exercise challenge. Previous reports from fish not chronically exposed to metals indicate brief decreases in these plasma ions after aerobic exercise (Postlethwaite and McDonald, 1995) and after five to six minutes of exhaustive exercise (Gonzalez and McDonald, 1992). In yellow perch, Nelson (1989) implicated ionoregulatory problems as the source of reduced swimming ability in acidic water. A 25-35% reduction of $Na^+$ and $Cl^-$ was discovered in brown trout (*Salmo trutta*) after the $U_{crit}$ test in soft acidic water at 5 and 30 $\mu$g Cu/L (Beaumont *et al.*, 1995). However, no distinction was made between the acute effects of acid (pH 5.0) versus the acute effects of copper in the former study. From the present study, chronic exposure to metals did not increase the ionoregulatory cost for exercise.

*Acute Copper Toxicity*

Yellow perch from Hannah Lake survived longer than those from Halfway Lake in an acute challenge to waterborne copper. However, their resistance was at least in part due to their greater whole body size (Table 2). A mechanism defining this type of metal tolerance (i.e., tolerating higher metal concentrations as a result of chronic exposure) has been referred to as acclimation (see review by McDonald and Wood, 1993). The fact that Hannah Lake fish were able to resist the toxic effects of copper during the lethal

challenge was supported by further physiological evidence, such as the absence of increased body copper concentration and decreased body sodium concentration. In contrast, these fish had higher gill copper concentrations at death, than yellow perch from the reference lake (about three times; Fig. 4B). Apparently, wild yellow perch can tolerate copper on the gill without the usual toxic consequences.

The mechanism of acute copper toxicity is to disrupt ion regulation beyond the homeostatic range, in part by stimulating sodium efflux (Laurén and McDonald, 1985). When the present study evaluated the rates of sodium loss, the larger yellow perch from the contaminated lake had lower rates of loss than those from the reference site (Fig.5). The fact that these yellow perch were able to resist sodium loss was likely a contributing factor to their longer survival at acutely lethal copper concentrations. This resistance to copper-induced sodium loss was the same mechanism of copper tolerance subsequently observed in laboratory yellow perch in comparison to rainbow trout, the latter species being much more sensitive to copper (Taylor *et al.*, submitted).

*Gill-Copper Binding*

The binding of copper to the gills has been characterized in relation to acute toxicity (MacRae *et al.*, 1999). Little is known, however, about the effects of chronic metal exposure on the binding properties of the gill. Only three studies exist on the subject, all of which used hatchery-reared rainbow trout exposed to metals in the laboratory. The first two studies by Taylor *et al.* (2000) and Kamunde *et al.* (2002) evaluated short-term copper binding and the third by McGeer *et al.* (2000b) reported the

long-term (kinetic) properties of the gill for copper. The present study is novel in that a more ecologically-relevant fish species was assessed, one that actually exists in metal contaminated lakes, and the chronic pre-exposure conditions were 'natural' (i.e., included waterborne plus dietary routes of metal uptake).

The binding of copper to the gills at sublethal Cu concentrations (i.e., sublethal over three hours) did not reveal large differences between the two populations of yellow perch. In fact, below 300 $\mu$g Cu/L the binding of copper was identical to the gills (Fig. 6). However, the application of non-linear regression revealed an almost three fold difference in the level of projected gill copper saturation. The corresponding affinity term (log K) of the gill for copper ions was estimated to be ~5 to 5.6 for both populations of yellow perch, assuming that at pH 6.7 in distilled water the copper was likely 100% free cupric ion. This log K is relatively low in comparison to other reports of gill-copper affinities (log $K_{gill\text{-}Cu}$ was 7.4 for fathead minnows, Playle *et al.*, 1993a; log $K_{gill\text{-}Cu}$ was 7.1 for brook trout, MacRae *et al.*, 1999; log $K_{gill\text{-}Cu}$ 7.9 for rainbow trout, Taylor *et al.*, 2000), however, the higher water copper concentrations are the likely cause of this discrepancy. Logically, at low copper concentrations the high affinity sites are filled first, then as waterborne copper concentration is increased the lower affinity sites are filled. There was no effect of chronic metal exposure on the binding properties of the gill over the range of 10 to 300 $\mu$gCu/L, however, and more importantly, the accumulation occurring at death was affected. Yellow perch from the contaminated lake bound more copper while resisting the toxic effects on sodium balance.

## CONCLUSIONS

The acclimatory processes which allow yellow perch to live under metal contaminated conditions may have contributed to a slightly improved sprint performance in fish inhabiting metals-contaminated lakes, but did not alter the binding properties of the gill. Chronic metal exposure did however alter the accumulation of gill copper upon death, an important finding with implications for the development of a chronic biotic ligand model.

## ACKNOWLEDGMENTS

We thank Debbie Matsuda and Daniel Baker for their technical assistance in sample analysis at McMaster University. We also thank James Rajotte, Puja Kumar and Angéle Brabant for their assistance in fish sampling at Laurentian University, and Chris Wood and Richard Playle for comments on the manuscript. This research was supported by the Metals in the Environment Research Network (MITE-RN), the International Copper Association Inc., Falconbridge Ltd., Cominco Ltd., International Lead Zinc Research Organization, Noranda Inc., Nickel Producers Environmental Research Association and a Strategic Research Grant from the Natural Sciences and Engineering Research Council of Canada.

Table 1. Water quality characteristics for each source of experimental yellow perch. Caledonia hatchery perch were held at McMaster University, Hamilton, Ontario, Canada. A '–' means not measured.

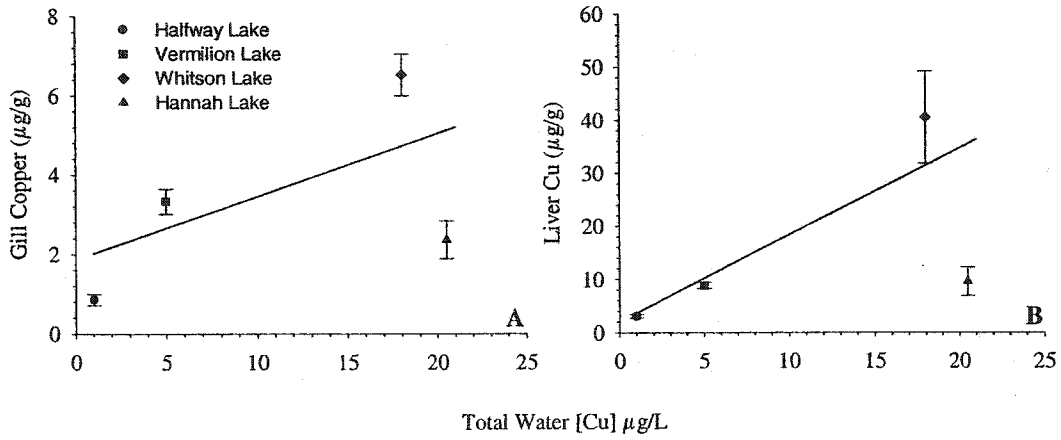
| *Source* | *pH* | *Cu* (μg/L) | *Na* (mg/L) | *Ca* (mg/L) | *Zn* (μg/L) | *Cd* (μg/L) |
|---|---|---|---|---|---|---|
| Caledonia Hatchery | 8.0 | 3 | 13.8 | 40.0 | - | - |
| Vermilion Lake | 7.5 | 5 | - | - | - | - |
| Whitson Lake | 6.5 | 18 | 14.5 | 7.0 | - | - |
| Halfway Lake | 6.7 | 1 | 3.3 | 4.3 | 69.0 | 0.025 |
| Hannah Lake | 7.7 | 21 | 6.6 | 20.6 | 67.3 | 0.152 |

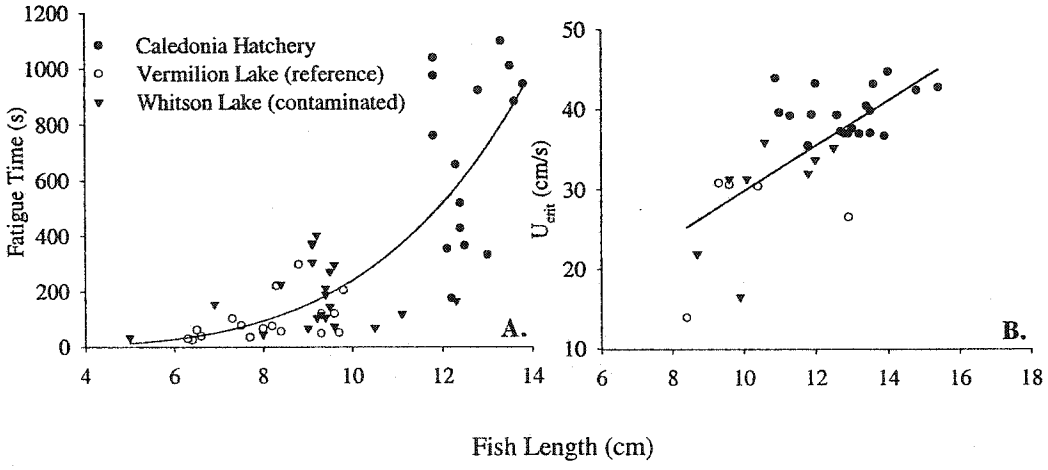
Table 2. Ten measures of physiological status for the test groups of yellow perch. Condition factor was calculated for all fish using the scaling exponent from the power fit of length *vs.* weight for all control/reference yellow perch (i.e., Caledonia hatchery, Vermilion Lake and Halfway Lake). Tissue copper and sodium levels represent the background concentrations determined on a wet weight basis. Carcass Na and Cu concentrations are the whole body minus the gills. Muscle glycogen levels were sampled from resting fish. Plasma Na and Cl levels (from the swimming performance experiment) were pooled values from each swim trial since there was no significant effect on swimming on plasma ion levels. Data represents the mean ± 1SEM (N). Statistical comparison was run within each experiment type. Values assigned a different letter were significantly different ($p<0.05$).

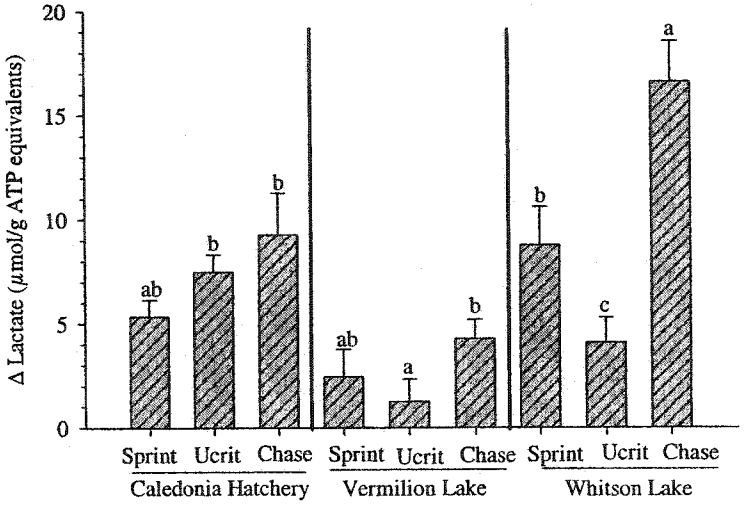
| | *Swimming Performance Experiment* | | | *Gill Binding Experiment* | |
|---|---|---|---|---|---|
| Condition Indicator | Caledonia Hatchery | Vermilion Lake (Reference) | Whitson Lake (Contaminated) | Halfway Lake (Reference) | Hannah Lake (Contaminated) |
| Weight (g) | 29.9 ± 1.1 (68)a | 9.6 ± 1.1 (38)b | 9.8 ± 0.5 (49)b | 7.1 ± 0.6 (70)a | 17.2 ± 0.9 (70)b |
| Length (cm) | 13.6 ± 0.2 (68)a | 9.3 ± 0.3 (38)b | 9.8 ± 0.2 (49)b | 8.6 ± 0.2 (70)a | 11.2 ± 0.2 (70)b |
| Condition Factor | 0.63 ± 0.01 (68)a | 0.64 ± 0.02 (38)a | 0.58 ± 0.01 (49)b | 0.60 ± 0.01 (70)a | 0.65 ± 0.01 (70)b |

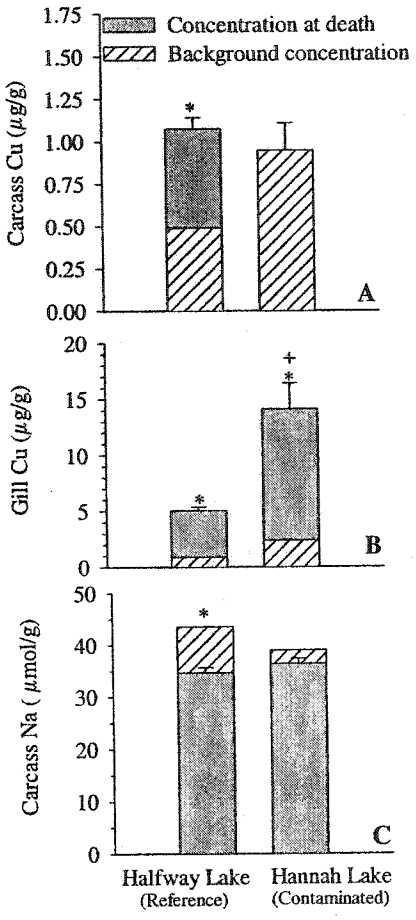
| | *Swimming Performance Experiment* | | | *Gill Binding Experiment* | |
|---|---|---|---|---|---|
| Condition Indicator | Caledonia Hatchery | Vermilion Lake (Reference) | Whitson Lake (Contaminated) | Halfway Lake (Reference) | Hannah Lake (Contaminated) |
| Gill Cu ($\mu$g/g) | 0.19 ± 0.01 (10)a | 3.33 ± 0.32 (10)b | 6.53 ± 0.53 (10)c | 0.85 ± 0.14 (10)a | 2.36 ± 0.48 (10)b |
| Liver Cu ($\mu$g/g) | 1.44 ± 0.35 (10)a | 8.86 ± 0.57 (10)b | 40.53 ± 8.72 (10)c | 3.08 ± 0.32 (10)a | 9.56 ± 2.68 (10)b |
| Carcass Cu ($\mu$g/g) | - | - | - | 0.49 ± 0.05 (10)a | 1.13 ± 0.20 (10)b |
| Carcass Na ($\mu$mol/g) | - | - | - | 34.6 ± 1.0 (10) | 36.4 ± 1.0 (10) |
| Plasma Na (mM) | 122.3 ± 2.6 (49)a | 139.7 ± 1.7 (17)b | 135.7 ± 3.0 (25)b | 125.9± 4.4 (6) | 123.7 ± 3.6 (5) |
| Plasma Cl (mM) | 118.7 ± 2.9 (52)a | 124.0 ± 3.4 (20)b | 111.2 ± 3.3 (25)b | - | - |
| Muscle Glycogen ($\mu$mol/g) | 11.07 ± 1.74 (14)a | 20.81 ± 0.97 (8)b | 25.52 ± 0.96 (10)c | - | - |

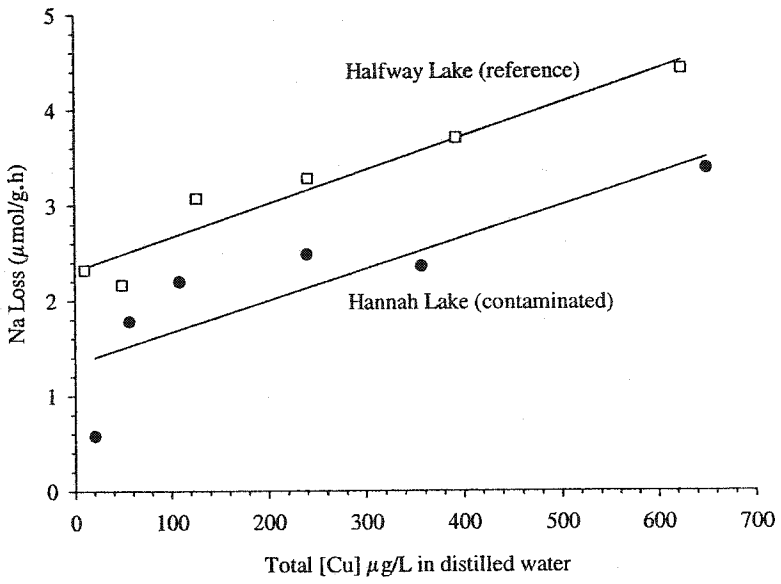
Table 2 (cont.)

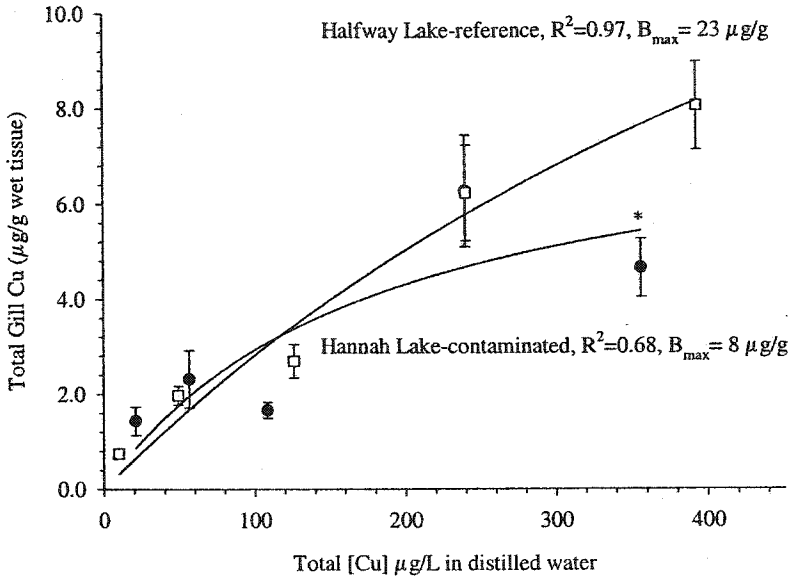

Halfway Lake
Vermilion Lake
Whitson Lake
Hannah Lake
Gill Copper (μg/g)
Liver Cu (μg/g)
A
B
0
2
4
6
8
10
20
30
40
50
60
0
5
10
15
20
25
Total Water [Cu] μg/L


Caledonia Hatchery
Vermilion Lake (reference)
Whitson Lake (contaminated)
Fatigue Time (s)
1200
1000
800
600
400
200
0
4
6
8
10
12
14
A.
$U_{crit}$ (cm/s)
50
40
30
20
10
6
8
10
12
14
16
18
B.
Fish Length (cm)


20
15
10
5
0
Δ Lactate (μmol/g ATP equivalents)
ab
b
b
ab
a
b
b
c
a
Sprint
Ucrit
Chase
Sprint
Ucrit
Chase
Sprint
Ucrit
Chase
Caledonia Hatchery
Vermilion Lake
Whitson Lake


Concentration at death
Background concentration
Carcass Cu (μg/g)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
A
Gill Cu (μg/g)
20
15
10
5
0
B
Carcass Na ( μmol/g)
50
40
30
20
10
0
C
Halfway Lake
(Reference)
Hannah Lake
(Contaminated)


Halfway Lake (reference)
Hannah Lake (contaminated)
Na Loss (μmol/g.h)
Total [Cu] μg/L in distilled water
0
1
2
3
4
5
0
100
200
300
400
500
600
700


Halfway Lake-reference, $R^2$=0.97, $B_{max}$= 23 μg/g
Hannah Lake-contaminated, $R^2$=0.68, $B_{max}$= 8 μg/g
Total Gill Cu (μg/g wet tissue)
Total [Cu] μg/L in distilled water
10.0
8.0
6.0
4.0
2.0
0.0
0
100
200
300
400
*

# CHAPTER 7

# OVERALL CONCLUSIONS

This thesis, which consisted of mainly laboratory-based experiments, set out to reduce the uncertainty associated with extrapolating from acute to chronic effects, from laboratory model organisms to resident species and from controlled laboratory exposures to situations in contaminated lakes. By reducing the uncertainty in these three areas, the role of the precautionary principle in the environmental management of copper becomes less unempirical and arbitrary. The present research provides a clearer understanding of which mitigating factors exist and how they may influence toxicity. Most importantly, this research will allow the incorporation of these factors into future experimental study design. Namely, the role of soft water acclimation, ration quantity and quality, individual variation, individual susceptibility, tolerance differences among species and the variable impact of water chemistry on different aspects of copper toxicity. Taking these issues into consideration will only improve the quality of the available science, especially in determining the compensatory costs associated with chronic copper toxicity.

Understanding chronic copper toxicity is complicated by the essentiality of copper to aquatic organisms. The 'threshold' of chronic toxicity, which is beyond homeostatic control, can be described more accurately as a 'window' of toxicity. Some organisms will be affected over a range of copper concentrations, while others may not, depending on their physiological status and past exposure(s). The state and history of the organism

will also influence the tools used to detect chronic effects. Traditional biomarkers or indicators of acute toxicity may be of limited value, in their current form, in documenting chronic exposures. For risk assessors, this means the task of predicting chronic effects, which may translate into effects on populations or food chains, is progressively more difficult for fish in their natural environment.

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